

A TALE of Transposition: Tn3-Like Transposons Play a Major Role in the Spread of Pathogenicity Determinants of *Xanthomonas citri* and Other Xanthomonads

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ABSTRACT Members of the genus *Xanthomonas* are among the most important phytopathogens. A key feature of *Xanthomonas* pathogenesis is the translocation of type III secretion system (T3SS) effector proteins (T3SEs) into the plant target cells via a T3SS. Several T3SEs and a murein lytic transglycosylase gene (*mlt*, required for citrus canker symptoms) are found associated with three transposition-related genes in *Xanthomonas citri* plasmid pXAC64. These are flanked by short inverted repeats (IRs). The region was identified as a transposon, Tn*Xax1*, with typical Tn3 family features, including a transposase and two recombination genes. Two 14-bp palindromic sequences within a 193-bp potential resolution site occur between the recombination genes. Additional derivatives carrying different T3SEs and other passenger genes occur in different *Xanthomonas* species. The T3SEs include transcription activator-like effectors (TALEs). Certain TALEs are flanked by the same IRs as found in Tn*Xax1* to form mobile insertion cassettes (MICs), suggesting that they may be transmitted horizontally. A significant number of MICs carrying other passenger genes (including a number of TALE genes) were also identified, flanked by the same Tn*Xax1* IRs and delimited by 5-bp target site duplications. We conclude that a large fraction of T3SEs, including individual TALEs and potential pathogenicity determinants, have spread by transposition and that Tn*Xax1*, which exhibits all of the essential characteristics of a functional transposon, may be involved in driving MIC transposition. We also propose that TALE genes may diversify by fork slippage during the replicative Tn3 family transposition. These mechanisms may play a crucial role in the emergence of *Xanthomonas* pathogenicity.

IMPORTANCE *Xanthomonas* genomes carry many insertion sequences (IS) and transposons, which play an important role in their evolution and architecture. This study reveals a key relationship between transposons and pathogenicity determinants in *Xanthomonas*. We propose that several transposition events mediated by a Tn3-like element carrying different sets of passenger genes, such as different type III secretion system effectors (including transcription activation-like effectors [TALEs]), were determinant in the evolution and emergence of *Xanthomonas* pathogenicity. TALE genes are DNA-binding effectors that modulate plant transcription. We also present a model for generating TALE gene diversity based on fork slippage associated with the replicative transposition mechanism of Tn3-like transposons. This may provide a mechanism for niche adaptation, specialization, host-switching, and other lifestyle changes. These results will also certainly lead to novel insights into the evolution and emergence of the various diseases caused by different *Xanthomonas* species and pathovars.

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The genus *Xanthomonas* represents an important group of bacterial plant pathogens with global economic agricultural impacts. *Xanthomonas* species are clearly distinctive in their pathology and plant host ranges (1). Among the more economically important are *Xanthomonas citri*, causing citrus canker, *Xanthomonas oryzae* pathovars, causing bacterial blight and leaf streak in rice (*Oryza sativa*) (2, 3), *Xanthomonas campestris* pv. vesicato-

ria and *Xanthomonas arboricola* pv. pruni, responsible for bacterial spot on a wide range of commercial plants, including pepper, tomato, and *Prunus* species (1, 4), and *Xanthomonas fuscans* subsp. *fuscans* and *Xanthomonas axonopodis* pv. manihotis, responsible for common bean blight and bacterial cassava blight, respectively (1, 5).

Citrus canker is an important disease affecting many citrus

species. It is endemic in many parts of the world and has a severe economic impact on the citrus-producing industry (2, 6). Asiatic type A citrus canker caused by *X. citri* subsp. *citri* pathotype A infection is the most widespread and destructive citrus canker type (2). Types B and C result from infection by different strains of *X. fuscans* subsp. *aurantifolii*, and these are restricted to South America (2, 7). Citrus canker can be easily transmitted by wind-blown rain. Infection causes hyperplasia and necrosis, leading to cell lysis (8). As is the case for other diseases caused by the *Xanthomonas* genus, citrus canker involves a complex network of interactions implicating bacterial type II (T2SS) and type III secretion systems (T3SS) and biofilm formation (8). As observed in other pathosystems (9, 10), T3SS proteins are an important part of the *Xanthomonas* pathogenesis repertoire (11, 12), creating a bridge between the bacterium and the plant host cell and permitting the injection of bacterial T3SS effector proteins (T3SEs) into the plant cell cytoplasm (6). Over 60 type III effector proteins (<http://www.xanthomonas.org/t3e.html>), including transcription activator-like effectors (TALEs; see below) (13, 14), have been identified.

Xanthomonas genome sequences have proved a rich source of information concerning the evolution, lifestyle, and pathogenicity of members of the genus. The genome of *X. citri* subsp. *citri* pathotype A was deciphered over a decade ago (6), and the genomes of four other citrus canker-related species (*X. fuscans* subsp. *aurantifolii* strains ICPB 11122 and ICPB 10535, *X. citri* subsp. *citri* strain A^w 12879, and *X. axonopodis* pv. *citrumelo* strain F1) (2, 7) were partially or completely sequenced recently. Those of several additional species and pathovars are currently available or in process (<http://www.xanthomonas.org>).

