Genomic and Evolutionary Features of the SPI-1 Type III Secretion System That Is Present in *Xanthomonas albilineans* but Is Not Essential for Xylem Colonization and Symptom Development of Sugarcane Leaf Scald

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Xanthomonas albilineans is the causal agent of sugarcane leaf scald. Interestingly, this bacterium, which is not known to be insect or animal associated, possesses a type III secretion system (T3SS) belonging to the injectisome family *Salmonella* pathogenicity island 1 (SPI-1). The T3SS SPI-1 of *X. albilineans* shares only low similarity with other available T3SS SPI-1 sequences. Screening of a collection of 128 plant-pathogenic bacteria revealed that this T3SS SPI-1 is present in only two species of *Xanthomonas*: *X. albilineans* and *X. axonopodis* pv. *phaseoli*. Inoculation of sugarcane with knockout mutants showed that this system is not required by *X. albilineans* to spread within xylem vessels and to cause disease symptoms. This result was confirmed by the absence of this T3SS SPI-1 in an *X. albilineans* strain isolated from diseased sugarcane. To investigate the importance of the T3SS SPI-1 during the life cycle of *X. albilineans*, we analyzed T3SS SPI-1 sequences from 11 strains spanning the genetic diversity of this species. No nonsense mutations or frameshifting indels were observed in any of these strains, suggesting that the T3SS SPI-1 system is maintained within the species *X. albilineans*. Evolutionary features of T3SS SPI-1 based on phylogenetic, recombination, and selection analyses are discussed in the context of the possible functional importance of T3SS SPI-1 in the ecology of *X. albilineans*.

one species, *Stenotrophomonas maltophilia*, is known to be an opportunistic human pathogen.

Xanthomonas albilineans is a systemic, xylem-invading pathogen that causes leaf scald—a lethal disease of sugarcane (interspecific hybrids of *Saccharum* spp.) (Rott and Davis 2000). Leaf scald symptoms vary from a single, sharply defined narrow white stripe to complete wilting and necrosis of infected leaves leading to plant death. The pathogen is transmitted mainly by infested tools and infected sugarcane cuttings. *X. albilineans* is not known to be insect transmitted but there is evidence of aerial transmission under certain environmental conditions (Champoiseau et al. 2009; Daugrois et al. 2003; Davis et al. 1997; Saumtally et al. 1996). The only pathogenicity factor of *X. albilineans* that has been studied extensively to date is allicidin—a secreted small molecule with phytotoxic and antibiotic properties (Birch 2001). Allicidin is a potent DNA gyrase inhibitor that targets chloroplastic DNA gyrase A, inhibits chloroplast DNA replication, and blocks chloroplast differentiation, resulting in the white foliar stripe symptom (Birch 2001; Hashimi et al. 2007).

Type III secretion systems (T3SS), also called injectisomes, produce nanomachines that deliver effector proteins specifically into the cytoplasm of host cells. These effectors then target key functions of the host, thus explaining the major role played by T3SS in pathogenesis and symbiosis (Cornelis 2006; He et al. 2004; Lewis et al. 2009; Saijo and Schulze-Lefert 2008). The mechanisms whereby T3SS effectors interact with host cells have been well documented for animal-infecting bacteria and plant pathogens (Croxen and Finlay 2010; Kay and Bonas 2009; McGhie et al. 2009). Evolutionary trees of injectisome T3SS gene clusters are completely different from bacterial evolutionary trees based on 16S rRNA analysis (Gupta 2000), implying that T3SS gene clusters have been distributed among bacteria through lateral gene transfer. Based on phylogenetic analyses, T3SS were divided into seven families (Cornelis 2006), including three that are found mainly in free-living animal pathogens (*Yersinia* secretion [Ysc] and *Salmonella* pathogenicity islands 1 and 2 [SPI-1 and SPI-2, respectively]) and two that occur mainly in plant-invading bacteria (hypersensitive response and pathogenicity 1 and 2 [Hrp1 and Hrp2, respectively]). The global architecture of the structural components within the bacterial membrane is well conserved among the different T3SS families. However, the length of the

The *Xanthomonadaceae* are a family of gram-negative bacteria belonging to the order Xanthomonadales in the gamma subdivision of the *Proteobacteria* (Saddler and Bradbury 2005). Members of this family occupy diverse ecological niches in soil and water, as well as being present in plant tissues. Many *Xanthomonadaceae*, especially species from the genera *Xanthomonas* and *Xylella*, cause plant diseases, and

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pilus that extends from the basal structure varies from 40 to 80 nm in animal pathogens (T3SS SPI-1, SPI-2, or Ysc) and up to several microns in plant-invading bacteria (T3SS Hrp1 or Hrp2), allowing it to protrude into the thick plant cell wall (Cornelis 2006).

Recent sequencing of the complete genome of *X. albilineans* GPE PC73 confirmed the absence of a T3SS of the Hrp1 or Hrp2 type but revealed the presence of T3SS SPI-1 (Pieretti et al. 2009). T3SS SPI-1, which is found mostly in bacteria associated with mammals or insects, also occurs in four plant-invading bacteria: *Erwinia amylovora* Ea273 (the causal agent of fire blight of apple, pear, and related plants), *E. tasmaniensis* Et1/99 (a strictly epiphytic bacterium isolated from apple flowers), *E. pyrifoliae* (the causal agent of black stem blight of pear), and *Pantoea stewartii* subsp. *stewartii* (the causal agent of Stewart's wilt of maize) (Correa et al. 2010; Kube et al. 2008, 2010; Sebahia et al. 2010; Smits et al. 2010). Moreover, three DNA fragments of a putative T3SS SPI-1 were disclosed by subtractive hybridization in strains belonging to one (lineage 1) of the four genetic lineages of *X. axonopodis* pv. *phaseoli* (the causal agent of common bacterial blight of bean) (Alavi et al. 2008). Similarly, T3SS Ysc, which is found preferentially in animal pathogens, has also been found in the genome of the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* 1448A (the causal agent of halo blight disease in common bean) (Joardar et al. 2005). The role of T3SS SPI-1 or Ysc in these plant-invading bacteria remains unknown but it could be hypothesized that T3SS SPI-1 or Ysc is required for interaction of these plant-invading bacteria with an insect or an animal. In *Pantoea stewartii* subsp. *stewartii*, it was shown recently that T3SS SPI-1 was required for persistence in its flea beetle vector, *Chaetocnema pulicaria*, whereas T3SS Hrp was involved in plant colonization (Correa et al. 2010).

The objectives of the present study were to i) perform a functional analysis of the T3SS SPI-1 of *X. albilineans* in order to confirm that this system is not required for sugarcane infection, ii) analyze the distribution of this T3SS SPI-1 within a collection of plant-pathogenic bacteria, and iii) analyze the genetic diversity of the T3SS SPI-1 gene cluster within the species *X. albilineans* in order to shed some light on its functional importance during the life cycle of the causal agent of sugarcane leaf scald.

RESULTS

The genome of *X. albilineans* GPE PC73 harbors a T3SS SPI-1.