An intriguing feature of the xanthomonads is their extraordinary genome plasticity and highly mosaic architecture (8), largely due to genomic rearrangements involving genomic islands and insertion sequences (IS) (15, 16). Indeed, IS are important landmarks of some *Xanthomonas* genomes. *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are rich in IS transposases, which can represent up to 10% of their genomes, whereas in *X. citri* subsp. *citri* strain 306 and *X. campestris* pv. *vesicatoria*, IS transposases represent less than 2% of the genome (17).

It has been noted that several *Xanthomonas* T3SEs are flanked by short (<100 bp) inverted repeat (IR) sequences (18) that form genetic structures typical of mobile insertion cassettes (MIC) (19). These are composed of passenger genes flanked by IS-related inverted repeats. MICs do not encode their own transposases but can be activated by a cognate transposase from a related IS (19). It has been suggested that these effector modules may have been transmitted by transposition and lateral gene transfer (18), although no specific IS family repeats have been reported to be predominantly associated with T3SEs (3).

The T3SEs found in the MIC structures include TALE genes. The products of these are transcription factors which modulate plant host gene expression to the benefit of the pathogen by interacting with various host cell factors and interfering with cell growth and the defense response (20, 21). Members of this class of DNA binding protein are found in a number of xanthomonads and are a key pathogenic feature (8, 13, 18, 22). Host DNA sequence specificity is determined by conserved near-identical tandem repeats, usually 34 amino acids long, that include a repeat-variable diresidue (RVD) at positions 12 and 13 (23). Each RVD recognizes a given nucleotide, and the string of tandem repeats

therefore recognize a specific DNA binding sequence (20, 23, 24). TALEs are becoming powerful DNA-targeting tools in biotechnology (23, 25).

One of the largest and most diverse transposon groups is the Tn3 family. Members range in size and structure from 3,000 bp to more than 50,000 bp. All include a transposase (TnpA) of the DDE motif (RNase H fold) class (26, 27), and most carry a site-specific recombinase system, as well as passenger genes for functions such as resistance to antibiotics and heavy metals like mercury (28, 29) or copper (30), which have been sprayed for many years on vegetable and fruit crops to limit the spread of plant-pathogenic bacteria and fungi (31). They transpose by replicative replicon fusion, generating a cointegrate with a copy of the transposon in direct orientation at each junction between the fused donor and target replicons (26). Transposition terminates by recombination (resolution) between the two directly repeated Tn copies at a particular site, *res* or *rst*, in a reaction catalyzed by a transposon-encoded site-specific recombinase. In many Tn3 family members, this is a serine recombinase, the resolvase (TnpR) (26). In others, it may be a single tyrosine recombinase related to phage integrases (TnpI) (32) or a pair of genes encoding TnpT and TnpS (33). TnpS is related to the bacteriophage P1 Cre tyrosine recombinase, whereas TnpT shows no similarity to any known protein. Shorter derivatives have also been identified that encode only the transposase and are devoid of the resolvase (e.g., *ISVsa19*, *ISShfr9*, *ISBusp1*, and *IS1071D*) (34). These therefore resemble simple bacterial ISs.

In the studies reported here, high-quality IS annotation coupled with comparative genomics revealed an important group of xanthomonad Tn3 family transposons and derivatives with associated T3SEs in general and TALE genes in particular. This includes a complete Tn3 family transposon in the *X. citri* subsp. *citri* pathotype A genome and a large number of derivatives in plasmids and chromosomes of other *Xanthomonas* species.

The *X. citri* subsp. *citri* pathotype A Tn3-like transposons carry T3SE passenger genes, together with a highly conserved murein lytic transglycosylase gene (*mlt*). The analysis also underlined the importance of related transposon-like structures in the transmission of other pathogenicity factors between different xanthomonads. Not only were the four previously known *X. citri* subsp. *citri* pathotype A plasmid (pXAC33 and pXAC64) TALE genes located in a MIC structure flanked by the same Tn3-like repeats as the *TnXax1* transposon, but many other TALE and pathogenicity genes from other xanthomonads were observed to be associated with similar structures. These results provide strong evidence that, at least in the case of *X. citri* subsp. *citri* pathotype A and other closely related species, key pathogenicity and virulence genes are spread by a Tn3-like transposition mechanism and use (or, in the case of *X. oryzae*, have used) conjugative plasmids as intercellular vectors. We propose that the transposition process can inherently generate TALE gene diversity and is a key agent in modulating bacterial virulence and host specificity in this group of plant pathogens.

RESULTS

Tn3 family elements in the pXAC64 plasmid and the distribution of their specific IRs among the xanthomonads. Plasmid pXAC64 and closely related plasmids are widely distributed in xanthomonads (4, 7), including *X. oryzae* strains isolated from North America, although they appear to be absent in the sequenced *X. oryzae* and *X. oryzae* pv. *oryzicola* genomes (3, 35).

that of TnPa43). The second, XACb0009, shows high similarity to the TnPa43 tyrosine recombinase, TnpS (49% identity), and the third, XACb0010, is related to the Tn4651 auxiliary cointegrate resolution protein T (TnpT) (43% identity) (33). TnpS and TnpT are oriented divergently in both Tn4651 and TnXax1.

For Tn4651, the intergenic region between the divergent *tnpS* and *tnpT* genes is 188 bp and includes two short palindromes within a 136-bp functional resolution recombination site (33), the Tn3 family *rst* site (26). One pair are putative binding sites for TnpT, whereas the other pair are part of the core site at which recombination occurs (33).

The TnXax1 *tnpT-tnpS* intergenic region is 193 bp and also includes two pairs of palindromic sequences; one pair, IR1 and IR2, are 14 bp with a potential core recombination site of 5 bp (Fig. 1A), and the other pair are two short IRs, IRa and IRb, which could represent a putative TnpT binding site (33).

Other TnXax1-related derivatives. A TnXax1-related structure was previously identified in plasmid pXap41, which is present in a variety of *X. arboricola* pv. *pruni* species (4). We have identified a number of additional TnXax1-related structures in a variety of xanthomonads (Fig. 2), both in plasmids and chromosomes. One striking feature of these is that, while the right ends are quite similar (Fig. 2B), the left ends exhibit a large degree of variability (Fig. 2A). Clearly the left end can act as a platform for accommodating various passenger genes, as well as different IS and other Tn3 family transposons. Additional IR copies are also observed in some cases, permitting the transposition of alternative structures using alternative combinations of ends. Many contain mutations which would render them unable to transpose (e.g., an in-frame stop codon in *tnpT* and the absence of a right IR [IRR] in *X. campestris* pv. *vesicatoria* strain 85-10 chromosomal copy B) (Fig. 2A). In addition, it seems probable that intertransposon recombination has also played a role in generating these structures. For example, the *X. citri* subsp. *citri* strain 29-1 chromosomal TnXax1 derivative (called XccA-29-1 herein) includes *xopE2* and *xopAI* effector genes and two left IRs (IRLs). It resembles a chimera derived by recombination between the TnXax1 from the pXAC64 plasmid and the *X. citri* subsp. *citri* pathotype A chromosomal TnXax1 copy (Fig. 2A).

Passenger genes: effector proteins and Mlt. These transposon-like structures were initially identified in regions containing a number of T3SE pathogenicity genes. The annotated regions shown in Fig. 2 show that these occur as passenger genes within many of the TnXax1 derivative structures. These are principally the effector genes *xopE2*, *xopE3*, *xopAI*, *xopC*, and *mlt*, which have received much attention over the past few years. They are central for pathogenicity (38–40).

The *mlt* gene is not considered to be a T3SE family member (4), but except for a single case from *X. campestris* pv. *vesicatoria* 85-10 chromosomal copy B, it is consistently found in these structures (Fig. 2B). Laia and coauthors previously suggested that Mlt may be important for T3SS apparatus assembly and activity (41). This conservation suggests that *mlt* is maintained by a significant selective pressure. It is noteworthy that *mlt* homologues are also present in a variety of xanthomonads (see Tables S3 and S4 in the supplemental material) and in other genera, such as *Pseudomonas* and *Ralstonia*. Mlt shares 63% sequence identity with HopAJ1 from *Pseudomonas syringae* pv. *tomato* DC3000 (4, 41). Although HopAJ1 is not a T3SS substrate, it probably enables the T3SS to penetrate the peptidoglycan layer in the bacterial periplasm and

deliver virulence proteins into host cells (42). In *X. citri* subsp. *citri* pathotype A, it appears to be expressed specifically during *in vivo* multiplication, and its deletion reduces the severity of citrus canker (41, 43).

TnXax1 from plasmid pXAC64 and from the *X. citri* subsp. *citri* pathotype A chromosome carry two different T3SE genes. That from pXAC64 includes *xopE2*, a putative transglutaminase that acts on the plant cell plasma membrane, suggesting a role in virulence and in the suppression of the hypersensitive response of the host (40). The *X. citri* subsp. *citri* pathotype A chromosome copy includes *xopAI*, homologous to the *P. syringae* pv. *tomato* virulence factors *hopU1*, *hopO1-1*, and *hopO1-2*. These probably have a posttranslational modification function (44) (Fig. 2A). Interestingly, the two effectors share about 200 bp at their 5' ends. The C-terminal end of XopAI shows some similarity to the arginine ADP-ribosyltransferase family (PF01129). The chromosomal *X. citri* subsp. *citri* pathotype A TnXax1 derivative also includes an additional 274 bp of noncoding DNA between *xopAI* and the IRL, whereas the derivative *X. citri* subsp. *citri* strain A^w 12879 has 204 bp of noncoding DNA between *xopE2* and IRL. Other differences between the TnXax1 structures are much more complex, suggesting that a high level of recombination may be involved in their formation (Fig. 2A).