Annotation of the genome of *X. albilineans* GPE PC73 revealed the presence of a T3SS similar to the *bsa* T3SS of several *Burkholderia* spp. (e.g. *bsa* T3SS of *Burkholderia pseudomallei*, which belongs to the injectisome family SPI-1 and is a causal factor in human melioidosis) (Stevens et al. 2002). In *X. albilineans* GPE PC73, T3SS SPI-1 is present in a region of 43.6 kb flanked by IS elements and which has an overall G+C base composition (52%) lower than that of the rest of the genome (63%). Both these latter features suggest that the 43.6-kb region represents a genetic island acquired by lateral gene transfer. This region is located next to the albicidin biosynthesis gene cluster near the terminus of replication (Pieretti et al. 2009). Phylogenetic reconstruction using a concatenation of six T3SS SPI-1 structural proteins from bacteria associated with mammals, insects, or plants showed that the T3SS SPI-1 of *X. albilineans* belongs to the same phylogenetic group as the T3SS SPI-1 present in the plant-invading bacteria *E. tasmaniensis*, *E. amylovora*, *E. pyrifoliae*, and *Pantoea stewartii* subsp. *stewartii* (Fig. 1, group A). All *B. pseudomallei* genes encoding the components of the SPI-1 injectisome and translo-

con are conserved in *X. albilineans* (Fig. 2; Table 1). Genes required for injectisome assembly were annotated with reference to their *bsa* orthologous genes in *Burkholderia* spp. (*xsaj*, *xsak*, *xsal*, *xsam*, *xso*, *xsap*, *xsaq*, *xsar*, *xsas*, *xsav*, *xsw*, *xsax*, *xsay*, and *xsaZ*) (Fig. 2). Genes encoding components of the SPI-1 translocon were annotated *xipB*, *xipC*, and *xipD* with reference to their *bip* and *sip* orthologous genes in the genera *Burkholderia* and *Salmonella*, respectively. The *B. pseudomallei* gene *bsaN*, which encodes a transcriptional regulator of the AraC family, is conserved in *X. albilineans*, in which it was annotated as *xsaN*. *B. pseudomallei* gene *bicA* (encoding a chaperone protein) is also conserved in *X. albilineans* (annotated as *xicA*). Two *B. pseudomallei* genes encoding putative oxygen-regulated invasion proteins are also conserved in *X. albilineans*, and were annotated as *orgA* and *orgB*. Interestingly, *B. pseudomallei* genes *bsaT* and *bsaU*, which are specific to T3SS SPI-1 of *Burkholderia* spp., are not conserved in *X. albilineans*. *B. pseudomallei* genes encoding T3SS SPI-1 effectors are also not conserved in *X. albilineans*. BLAST analyses with the entire genome of *X. albilineans* confirmed that this species does not possess any of the known T3SS secreted effectors (T3SEs). However, T3SS SPI-1 of *X. albilineans* GPE PC73 encodes 15 hypothetical proteins specific to this species that represent candidate T3SEs. In other T3SS SPI-1 described to date, several genes encoding T3SEs are present in the same genetic island as the genes encoding structural components. Genes encoding candidate T3SEs SPI-1 of *X. albilineans* were annotated as *Xanthomonas*-associated protein (*xap*)A through *xapO*. The presence of an F-box-like domain in the hypothetical protein encoded by *xapN* supports the hypothesis that this protein is a T3SE because such a domain was described in T3SEs of *Ralstonia solanacearum* (Angot et al. 2006). The similarity of the hypothetical protein encoded by *xapM* with the toxin RtxA of *Photobacterium luminescens* is also evidence that this protein may be a T3SE.

Screening of 128 plant-pathogenic bacteria for the presence of T3SS SPI-1.

It was shown recently by subtractive hybridization that strains belonging to lineage 1 of *X. axonopodis* pv. *phaseoli* harbor at least three DNA fragments encoding putative components of a T3SS SPI-1 (Alavi et al. 2008). These fragments share >85% nucleotide identity and 83% amino acid identity with the sequence of the T3SS SPI-1 gene cluster of *X. albilineans*. To determine whether the T3SS SPI-1 gene cluster was fully or partially present in *X. axonopodis* pv. *phaseoli* strains, we used 35 primer pairs designed to amplify the complete T3SS SPI-1 from *X. albilineans* (Supplementary Table 1). Polymerase chain reaction (PCR) products of the expected size were obtained with only eight primer pairs (T3SS 6, 9, 13, 14, 15, 17, 18, and 22) in three *X. axonopodis* pv. *phaseoli* strains (CFBP 2534, CFBP 6164, and CFBP 6982). Sequencing of these PCR products revealed between 77 and 87% nucleotide identity with the sequence of the T3SS SPI-1 of *X. albilineans*. Moreover, no nucleotide diversity was seen between the three tested *X. axonopodis* pv. *phaseoli* strains. The amplified PCR products correspond to genes required for injectisome assembly (*xsaj*, *xsak*, *xsal*, *xsam*, *xso*, *xsap*, *xsar*, *xsw*, *xsax*, or *xsay*) and one gene encoding a component of the SPI-1 translocon (*xipB*). However, other T3SS SPI-1 genes, including *xipD* and candidate T3SEs genes, were not detected by PCR in any strain of *X. axonopodis* pv. *phaseoli*.

To see whether this T3SS SPI-1 gene cluster is indeed restricted to only two xanthomonads (*X. albilineans* and *X. axonopodis* pv. *phaseoli*), a collection of 128 strains representing the main genera of plant-pathogenic bacteria (Supplementary Table 2) was screened by PCR and dot-blot hybridization. Both

methods yielded the same result and revealed that the distribution of T3SS SPI-1 is very restricted. Indeed, no DNA fragments homologous to T3SS SPI-1 were detected in any of the 128 strains, with the exception of the 14 strains belonging to *X. albilineans* and genetic lineage 1 of *X. axonopodis* pv. *phaseoli* (Supplementary Fig. 1).

Is T3SS SPI-1 required

for *X. albilineans* to infect sugarcane and cause symptoms?

Sugarcane cv. CP68-1026 was inoculated with wild-type strain GPE PC73 R5 and three *xsaO* mutants of GPE PC73 R5 (six plants inoculated per strain at 10^8 CFU/ml). Detailed results are presented in Supplementary Figure 2. Within 2 to 4

Fig. 1. Tree of the concatenated amino acid sequence of six type III secretion system (T3SS) *Salmonella* pathogenicity island 1 (SPI-1) structural proteins from bacteria associated with mammals (*Burkholderia pseudomallei*, *B. thailandensis*, *Yersinia enterocolitica*, *Chromobacterium violaceum*, *Salmonella typhimurium*, *Escherichia coli* 0157:H7, *Shigella flexeneri*, and *Proteus mirabilis*), insects (*Sodalis glossinidius* and *Hamiltonella defensa*), or plants (*Xanthomonas albilineans*, *Erwinia tasmaniensis*, *E. amylovora*, *E. pyrifoliae*, and *Pantoea stewartii* subsp. *stewartii*) using the maximum likelihood method and LG as the substitution model. Six T3SS SPI-1 structural proteins were used in this analysis in *X. albilineans*: XsaS/InvC/SpaI, XsaP/InvE/MxiC, XsaQ/InvA/MxiA, XsaX/SpaQ, XsaZ/SpaS, and XsaY/InvN/SpaR. The tree was constructed with sequences of the T3SS SPI-2 structural proteins of *B. pseudomallei* and *H. defensa* as an outgroup. Bootstrap percentages retrieved in 500 replications are shown at the nodes. The scale bar (0.2) indicates the number of nucleotide substitutions per site.

weeks, the wild-type strain and all three mutants produced pencil-line stripes characteristic of leaf scald symptoms on inoculated leaves. On average, 8 stripes per inoculated leaf were observed with strain GPE PC73 R5, whereas the average number of stripes varied between 8 and 10 for the three *xsaO* mutants (18 leaves observed per strain). In the six plants inoculated with the wild-type strain, 16 of 19 (84%) sampled stalk internodes were colonized by the pathogen. In plants inoculated with the three *xsaO* mutants, the pathogen was recovered in 15 of 20 (75%), 15 of 19 (79%), and 21 of 23 (91%) sampled stalk internodes, respectively. Thus, the capacity of the *xsaO* mutants to spread through sugarcane xylem and cause leaf symptoms was similar to that of the wild-type strain.

Sequencing of the T3SS SPI-1 gene cluster from *X. albilineans* Xa23R1.

A 56-kb DNA region containing the complete albicidin biosynthesis gene cluster from *X. albilineans* Xa23R1 was previously sequenced (Royer et al. 2004). This region also included the five hypothetical protein genes *xapM*, *xapL*, *xapK*, *xapJ*, and *xapI*. The rest of the T3SS SPI-1 gene cluster, which corresponds to a 37-kb region, was amplified by PCR from strain Xa23R1, with primers generating 35 overlapping PCR fragments (Fig. 2C). Assembling the sequences of the PCR fragments and the previously sequenced 56-kb region reconstituted the sequence of the entire T3SS SPI-1 gene cluster from *X.*

albilineans Xa23R1 (Fig. 2C). All genes identified in strain GPE PC73 are conserved in strain Xa23R1. The most notable differences between the two sequences are i) an internal deletion of 30 nt in *xapH* of Xa23R1; ii) a mutation in the start codon of *xapB* making the open reading frame (ORF) 45 nt shorter in Xa23R1; and iii) the presence, upstream of a sequence similar to *bipD* of *B. pseudomallei*, of two different sequences of 963 and 1,143 bp in GPE PC73 and Xa23R1, respectively. These two latter sequences showed no significant alignment with any nucleotide or amino acid sequence available in GenBank. Further analysis of these two unique sequences suggested that the XipD translocase proteins encoded by *X. albilineans* strains GPE PC73 and Xa23R1 each have their own unique features (Fig. 3). In both strains, the sequence similar to *bipD* is present in an ORF that is much longer (1,881 and 2,061 bp in strains GPE PC73 and Xa23R1, respectively) than orthologous genes described to date in other T3SS SPI-1 (855 to 1,020 bp). The first 363 bp of the *xipD* ORF of strains GPE PC73 and Xa23R1 are 98% identical and did not align significantly with any amino acid sequence available in GenBank. The last 555 bp of these *xipD* ORF are also 98% identical and exhibit 58% amino acid similarity with the orthologous gene *bipD* of *B. pseudomallei* (Fig. 3). The strain-specific internal sequences of 963 and 1,143 bp exhibit 37% amino acid identity and 59% amino acid similarity (Fig. 3; Supplementary Fig. 3), suggesting that the two allelic forms of *xipD*

are derived from the same ancestor gene and have a similar and unique function. Additionally, the 184 bp upstream of the two allelic forms of *xipD* are 97% identical, indicating that transcriptional regulatory sequences are conserved in both *X. albilineans* strains. The two allelic forms of *xipD* were designed *xipD-G* for GPE PC73 and *xipD-X* for Xa23R1.

Phylogenetic and recombination analyses of T3SS SPI-1 sequences from different strains of *X. albilineans*.

Sequence analyses were performed with a collection of 12 strains representative of the genetic diversity of *X. albilineans* previously identified using pulsed-field gel electrophoresis (PFGE) (Davis et al. 1997). Strains Xa23R1, GPE PC73, HVO082, REU173, FIJ080, HVO005, LKA070, PNG130, MTQ058, and REU209 belong to PFGE groups A, B, C, D, E, F, G, H, I, and J, respectively (Supplementary Table 3). Because strains involved in sugarcane leaf scald disease outbreaks since the late 1980s belong to group PFGE-B, two additional strains belonging to this group were included in our analysis (strains MTQ078 and BRA115). To study evolution of the core genome, multilocus sequence analysis (MLSA) was performed using a concatenated sequence of PCR fragments of seven housekeeping genes (*groEL*, *dnaK*, *gyrB*, *atpD*, *efp*, *recA*, and *glnA*) (Fig. 4). To study evolution of the T3SS SPI-1 gene cluster, five fragments were amplified by PCR: i) the large ORF *xipD*, except its 3' part; ii) the 3' part of *xipD* and complete sequence of *xapA* and *xapB*; iii) partial sequence of *xsam*, iv) partial sequence of *xsao*; and v) partial sequence of *xapH*, including the 30-nt deletion observed in Xa23R1 (Fig. 5B). No PCR product was obtained for strain PNG130, which probably lost T3SS SPI-1. All five PCR fragments were amplified from all other strains. Sequences of these PCR fragments were compared with the corresponding sequence of strains GPE PC73 and Xa23R1, allowing analysis of 11 strains in all. Interestingly, ORF *xipD* is present in all 11 strains but the distribution of the allelic forms *xipD-G* or *xipD-X* was not congruent with the core genome MLSA tree, suggesting the occurrence of recombination events (Fig. 4). To explain this unexpected

result, recombination analyses were performed with different algorithms (CHIMAERA, Recombination Detection Program [RDP], GENECONV, and DNA Sequence Polymorphism [DnaSP]) using a concatenated sequence of the five PCR fragments (Fig. 5B) for each of the 11 strains. Several recombination events were predicted with high probability (*P* values varying from 1×10^{-6} to 8×10^{-40}) (Fig. 5C), not only at the *xipD* locus but also along the entire concatenated sequence. Similar phylogenetic analyses were also conducted with these 11 strains using the entire coding sequences of *xipD*, *xapA*, and *xapB* and partial coding sequences of *xsam*, *xsao*, and *xapH*. The coding sequence of *xipD* was divided into three parts that were analyzed separately: *xipD/a* corresponds to the 5' part of *xipD* shared by strains GPE PC73 and Xa23R1, *xipD/b* corresponds to the internal sequence specific to each allelic form (*xipD-G* and *xipD-X*), and *xipD/c* corresponds to the sequence similar to *bipD* that is common to strains GPE PC73 and Xa23R1 (Fig. 3). Two distinct phylogenetic clades (clade 1 and clade 2) were obtained for each of the seven coding sequences used for phylogenetic analyses (*xipD/a*, *xipD/c*, *xapA*, *xapB*, *xsam*, *xsao*, and *xapH*) (Fig. 4). The distribution of the 11 strains in clade 1 or clade 2 differed according to the sequence used for phylogenetic analyses (Fig. 4). For example, strains GPE PC73 and LKA070, which possess two different allelic forms of *xipD*, are in the same clade (clade 1) in trees constructed with *xipD/a*, *xapB*, *xsam*, *xsao*, and *xapH* but in different clades in trees constructed with *xipD/c* and *xapA*. Similarly, strains FIJ080 and Xa23R1, which also possess two different allelic forms of *xipD*, are in the same clade (clade 2) in trees constructed with *xipD/a*, *xapA*, *xsam*, and *xapH* but in different clades in trees constructed with *xipD/c* and *xsao*. Distribution of the 11 strains in phylogenetic trees obtained with housekeeping genes (core genome) or coding sequences *xsao*, *xapA*, *xipD/b*, and *xipD/c* also provided evidence of recombination events (Fig. 5D). The existence of only two allelic forms of *xipD* (*xipD-G* and *xipD-X*) (Fig. 3) and the distribution of T3SS SPI-1 sequences in only two clades (clade 1 and clade 2) (Fig. 4) indicate that recombination events have mobilized only two ancestral alleles of T3SS SPI-1.