Both *X. axonopodis* pv. *citrumelo* strain F1 TnXax1 relatives (A and B) carry a disrupted *xopC* gene whose product is secreted normally and translocates into the plant cell (Fig. 2A) (38). The *xopC* copies found in the *X. axonopodis* pv. *citrumelo* F1 A TnXax1 derivative are disrupted by IS404 and IS1389 insertions (Fig. 2A) that are not present in *X. axonopodis* pv. *citrumelo* F1 B.

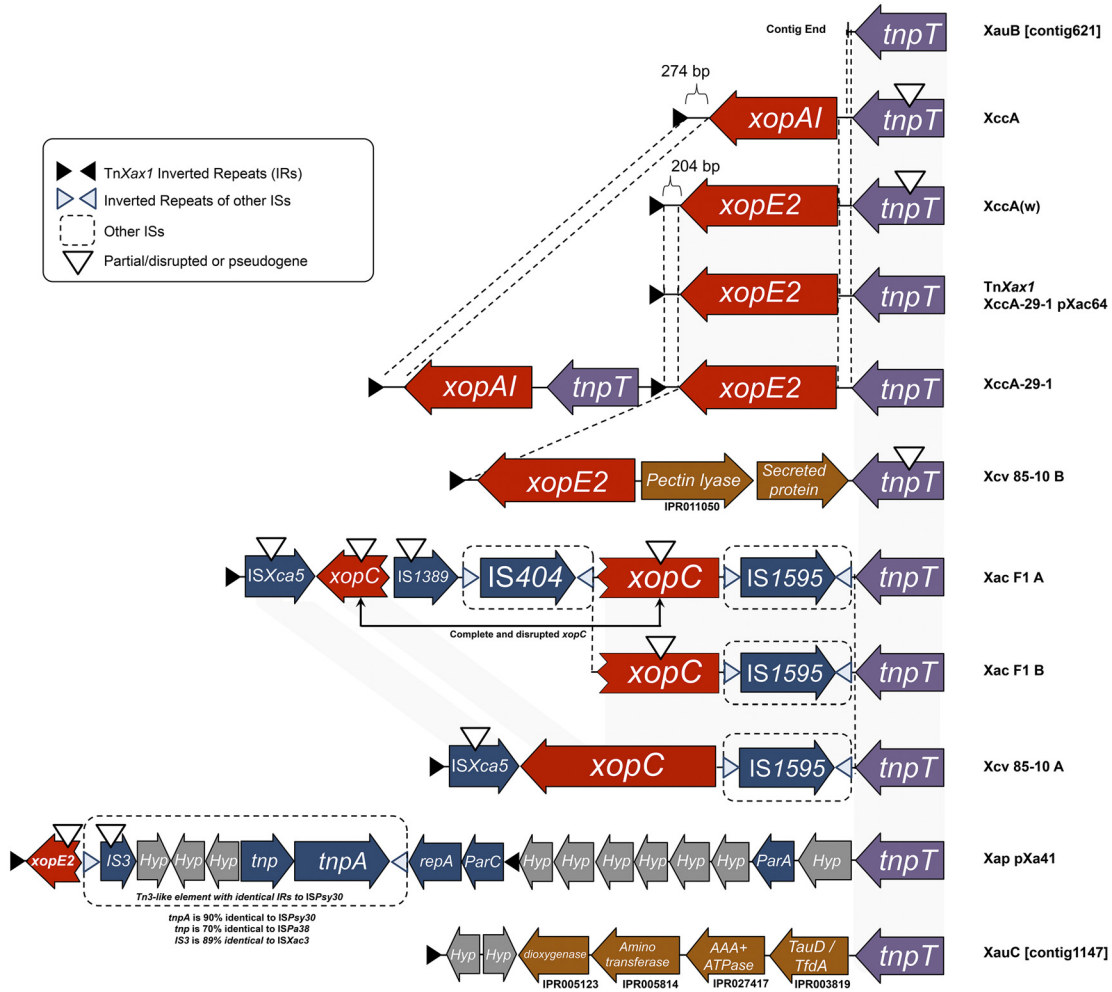
As shown in Fig. 2 and in Table S2 in the supplemental material, many other passenger genes carried by these transposon-like structures are also related to virulence.

TALE gene distribution among the Xanthomonadales: a story of transposition. Although several of the >264 different IR occurrences (see Table S1 in the supplemental material) were found to form part of TnXax1 derivatives, many occurred as inverted repeats flanking passenger genes without associated transposition genes and form putative MIC elements.

Analysis of the passenger genes neighboring the 92 bp IR showed that many were TALE gene family members. TALE genes have attracted attention by their simple and predictable specific DNA sequence recognition readout, which uses a number of repeating peptide blocks, some of which include a dipeptide with specific recognition properties for a given base (20, 24). In their original xanthomonad hosts, TALE proteins are transmitted to the host plant cell nucleus, where they activate or inactivate important genes, thus contributing directly to pathogenicity. Indeed, a single amino acid substitution in a TALE sequence can affect citrus canker development (22). However, the detailed mechanism of transcriptional regulation by TALEs is still not fully understood (20).

Previous studies had revealed that a number of these in different *Xanthomonas* strains are flanked by 62 bp IRs, and it was suggested that they may have been transmitted as mobile cassettes (18). Two were identified on each of the two *X. citri* subsp. *citri* strain 306 plasmids, pXAC33 (carrying *pthA1* and *pthA2*) and pXAC64 (carrying *pthA3* and *pthA4*) (18). The four genes are slightly different, with the principal variations located in the repeating peptide blocks. The analysis performed here showed that they are flanked by the same 92 bp (rather than 62 bp) IR sequence

A Left end – 5'



B Right end – 3'

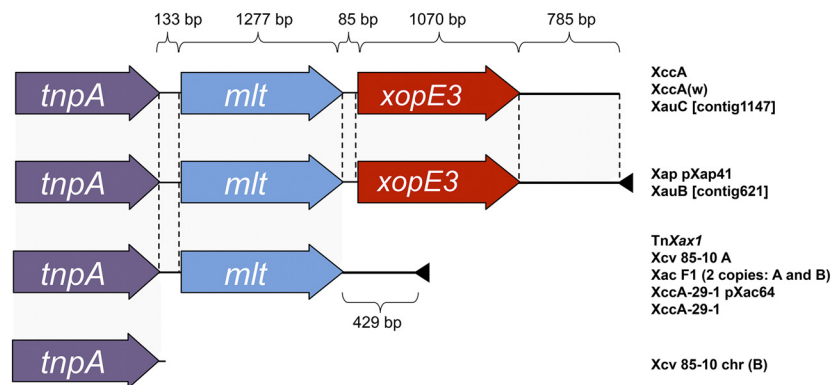


FIG 2 Genetic organization of the left (A) and right (B) ends of the TnXax1-related structures found in other *Xanthomonas* species. Abbreviations for the TnXax1-related structures: Xap pXap41, *X. arboricola* pv. pruni, plasmid pXap41; XccA, *X. citri* subsp. *citri* pathotype A, chromosomal copy; TnXax1, *X. citri* subsp. *citri* pathotype A, plasmid copy and canonical element; Xac F1 A and Xac F1 B, *X. axonopodis* pv. citrumelo strain F1, 2 chromosomal copies; Xcv A and Xcv B, *X. campestris* pv. vesicatoria strain 85-10, chromosomal copies A and B; XccA(w), *X. citri* subsp. *citri* strain A^w 12879, chromosomal copy; XccA-29-1, *X. citri* subsp. *citri* strain 29-1, chromosomal copy; XccA-29-1 pXac64, *X. citri* subsp. *citri* strain 29-1, plasmid copy; XauB ctg 621_0, *X. fuscans* subsp. *aurantifolii* strain ICPB 11122; XauC ctg 1147_0, *X. fuscans* subsp. *aurantifolii* strain ICPB 10535.