Table 1. Comparative analysis of type III secretion system *Salmonella* pathogenicity island 1 (SPI-1) genes conserved in *Xanthomonas albilineans* and *Burkholderia pseudomallei*

Putative function	Nomenclature ^a	<i>X. albilineans</i> (strain GPE PC73)			<i>B. pseudomallei</i> (strain K96243)			Identity (%) ^c
		Accession	Protein	Length ^b	Accession	Protein	Length ^b	
Injectisome component	PrgK/MxiJ	XALc_1486	XsaJ	295	BPSS1550	BsaJ	316	100/181 (55)
Injectisome component	PrgJ	XALc_1487	XsaK	94	BPSS1549	BsaK	100	20/73 (27)
Injectisome component	PrgI/MxiH	XALc_1488	XsaL	97	BPSS1548	BsaL	89	44/90 (48)
Injectisome component	PrgH	XALc_1489	XsaM	467	BPSS1547	BsaM	428	115/441 (26)
Regulation	InvF	XALc_1490	XsaN	210	BPSS1546	BsaN	252	73/165 (44)
Injectisome component	InvG/MxiD	XALc_1491	XsaO	626	BPSS1545	BsaO	606	234/562 (41)
Injectisome component	InvE/MxiC	XALc_1492	XsaP	371	BPSS1544	BsaP	373	115/371 (30)
Injectisome component	InvA/MxiA	XALc_1493	XsaQ	703	BPSS1543	BsaQ	690	369/701 (52)
Chaperone	InvB	XALc_1494	XsaR	136	BPSS1542	BsaR	135	32/135 (23)
Injectisome component	InvC/SpaI	XALc_1495	XsaS	449	BPSS1541	BsaS	436	221/445 (49)
Injectisome component	InvK/SpaO	XALc_1498	XsaV	346	BPSS1538	BsaV	327	71/266 (26)
Injectisome component	InvL/SpaP	XALc_1499	XsaW	231	BPSS1537	BsaW	226	119/227 (52)
Injectisome component	SpaQ	XALc_1500	XsaX	88	BPSS1536	BsaX	84	42/82 (51)
Injectisome component	InvN/SpaR	XALc_1501	XsaY	256	BPSS1535	BsaY	256	102/244 (41)
Injectisome component	SpaS	XALc_1502	XsaZ	358	BPSS1534	BsaZ	411	156/350 (44)
Hypothetical protein	OrgA	XALc_1485	OrgA	198	BPSS1551	...	195	52/186 (27)
Hypothetical protein	OrgB	XALc_1484	OrgB	220	BPSS1552	...	260	37/176 (21)
Chaperone	SicA	XALc_1503	XicA	171	BPSS1533	BicA	171	80/166 (48)
Translocon component	SipB/IpaB	XALc_1504	XipB	534	BPSS1532	BipB	620	131/375 (34)
Translocon component	SipC/IpaC	XALc_1505	XipC	348	BPSS1531	BipC	419	68/263 (25)
Translocon component	SipD/IpaD	XALc_1477	XipD	626	BPSS1529	BipD	285	66/199 (33)

^a Frequently used nomenclature.

^b Length in amino acids.

^c Amino acid identity.

Selection analysis of T3SS SPI-1 coding sequences in 11 strains of *X. albilineans*.

Despite the occurrence of several recombination events, no nonsense mutations or frameshifting indels were observed in any of the five PCR fragments from nine *X. albilineans* strains or in the complete sequence of T3SS SPI-1 from strains GPE PC73 and Xa23R1. The 30-nt internal deletion in *xapH* found in strain Xa23R1 was not detected in any other strain; this deletion does not modify the coding phase and, thus, may not affect the function of *xapH*. The mutation in the start codon of *xapB* in Xa23R1, which shortens the ORF by 45 nt, was also observed in strains HVO082, HVO005, and REU209. This codon, which was identified as a start codon by annotators of the genome of strain GPE PC73, is not preceded by a known ribosome binding site and is most likely not a real start codon. Thus, *xapB* may be 45 nt shorter than previously thought in all *X. albilineans* strains. All T3SS SPI-1 genes are conserved in strains GPE PC73 and Xa23R1, which belong to two distinct core genome (MLSA) clades. All these data suggest that T3SS SPI-1 is still functional in all 11 *X. albilineans* strains.

Estimation of the ω = nonsynonymous to synonymous substitution (d_N/d_S) ratio is usually used to quantify the selective pressure acting on genes. Genes evolving under no selective pressure evolve neutrally ($\omega = 1$), whereas genes evolving under selection pressure have $\omega \neq 1$. Purifying selection ($\omega < 1$) is observed when amino acid replacement is counterselected

and positive selection ($\omega > 1$) occurs when amino acid replacement is favored by natural selection. We estimated the ω ratio for each coding sequence of the T3SS SPI-1 cluster retrievable from the five PCR fragments (Table 2). Four coding sequences (*xipD/c*, *xapA*, *xapB*, and *xsaM*) have an ω ratio ranging from 0.248 to 1.956, which was not statistically different from 1 according to likelihood ratio tests (LRT) (P value = 0.1512, 0.2073, 0.3638, and 0.6825, respectively) (Table 2). Therefore, the corresponding genes are thought to have evolved neutrally, under no selection pressure. The coding sequences *xipD/a* and *xsaO* have an ω ratio of 0.252 and 0.113, respectively, which was statistically significant at $P < 1$ (P value = 0.0158 and P value < 0.001 , respectively) (Table 2). This result suggests the presence of purifying selection (i.e., selection still acting on *xsaO* and *xipD/a* to prevent amino acid replacement), indicating that the 5' part of *xipD* (*xipD/a*) is translated in *X. albilineans* and supporting the hypothesis that *xipD* is longer than orthologous genes.

Unlike other analyzed T3SS SPI-1 coding sequences, *xapH* has an $\omega > 1$ ($\omega = 4.552$; P value = 0.0125) (Table 2), suggesting that *xapH* evolved under positive selection. The presence of positive selection was analyzed in detail using a codon-by-codon approach (Table 3). Positive selection in a few codons of a sequence may increase the estimated average ω ratio for the whole gene, thus leading to an artefactual signature of neutral evolution. We observed that *xapH* evolved under positive selection (P value = 0.044) and that no other genes of the

Fig. 3. A, Comparison of the physical maps of the allelic forms of *xipD* present in *Xanthomonas albilineans* strains GPE PC73 and Xa23R1. Black and gray hatched boxes indicate parts of coding sequence of *xipD* shared by both strains (parts called *xipD/a* and *xipD/c*, respectively). Gray hatched box (part *xipD/c*) indicates the part of *xipD* that is similar to the *bipD* gene of *Burkholderia pseudomallei*. White and black dotted boxes indicate the region of the coding sequence of *xipD* that is not shared by strains GPE PC73 and Xa23R1 (*xipD/b*). Boxes with bricks upstream of the ATG codon indicate the noncoding 5' region shared by strains GPE PC73 and Xa23R1. The lengths of the corresponding nucleotide sequences are indicated below each box. Putative start codon (ATG), stop codon (TAA), and a ribosome binding site (GAGAA) located 8 bp upstream of the start codon are indicated for the two allelic forms of *xipD*. IS = insertion sequence. **B**, Data from BLAST alignments of the two allelic forms of *xipD* of strains GPE PC73 and Xa23R1. **C**, Data from BLAST alignments of the two allelic forms of *xipD* with the orthologous gene *bipD* of *B. pseudomallei*. Vertical gray zones indicate the three parts of coding sequence of *xipD* (*xipD/a*, *xipD/b*, and *xipD/c*).