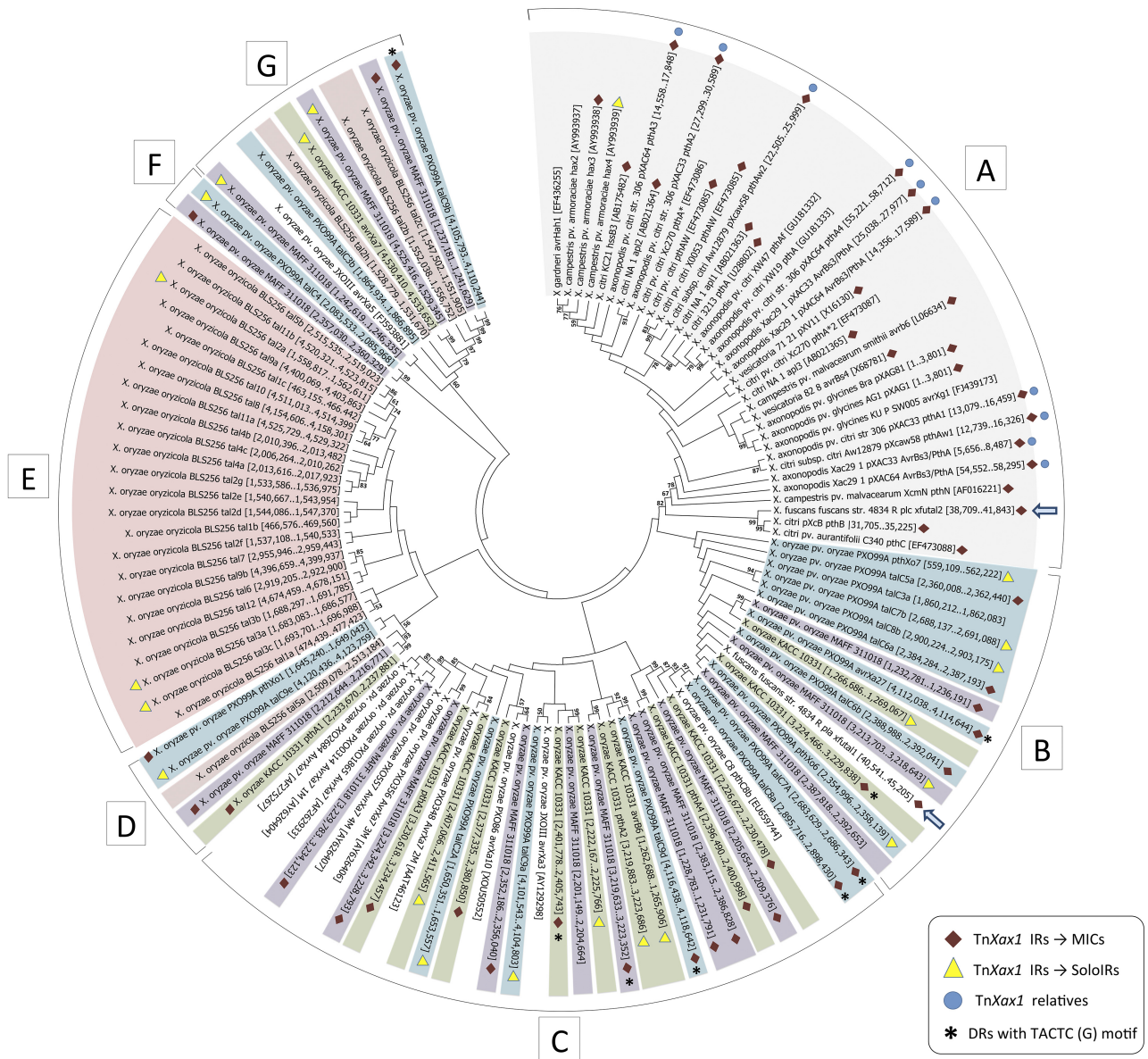


FIG 3 Molecular phylogenetic analysis by maximum-likelihood method of the TALEs genes found in completely sequenced *Xanthomonas* genomes. Each TALE gene is shown with its respective genomic coordinates (for TALEs extracted from complete genomes) or GenBank accession number inside the brackets. Symbols: red diamonds, TALE genes associated with MIC structures; yellow triangles, TALE genes associated with a solo IR flanking one of their extremities; blue circles, genomes which carry *TnXax1* or related structures; asterisks, MICs with the direct TACTC(G) target repeat; blue arrow, the TALE gene from *X. fuscans* subsp. *fuscans* strain 4834-R plasmids *pla* and *plc*. TALEs from different *X. oryzae* pathovars are highlighted as follows: blue, *X. oryzae* pv. *oryzae* strain PXO99A; green, *X. oryzae* pv. *oryzae* strain KACC 10331; purple, *X. oryzae* pv. *oryzae* strain MAFF311018; pink, *X. oryzae* pv. *oryzicola* strain BLS256. TALEs from *X. citri*, *X. axonopodis*, *X. campestris* pv. *vesicatoria*, and *X. campestris* are highlighted in gray.

as the *Xanthomonas*-specific *TnXax1* IR. This suggests that the Tn3-like element may provide transposition functions for these MIC elements.

Boch and Bonas described 113 known TALEs in different *Xanthomonas* species (18) (see also Bogdanove et al. [14]). Here, we analyzed these genes together with newly identified TALE genes deposited in the public databases, focusing principally on those from complete and fully sequenced genomes. A maximum-likelihood tree constructed from the full-length DNA sequences of 122 different TALEs is shown in Fig. 3. The topology shows well-defined groups, including two major groups (Fig. 3B and C) and

three minor groups (Fig. 3D, F, and G) composed of TALEs from various *X. oryzae* strains (Fig. 3, purple, green, blue, and white), a group composed of TALEs from *X. citri*, *X. axonopodis*, *X. campestris* pv. *vesicatoria*, and *X. campestris* (Fig. 3A, gray), and a group representing most of the *X. oryzae* pv. *oryzicola* BLS256 TALEs (pink) (Fig. 3E). A similar topology was obtained using the set of full-length proteins (data not shown). The general topology is consistent with that obtained in other studies, which used nucleotide sequences without the RVD repeats (14), concatenated N- and C-terminal portions of the proteins (45), or C-terminal regions alone (46).

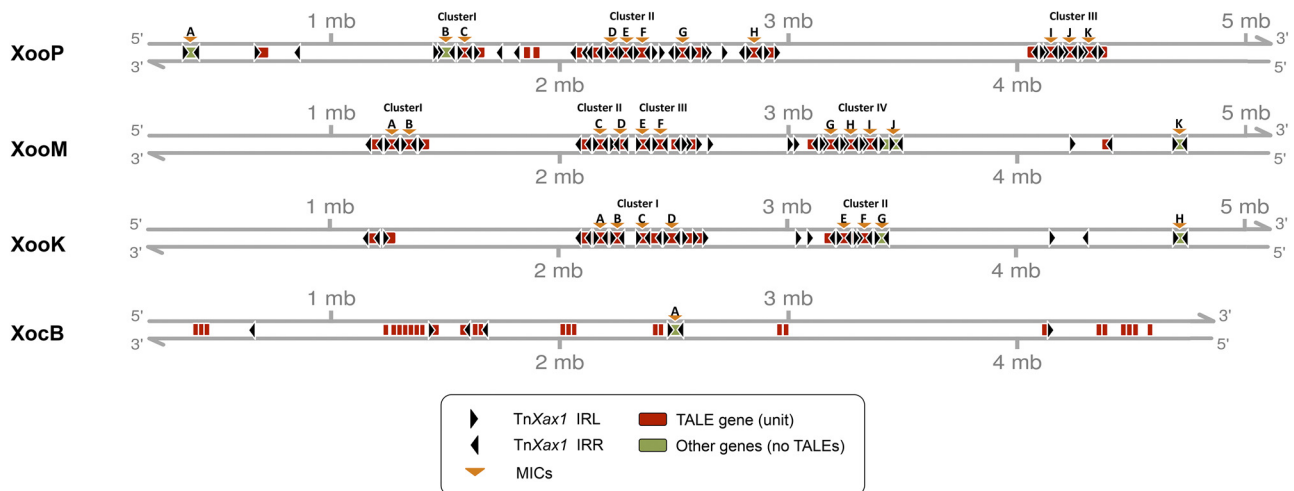


FIG 4 Linear genomic representation of the distribution of the IRs and tandemly repeated MIC clusters in *X. oryzae* genomes. Abbreviations: XooP, *X. oryzae* pv. *oryzae* strain PXO99A; XooM, *X. oryzae* pv. *oryzae* strain MAFF311018; XooK, *X. oryzae* pv. *oryzae* strain KACC 10331; XocB, *X. oryzae* pv. *oryzicola* strain BLS256.

Association of TALE genes with TnXax1 IR. A substantial number of TALE genes from this collection (55 or 45%) (Fig. 3) proved to be associated with the TnXax1 IRs and to form MIC structures. These include both MICs with full-length IRs and MICs with IRs ranging from 33 bp to 97 bp but always maintaining the GAGGG tip (see Table S3 in the supplemental material). MIC-associated TALEs are found in a majority of the Asian *X. oryzae* and the *X. citri/axonopodis/campestris/campestris* pv. *vesicatoria* group, suggesting that transposition by a Tn3 mechanism may be an important driver of TALE dispersion and diversification in these groups. However, they are much less frequent in the *X. oryzae* pv. *oryzicola* strains.

In addition to the MIC structures, 20 solo IRs were identified next to TALEs from the *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* groups, and a potential solo IR was identified in the *X. citri/axonopodis/campestris/campestris* pv. *vesicatoria* group (Fig. 3; see Table S1 in the supplemental material). Solo IRs may reflect previous transposition or recombination events involving the adjacent TALE gene. The chromosomal TALE-containing MICs are particularly notable in the Asian *X. oryzae* group (Fig. 4), where, assuming that there have been no major errors in genome assembly, there are small clusters of tandemly repeated MIC units. Figure 4 and Fig. S1 show the distribution of all MIC structures and their association with the TALE genes and solo IRs. Table S3 lists these elements in more detail. In all three *X. oryzae* strains, the tandemly repeated MIC units are distributed in a complex cluster organization that is distinct for each strain (Fig. 4).

Moreover, the DNA located between the MICs of each cluster shows a higher-than-average percentage of genes encoding transposases from other transposable element (TE) families, integrases, and phage-related functions and could represent a plasticity region in which insertions and rearrangements do not adversely affect cell viability. Since more than one MIC structure and IR are associated with each locus, transposition of alternative structures using different combinations of neighboring ends may be possible. In addition, these *X. oryzae* genomes also carry other isolated TALE genes and solo IRs which may also act as substrates for recombination and transposition of alternative structures.

For some MICs, it was possible to identify 5 bp target duplications that are commonly generated by Tn3 family elements, indicating that these insertions represent true transposition events (Fig. 3; see Table S3 in the supplemental material). These DRs are diverse in sequence (Table S3), but a particular sequence, TACTC(G), is shared by a number of MIC elements of all three *X. oryzae* strains, although the associated TALEs are different and grouped in different *X. oryzae* subgroups (Fig. 3B, C, and G, asterisks). This may indicate either that these insertions exhibit a preferred target sequence or that the TALEs have evolved from a single MIC which has been displaced and amplified and has diverged as part of a larger structure (Table S3).