Fig. 4. Phylogenetic trees of *Xanthomonas albilineans* based on sequences of 11 or 12 strains of the pathogen. The multilocus sequence analysis tree was constructed with the concatenated nucleotide sequences of polymerase chain reaction (PCR) fragments from seven housekeeping genes (*gyrB*, *atpD*, *dnaK*, *efp*, *groEL*, *glnA*, and *recA*) using the maximum likelihood method and GTR as the substitution model. The tree was constructed with *X. albilineans* XsaS3 as outgroup. Strain XsaS3, which was isolated in Guadeloupe from sugarcane leaf surfaces, failed to induce symptoms in sugarcane after plant inoculation (Daugrois et al. 2003) and lacks most of the sequences identified in the complete genome sequence of GPE PC73 as being specific to *X. albilineans*, including type III secretion system (T3SS) *Salmonella* pathogenicity island 1 (SPI-1) (M. Royer, *unpublished data*). Bootstrap percentages retrieved in 1,000 replications and >70% are shown at the nodes. Scale bar (0.005) indicates the number of nucleotide substitutions per site. Distribution of the two allelic forms of gene *xipD* (*xipD-G* or *xipD-X*) in different strains of *X. albilineans* is indicated to the right of the tree. No PCR product was obtained with primers specific to T3SS SPI-1 for strain PNG130. Other trees were constructed with the complete or partial coding sequence of six genes from the T3SS SPI-1 gene cluster of *X. albilineans* using the maximum likelihood method and GTR as the substitution model. Trees *xapA* and *xapB* resulted from alignment of complete genes *xapA* and *xapB*, respectively. Trees *xsaM*, *xsaO*, and *xapH* resulted from alignment of the partial coding sequences of genes *xsaM*, *xsaO*, and *xapH*, respectively. Trees *xipD/a* and *xipD/c* resulted from alignment of the 5' and 3' part of *xipD*. Major clades (1 and 2) are indicated on top of the left branch of each tree (see text for details). Bootstrap percentages retrieved in 1,000 replications and >50% are shown at the nodes. Scale bar (0.001 or 0.002) indicates the number of nucleotide substitutions per site.

Fig. 5. Detection of recombination events in the type III secretion system (T3SS) *Salmonella* pathogenicity island 1 (SPI-1)-1 of *Xanthomonas albilineans*. **A**, Genomic organization of the *X. albilineans* T3SS SPI-1 gene cluster. Colored boxes indicate sequences amplified by polymerase chain reaction (PCR) from 10 *X. albilineans* strains and retrieved from GenBank for *X. albilineans* GPE PC73: orange box = two overlapping PCR fragments amplified by primer pairs TTSS28/XipDF and TTSS27/TTSS27bis, green box = PCR fragment amplified by primer pair TTSS20/TTSS20bis, blue box = PCR fragment amplified by primer pair TTSS18/TTSS18bis, and purple box = PCR fragment amplified by primer pair TTSS3/TTSS3bis. IS = insertion sequence, XALB1 = albicidin biosynthesis gene cluster, and T = terminus of replication. **B**, Concatenation of the five PCR fragments used for recombination analyses. Concatenated sequence including coding and noncoding sequences. Names of following coding sequences are indicated below boxes: complete *xipD* gene (parts a, b, and c); complete *xapA* and *xapB* genes; and partial sequences of genes *xsaM*, *xsaO*, *xipC*, and *xapH*. Scale bar = 500 bp. **C**, Recombination analyses. Horizontal bars indicate recombinant regions detected in the concatenated sequence by the method listed to the left of the figure. Reported *P* value, indicated above each bar, corresponds to the method in bold type and is the best calculated *P* value. **D**, Phylogenetic analyses. The tree corresponds to the multilocus sequence analysis tree shown in Figure 4, except that branch lengths are not proportional to the number of nucleotide substitutions per site. Numbers 1 and 2 indicate the clade to which each strain belongs in trees obtained for each coding sequence included in the concatenated sequence. Distribution of the two allelic forms of gene *xipD* (*xipD*-G or *xipD*-X) in the different strains of *X. albilineans* is indicated for the coding sequence *xipD*/b. Vertical gray zones indicate the coding sequences used to construct each tree with the corresponding clade.

Table 2. Nonsynonymous to synonymous (d_N/d_S ; ω) ratio estimates and likelihood ratio tests of coding sequences of the type III secretion system *Salmonella* pathogenicity island 1 (SPI-1) of *Xanthomonas albilineans*

Models ^a	Coding sequences						
	<i>xipD</i> /a	<i>xipD</i> /c	<i>xapA</i>	<i>xapB</i>	<i>xsaM</i>	<i>xsaO</i>	<i>xapH</i>
Model1, $\omega = 1$ (fixed)							
np	20	20	20	20	20	20	20
LnL	-555.430	-839.852	-225.446	-375.089	-1,591.944	-1,717.916	-1,188.978
ω	1	1	1	1	1	1	1
Model2, ω estimated							
np	21	21	21	21	21	21	21
LnL	-552.517	-838.822	-224.650	-374.677	-1,591.860	-1,707.969	-1,185.860
ω	0.252	0.456	0.248	1.956	0.826	0.113	4.552
d_N	0.020	0.021	0.018	0.058	0.022	0.006	0.043
d_S	0.079	0.045	0.070	0.029	0.026	0.051	0.010
LRT model1 vs. model2							
2 Δ LnL	5.828	2.060	1.590	0.825	0.167	19.894	6.236
df	1	1	1	1	1	1	1
<i>P</i> value	0.0158	0.1512	0.2073	0.3638	0.6825	8.18E-06	0.0125
Significance ^b	*	NS	NS	NS	NS	**	*

^a LnL, log-likelihood of the model; np, number of parameters of the model; 2 Δ LnL, twice the likelihood of the two compared models; df, number of degree of freedom; LRT, likelihood ratio test.

^b Significance: * and ** = significant at the 5 and 0.1% level, respectively; NS = not significant.

T3SS SPI-1 evolved under positive selection (P values obtained for genes other than *xapH* were not significant: $0.662 < P$ value < 1.000), thus confirming that averaged ω estimations were not biased by overestimation.

DISCUSSION

Recent sequencing of the genome of the sugarcane leaf scald pathogen *X. albilineans* GPE PC73 revealed the presence of a complete T3SS SPI-1 gene cluster (Pieretti et al. 2009). Data described herein reveal that this complete gene cluster is also present in *X. albilineans* Xa23R1, which belongs to another MLSA group. This T3SS SPI-1, which shows low similarity with other T3SS SPI-1 sequences available in data libraries, encodes a translocase protein, XipD (= SipD), exhibiting a unique and large additional sequence. A collection of 128 strains belonging to the main genera of plant-pathogenic bacteria, including 42 species of *Xanthomonas* to ensure that the genetic diversity of this genus was fully represented, was screened for the presence of the T3SS SPI-1 gene cluster of *X. albilineans*. This gene cluster was found in only two phylogenetically distant species of *Xanthomonas*: *X. albilineans* and *X. axonopodis* pv. *phaseoli*. Interestingly, the T3SS SPI-1 was not found in *X. translucens* and *X. hyacinthi*—the two species most related to *X. albilineans* according to a phylogenetic study based on 1,314 amplified fragment length polymorphism markers (Bui Thi Ngoc et al. 2010).

The presence of the same T3SS SPI-1 in two species of *Xanthomonas* (*X. albilineans* and *X. axonopodis*) raises the question of the origin of this gene cluster. Did these species share the same ecological niche and exchange genetic material? Did these species acquire T3SS SPI-1 independently from the same bacterium? Was a T3SS SPI-1 present in the ancestor of

the genus *Xanthomonas* but subsequently lost by most *Xanthomonas* spp.? The lack of PCR detection of *xipD* and candidate T3SEs genes in *X. axonopodis* pv. *phaseoli* may be due to polymorphism between the two species of *Xanthomonas* at the level of these genes. Sequencing of the complete genome of a *X. axonopodis* pv. *phaseoli* strain harboring T3SS SPI-1 should allow some conclusions to be drawn about the maintenance or degradation of this gene cluster in this species.

The flanking regions of the complete T3SS SPI-1 are identical in strains GPE PC73 and Xa23R1, indicating the occurrence of a single integration event of T3SS SPI-1 in the genome of the common ancestor of all *X. albilineans* strains. However, phylogenetic and recombination analyses clearly indicate that several recombination events have occurred between two alleles of the same T3SS SPI-1 gene cluster in ancestors of *X. albilineans* strains. No remains of a second copy of the T3SS SPI-1 gene cluster were found in the genome sequence of *X. albilineans* GPE PC73, and none were amplified by PCR in other strains. Therefore, in order for these recombination events to have occurred, a second copy of T3SS SPI-1 must have been present in ancestors of *X. albilineans* strains, either on a non-replicative DNA molecule (hypothesis 1) or on an ancestral plasmid (hypothesis 2) that was subsequently lost. According to hypothesis 1, multiple lateral gene transfers of T3SS SPI-1 must have occurred either between ancestors of *X. albilineans* strains themselves or between ancestors of *X. albilineans* strains and an unknown bacterium living in the same environment and possessing the same T3SS SPI-1. According to hypothesis 2, the occurrence of recombination events in both MLSA I and MLSA II groups supposes that the plasmid harboring a second copy of T3SS SPI-1 was lost only after the divergence of strains LKA070 and Xa23R1, implying that this plasmid was maintained by ancestors of *X. albilineans* strains.

Table 3. Detection of positive selection in the analyzed coding sequences of the type III secretion system *Salmonella* pathogenicity island 1 (SPI-1) of *Xanthomonas albilineans*^a

Sequence model, LRT ^b	LnL	np	ω Max	Percent	2 Δ LnL	df	P	Sig
<i>xipD</i> /a								
M7	-552.518	22	...	...	...	...	...	...
M8	-552.519	24	...	...	...	...	...	...
LRT	...	...	...	...	0	2	1.000	NS
<i>xipD</i> /c								
M7	-838.823	22	...	...	...	...	...	...
M8	-838.823	24	1.000	0.00001	...	...	...	...
LRT	...	...	...	...	3.000E-05	2	1.000	NS
<i>xapA</i>								
M7	-224.651	22	...	...	...	...	...	...
M8	-224.651	24	1.000	0.00001	...	...	...	...
LRT	...	...	...	...	1.540E-04	2	1.000	NS
<i>xapB</i>								
M7	-375.089	22	...	...	...	...	...	...
M8	-374.677	24	...	...	...	...	...	...
LRT	...	...	...	...	8.248E-01	2	0.662	NS
<i>xsam</i>								
M7	-1591.837	22	...	...	...	...	...	...
M8	-1591.793	24	1.839	0.473	...	...	...	...
LRT	...	...	...	...	8.792E-02	2	0.957	NS
<i>xsao</i>								
M7	-1707.970	22	...	...	...	...	...	...
M8	-1707.970	24	1.000	0.00001	...	...	...	...
LRT	...	...	...	...	4.460E-04	2	0.999	NS
<i>xapH</i>								
M7	-1188.978	22	...	...	...	...	...	...
M8	-1185.860	24	4.552	1.000	...	...	...	...
LRT	...	...	...	...	6.24	2	0.044	*

^a LRT, likelihood ratio test; LnL, log-likelihood of the model; np, number of parameters of the model; ω , max value of nonsynonymous to synonymous (ω) ratio estimated for codons evolving under positive selection; Percent, percentage of the codons evolving at ω max value; 2 Δ LnL, twice the likelihood of the two compared models; df, number of degree of freedom; Sig, significance; *, significant at the 5% level; and NS, not significant.

^b Coding sequence, model, and LRT.

Interestingly, phylogenetic analyses of the T3SS Hrp gene clusters of *Pseudomonas syringae* or enterobacterial plant pathogens (*Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya*, and *Pantoea* spp.) also revealed conflicts between phylogenetic trees constructed with different genes of the same T3SS Hrp gene cluster (Guttman et al. 2006; Naum et al. 2009). Recombination events between the conserved copy of the T3SS Hrp gene cluster of *Pseudomonas syringae* and an ancestral lost copy was proposed to explain these conflicts (Guttman et al. 2006). Extensive lateral gene transfer of T3SS Hrp genes among enterobacterial plant pathogens was proposed to explain the occurrence of multiple recombination events (Naum et al. 2009). As in the case of the T3SS SPI-1 of *X. albilineans*, phylogenetic analyses of the T3SS Hrp of *Pseudomonas syringae* indicated that recombination events mobilized only two ancestral alleles. Interpretations of phylogenetic analyses of T3SS Hrps of enterobacterial plant pathogens are more complicated, probably because multiple recombination events mobilized multiple alleles.

Shigellae and enteroinvasive *Escherichia coli* both contain a virulence plasmid harboring a full T3SS SPI-1 (Buchrieser et al. 2000; Venkatesan et al. 2001). *X. albilineans* could have acquired a similar plasmid harboring a T3SS SPI-1, possibly by lateral gene transfer. In a second step, this T3SS SPI-1 could have been integrated into the chromosome of *X. albilineans* near the terminus of replication. Acquisition of genes by lateral gene transfer at the terminus of replication has been described in other bacteria. In *Bacillus subtilis*, the presence of a large SPb prophage at the terminus of replication has caused lateral gene transfer (Kunst et al. 1997). Similarly, in *E. coli*, lambdaoid prophages tend to cluster close to the terminus of replication (Campbell 1992), and it has been suggested recently that hyper-recombination in the *E. coli* terminus region is due largely to these prophage sequences (Corre et al. 2000). Interestingly, the *X. albilineans* replication terminus region contains seven phage-related coding sequences (CDS) that are orthologous of CDS present in a filamentous prophage Cf1C close to the terminus of replication in other *Xanthomonas* spp. The presence of these phage sequences probably caused hyper-recombination and facilitated integration of the full T3SS SPI-1 and the albicidin biosynthesis gene cluster. The presence of these phage sequences may indicate that the full T3SS SPI-1 and the albicidin biosynthesis gene cluster were acquired by lateral gene transfer.

Despite the occurrence of several recombination events, no nonsense mutations or frameshifting indels were observed in the five PCR fragments from nine *X. albilineans* strains or in the complete sequence of T3SS SPI-1 from strains GPE PC73 and Xa23R1, supporting the hypothesis that T3SS SPI-1 is maintained in *X. albilineans*. Inoculation of sugarcane with knockout mutants showed clearly that the T3SS SPI-1 of *X. albilineans* is not required for sugarcane xylem colonization and symptom development by the pathogen. This result is in agreement with the absence of the T3SS SPI-1 in strain PNG130 of *X. albilineans*, which is pathogenic to sugarcane. Alternatively, the T3SS SPI-1 may be required for interaction with an insect or animal host. An interaction of *X. albilineans* with an insect could have played or possibly could still play an important role in the dissemination of this bacterium, as is the case for *Xylella fastidiosa*. Various sap-feeding insects can acquire and transmit *X. fastidiosa*—a xylem-limited bacterium that causes Pierce's disease of grapevine and other important plant diseases (Chatterjee et al. 2008). Interestingly, *X. albilineans* and *Xylella fastidiosa* are derived from the same ancestor, and experienced convergent genome erosion during their descent from the progenitor of the *Xanthomonas* genus (Pieretti et al. 2009). A similar insect-associated lifestyle may have favored such genome erosion because most genes required for a plant-associated

lifestyle are most likely not required for an insect-associated lifestyle (Pieretti et al. 2009). Furthermore, the *chiA* gene recently characterized as required by *X. fastidiosa* to use chitin of insect hosts as a source of carbon (Killiny et al. 2010) is also present in the genome of *X. albilineans*. However, *Xylella fastidiosa* lacks any T3SS, showing that T3SS SPI-1 is not necessarily required for insect transmission.

Selection analysis indicated that purifying selection has driven the evolution of *xipD* and *xsao*. Further analyses will be necessary to determine whether purifying selection also drove evolution of the entire T3SS SPI-1 gene cluster. Interestingly, most synonymous mutations in *xsao* and *xipD/a* were acquired by recombination (Supplementary Table 4), indicating that the variability in T3SS SPI-1 revealed in this study corresponds to the variability of the two allelic forms of T3SS SPI-1. According to hypothesis 1 (multiple lateral genetic transfer of T3SS SPI-1), this variability could reflect evolution of T3SS SPI-1 either in two ancestors of *X. albilineans* or in the unknown bacterium harboring T3SS SPI-1 living in the same environment. According to hypothesis 2 (plasmid-borne second copy of T3SS SPI-1), this variability might reflect co-evolution of two copies of the same T3SS SPI-1 in the common ancestor of *X. albilineans* strains. Consequently, the T3SS SPI-1 should be considered to be a system involved in the lifecycle of *X. albilineans* or in the lifecycle of the unknown bacterium occupying the same environment as *X. albilineans*.

Selection analysis also indicated that diversifying selection drove the evolution of *xapH* which, like other hypothetical protein genes specific to the T3SS SPI-1 of *X. albilineans*, is proposed to encode a T3SE. The proposed function of *xapH* is compatible with diversifying selection acting on this gene, which may prevent detection of the T3SE by animal immune systems.

Both allelic forms of *xipD* probably conserved the function of T3SS translocase because they harbor the same 3' part, which is similar to the translocase genes *bipD* and *sipD* of *Burkholderia* and *Salmonella* spp., respectively. The translocase protein SipD was described as being involved in the attachment of *Salmonella enterica* sv. typhimurium to animal host cells (Lara-Tejero and Galan 2009). Similarly, the two allelic forms of *xipD* may be involved in attachment to two different types of animal cells. According to hypothesis 1 (multiple lateral genetic transfer), selection of one of the two allelic forms of *xipD* may be linked to an adaptation of *X. albilineans* strains to attachment to only one type of animal cell. According to hypothesis 2 (plasmid harboring a second copy of T3SS SPI-1), maintenance of the two allelic forms of *xipD* in the ancestor of *X. albilineans* may be linked to association with an animal or insect host involving attachment to these two different types of cells.

Demonstration of in vitro expression of T3SS SPI-1 genes could provide further evidence for the functional maintenance of this secretion system in *X. albilineans*. However, reverse-transcription PCR assays with primers specific for *xipD*, *xsao*, or *xsam* failed to show any transcription of T3SS SPI-1 in *X. albilineans* GPE PC73 grown in a rich medium (M. Royer, unpublished data). Furthermore, transformation with plasmids harboring a bidirectional promoter located between genes *xsaN* and *xsam* fused in both orientations to a green fluorescent protein gene failed to yield any fluorescing bacteria in *X. albilineans* GPE PC73 grown in minimal and rich medium supplemented with different samples prepared from sugarcane leaves, mice, insects, or primate- or insect-cell cultures (M. Royer, unpublished data).

To conclude, phylogenetic, recombination, and selection analyses all concur that the T3SS SPI-1 is maintained within the species *X. albilineans*, although this gene cluster is absent in

strain PNG130 and is not required for xylem colonization and symptom development in sugarcane. T3SS SPI-1 may play an important role in association with an insect or animal host or in the ecology of *X. albilineans*. PCR screening for the presence of *X. albilineans* in insects collected recently in sugarcane fields in Guadeloupe failed to identify any insect host for this sugarcane pathogen thus far (M. Royer, *unpublished data*). Further surveys of insects or animals may be necessary to identify candidate hosts and to shed light on the function of T3SS SPI-1 in *X. albilineans*. T3SS SPI-1 PCR screening of bacteria, including endophytic and uncultivable bacteria, living in the xylem of sugarcane may also help determine the origin of the T3SS SPI-1 in *X. albilineans* and *X. axonopodis* pv. *phaseoli*.

MATERIALS AND METHODS

Bacterial strains.

The 12 *X. albilineans* strains used in this study originated from the collection of plant-pathogenic bacteria of the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) (Montpellier, France) and are representative of the genetic diversity of *X. albilineans*. Eleven strains were previously described (Renier et al. 2007) and one—GPE PC73 (group PFGE B)—was recently sequenced (Pieretti et al. 2009). Strain GPE PC73 R5 is a spontaneous rifampicin-resistant strain derived from GPE PC73. Strains were grown for 48 h on modified Wilbrink's medium (Rott et al. 1994) or on XAS selective growth medium (Davis et al. 1994) at 28°C before use.

Construction of *xsaO* mutants.

A 698-bp fragment of gene *xsaO* was amplified with *xsaO* primers from strain GPE PC73 and cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA, U.S.A.), yielding pTOPO-*xsaO*. An *XhoI/SpeI* fragment of pTOPO-*xsaO* containing the *xsaO* fragment was then transferred into vector pVO155 (Oke and Long 1999), yielding pVO155-*xsaO*. Plasmid pVO155-*xsaO* was then introduced into *X. albilineans* GPE PC73R5 by triparental conjugation using pRK2073 as described by Royer and associates (2004). Transformants were plated on Wilbrink agar plates supplemented with rifampicin at 50 mg/liter and kanamycin at 20 mg/liter. Plates were incubated at 28°C and mutants that grew within 5 to 7 days were screened with *xsaO*-screen primers, which consisted of one primer specific for pVO155 and one primer upstream of the fragment of *xsaO* used to construct the mutant.

Inoculation of sugarcane with *X. albilineans* and determination of stalk colonization.

Pathogenicity experiments were conducted at CIRAD in an S3 containment facility using healthy plants of sugarcane cv. CP68-1026, which is susceptible to leaf scald. Inoculum of *X. albilineans* was prepared from 5-day-old agar cultures on XaPR medium (sucrose at 10 g/liter, $\text{CaCl}_2[\text{H}_2\text{O}]$ at 5 mg/liter, ferric ammonium citrate $[\text{C}_6\text{H}_5\text{O}_7 \cdot x\text{Fe}_3^+ \cdot y\text{NH}_3]$ at 50 mg/liter, NH_4Cl at 1 g/liter, $\text{MgSO}_4[7\text{H}_2\text{O}]$ at 0.5 g/liter, DL-methionine at 0.1 g/liter, and agar at 15 g/liter; pH adjusted to 6.8 with a volume-to-volume solution of Na_2HPO_4 $[12\text{H}_2\text{O}]$ and KH_2PO_4 at 1/15 M). Six sugarcane stalks with at least four to five internodes were inoculated per strain with bacterial suspension at 10^8 CFU/ml in sterile distilled water by the decapitation method as described by Rott and associates (1997). Leaf scald symptoms were recorded on three emerging leaves 1 month after inoculation. Stalk colonization by *X. albilineans* was determined by the stalk blot inoculation technique in three to four upper internodes that had grown 4 months after inoculation. Stalks were sampled individually and cut at the soil

level with pruning shears. The rind of stalk fragments was cleaned with absorbent paper and flamed with alcohol to ensure external sterilization. Each stalk location was then cut transversally, in the middle, with sterile pruning shears. Cut sections were pressed firmly onto two plates of XAS selective growth medium supplemented with rifampicin at 50 mg/liter for stalks inoculated with wild-type strain GPE PC73 R5, and on XAS selective growth medium supplemented with rifampicin at 50 mg/liter and kanamycin at 20 mg/liter for stalks inoculated with *xsaO* mutant strains of *X. albilineans*. Pruning shears were flamed with alcohol after each cut. Growth of *X. albilineans* was recorded after incubating the agar plated for 5 days at 28°C.

Design of PCR primers.

The 35 primer pairs used for amplification of the T3SS SPI-1 gene cluster were designed using the tool Primer 3. Primers were designed based on the sequence of the T3SS SPI-1 of strain GPE PC73 (GenBank accession no. FP565176), and 35 overlapping DNA fragments of 1.2 to 1.9 kb were defined. Primer pairs used for amplification of fragments of seven house-keeping genes (*gyrB*, *groEL*, *recA*, *dnaK*, *efp*, *atpD*, and *glnA*) were designed manually and validated using the Integrated DNA Technologies melting temperature calculator.

PCR conditions.

DNA templates were prepared by suspending a freshly grown colony in 100 μl of sterile nuclease-free water. PCR amplifications were performed in an automated thermal cycler PTC-100 (MJ Research, Inc., Watertown, MA, U.S.A.). The 25- μl PCR reaction mix consisted of 3 μl of bacterial suspension, 2.5 μl of 10 $\times$ PCR buffer without MgCl_2 (Sigma-Aldrich, St. Louis), 120 μM dNTP mix, 2.0 mM MgCl_2 (Sigma-Aldrich), 0.36 μM each primer, 1 unit of *Taq* DNA Polymerase (Sigma-Aldrich), and sterile nuclease-free water to final volume. The PCR program was 94°C for 4 min; 30 cycles at 94°C for 30 s, melting temperature for 1 min 30 s, and 72°C for 1 min; with a final 72°C extension for 8 min. A 5- μl aliquot of each amplified product was analyzed by electrophoresis through a 1% agarose gel.

DNA sequencing.

DNA sequencing was performed by Beckman Coulter Genomics (Takely, U.K.). PCR fragments were sequenced with the primers used for their amplification. Sequences were aligned using ClustalW implemented in Bioedit software version 2 (ClustalW2) and corrected manually as necessary.

Phylogenetic analyses.

The phylogenetic T3SS SPI-1 tree was reconstructed using the maximum likelihood method implemented in the PhyML program. The LG substitution model was selected, assuming an estimated proportion of invariant sites (of 0.01) and four gamma-distributed rate categories to account for rate heterogeneity across sites. The six loci analyzed (InvC/XsaS, InvE/SpaP/XsaP, InvA/XsaQ, SpaQ/XsaX, SpaS/XsaZ, and SpaR/XsaY) are the same six T3SS SPI-1 structural proteins used in previous phylogenetic analysis (Degnan et al. 2009). Multiple alignments of the amino acid sequences of the six structural proteins and for all taxa were performed using ClustalW. The phylogenetic tree was calculated with PhyML version 2.4.4 (Guindon and Gascuel 2003). Five hundred bootstrap replicates were performed with PhyML program.

The phylogenetic core genome MLSA tree was reconstructed using the maximum likelihood method implemented in the PhyML program. The GTR substitution model was selected assuming an estimated proportion of invariant sites (of 0.01)

and four gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data ($\alpha = 0.011$). One thousand bootstrap replicates were performed with the PhyML program. The seven analyzed loci (*gyrB*, *groEL*, *recA*, *dnaK*, *efp*, *atpD*, and *glnA*) are typical housekeeping genes in the chromosome of *X. albilineans* GPE PC73, located 0.004, 0.348, 1.369, 1.983, 2.245, 3.442, and 3.655 Mb, respectively, from the origin of replication. The total length of the partial CDS nucleotide sequences concatenated for each taxon was 3,972 bp. Multiple alignments of the nucleotide sequences of the seven housekeeping fragment genes and for all taxa were performed using ClustalW.

Phylogenetic trees were also constructed from several complete or partial coding sequences of the locus T3SS SPI-1 of *X. albilineans* with the same methods used to construct the MLSA tree. PCR fragments used to construct these phylogenetic SPI-1 trees or the phylogenetic core genome MLSA tree were obtained from the same samples of DNA templates. Additional PCR controls were performed with other samples of the same DNA templates to rule out accidental mixing of samples.

Recombination analyses.

DnaSP (current released version: 5.10.01) (Librado and Rozas 2009) and the Recombination Detection Program (RDP3) version 3 were used to detect potential recombinant sequences and localize possible recombination breakpoints in multiple sequence alignments. RDP3 is a Windows interface for a range of different recombination detection and analysis programs, including the RDP method (Martin et al. 2005), GENECONV (Padidam et al. 1999), and CHiMAERA (Posada and Crandall 2001) methods used in this study. Analyses were performed with default settings for all detection methods.

Selection analyses.

Estimation of the ratio $\omega = d_n/d_s$ was performed using the branch models of the codeml program implemented in PAML 4.3 (Yang 1997, 1998). The likelihood (L) of each model was estimated by PAML. i) To test whether two clades in a given phylogeny underwent distinct selective pressures, a null model assuming a common ω ratio for both clades was compared with an alternative model assuming one ω ratio per clade. The two models were then compared statistically by LRT. The test statistics $2\Delta\ln L$ (twice the difference of the log-likelihood between the two models) was compared with a χ^2 distribution with n degrees of freedom, n being the difference between the numbers of parameters of the two models. ii) To test whether a gene evolved under purifying selection ($\omega < 1$) or evolved neutrally ($\omega = 1$), the null model assuming a fixed $\omega = 1$ was compared using LRT to an alternative model with an estimated ω ratio. Positive selection was detected with codon models of the program codeml (PAML) (Yang 1998; Yang and Nielsen 1998). A neutral model (M7), using a discrete β distribution (range [0,1]) to model different ω ratios among sites, was compared by LRT to an alternative model (M8) assuming a supplementary class of codons with $\omega > 1$ (ω estimated). Comparison of evolutionary rates between two clades of a given evolutionary tree was assessed by using the program baseml of PAML. A neutral model assuming that the two given clades evolved at the same evolutionary rate was compared by LRT with an alternative local clock model (Yang and Yoder 2003; Yoder and Yang 2000) that assumed that each clade or subclade had its own evolutionary rate.

Screening of plant-pathogenic bacteria for the presence of the T3SS SPI-1 gene cluster of *X. albilineans*.

A collection of 128 strains belonging to the main genera of plant-pathogenic bacteria (*Acidovorax*, *Agrobacterium*, *Burk-*

holderia, *Clavibacter*, *Curtobacterium*, *Erwinia*, *Dickeya*, *Herbaspirillum*, *Pseudomonas*, *Pectobacterium*, *Ralstonia*, *Xanthomonas*, and *Xylophilus*) was screened by PCR with the primer pairs BSA Q, J, and M (based on the three DNA fragments subtracted from *X. axonopodis* pv. *phaseoli*) (Alavi et al. 2008) and dot-blot hybridizations as described previously (Hajri et al. 2009).

The 35 primer pairs used to amplify the complete T3SS SPI-1 from *X. albilineans* Xa23R1 were used to reveal whether this gene cluster is fully or partially present in *X. axonopodis* pv. *phaseoli* strains. The amplified PCR products from three *X. axonopodis* pv. *phaseoli* strains (CFBP 2534, CFBP 6164, and CFBP 6982) were purified from agarose gels with a NucleospinH extract II kit (Macherey-Nagel EURL, Hoerd, France) and sequenced by the Genoscreen genomic analysis platform (Lille, France).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The ATGC Bioinformatics PhyML database: atgc.lirmm.fr/phyml
 ClustalW2 database: www.ebi.ac.uk/Tools/clustalw2/index.html
 DnaSP (DNA Sequence Polymorphism) website: www.ub.es/dnasp
 Integrated DNA Technologies database:
eu.idtdna.com/analyzer/applications/oligoanalyzer/Default.aspx
 The Primer 3 website: biotools.umassmed.edu/bioapps/primer3_www.cgi
 Recombination Detection Program RDP3 software:
darwin.uvigo.es/rdp/rdp.html