Relationship between TALEs from different xanthomonads. Not all TALE genes are included in well-defined MICs. The *X. oryzae* pv. *oryzicola* strain BSL256 carries many such copies (Fig. 3, pink). The *X. oryzae* pv. *oryzicola* *tal5a* without IRs is located on the phylogenetic tree within a small group of *X. oryzae* MIC-associated TALEs (Fig. 3D, blue, purple, and green) in a branch separate from the main *X. oryzae* groups (Fig. 3B and C) and more closely related to *X. oryzae* pv. *oryzicola* strain BSL256 TALEs (Fig. 3E). Another subset of *X. oryzae* pv. *oryzicola* BSL256 TALEs without IRs, *tal2c*, *tal2b*, and *tal2h*, forms part of the second *X. oryzae* TALE clade (Fig. 3G), which also includes MIC-associated TALEs (Fig. 3G, blue, purple and green). It is possible that *X. oryzae* pv. *oryzicola* BSL256 first acquired these TALEs by transposition and has subsequently lost the IRs.

Although the majority of *X. oryzae* pv. *oryzicola* BSL256 TALEs are not associated with flanking IRs (Fig. 3C), three appear to have a single IR copy. This is also consistent with the notion of IR decay.

There are a number of unexpected incongruences in the phylogenetic tree. In particular, *X. fuscans* subsp. *fuscans* strain 4834-R carries both *X. oryzae* and *X. citri/axonopodis/campestris/campestris* pv. *vesicatoria*-related TALEs. These are plasmid located (5). The TALE from one of the three resident plasmids, *X. fuscans* subsp. *fuscans* plasmid A, is grouped with those of the *X. oryzae* strains (Fig. 3B, blue arrow), whereas that of *X. fuscans* subsp. *fuscans* plasmid C is grouped with those of the *X. citri/axonopodis/campestris/campestris* pv. *vesicatoria* strains (Fig. 3A,

blue arrow). The presence of both types of TALE in different clades is therefore most simply explained by plasmid-mediated horizontal transfer.

MICs that include other pathogenicity functions. In addition to TALE genes, MIC structures contain other T3SEs. The unique MIC of *X. oryzae* pv. *oryzicola* BLS256 includes several complete and partial TEs of different families and the effector gene *xopU* (see Table S3 in the supplemental material). This effector is exclusive to *X. oryzae* strains and is found in different MIC structures. Other potential effector proteins and pathogenicity functions are shown in Fig. S1 and Table S3. While MICs carrying TALE genes tend to be rather simple, those carrying other potential effector and pathogenicity factors appear more complex and can include more than two IRs. They vary in length from 3 to 19 kb. Closely related elements are present in different related strains. This is the case for the two 16.2 kb chromosomal Mic A from *X. citri* subsp. *citri* ATCC 33913 and *X. citri* subsp. *citri* 8004 (which are inverted with respect to each other in the genome) and the 18.2 kb *X. oryzae* MIC J (strain MAFF311018) and MIC G (strain KACC 10331). It should also be noted that MIC B (*X. oryzae* strain PXO99A) is nearly identical to the MAFF311018 and KACC 10331 copies but contains additional DNA, including an extra IR and two different T3SEs (*xopU* and *xopX*).

In general, all these MICs carry known T3SS effector genes, such as *xopJ2*, *xopJ5*, *xopT*, *xopU* and *xopX*, as well as *mlt* and genes for pectin lyase, protein tyrosine phosphatase, a resistance-nodulation-cell division (RND) efflux pump, and members of a two-component system. Type IA DNA topoisomerase, potential tyrosine recombinase, toxin/antitoxin, and acriflavin resistance genes are also represented, as well as many unrelated hypothetical protein genes (see Table S3 in the supplemental material). Moreover, MIC H from *X. oryzae* KACC 10331, carrying a polymerase/histidinol phosphatase-like protein, generated 5 bp target site duplication, clearly indicating that it had inserted into the genome by transposition.

Transposase sources for MIC mobility. Clearly, the presence of flanking target DR observed for a number of MIC structures in these *Xanthomonas* strains indicates that they have undergone true transposition events (e.g., those marked with asterisks in Fig. 3B, C, and G and in Table S3 in the supplemental material). This requires an external source of the cognate transposase. Many TALEs of the *X. citri/axonopodis/campestris/campestris* pv. *vesicatoria* group are located on plasmids. In these cases, transposition functions could be supplied either from the plasmid *TnXax1* derivatives or, when present, from a chromosomal *TnXax1* derivative. On the other hand, Asian *X. oryzae* and *X. oryzae* pv. *oryzicola* group strains, whose TALEs are uniquely chromosomal, do not carry plasmid or chromosomal *TnXax1* derivatives or indeed isolated transposition functions. Thus, further MIC transposition is unlikely. To explain the appearance of TALEs in Asian *X. oryzae* and *X. oryzae* pv. *oryzicola* group strains, it seems probable that they were acquired by transmission from an ancestral plasmid which has subsequently been lost. The loss of this type of plasmid has been observed in some North American *X. oryzae* strains (which are more closely related to *X. oryzae* pv. *oryzicola* [35]). Most contain a plasmid similar to that of *X. campestris* pv. *vesicatoria* pXCV38, with a Tn3-like element (*ISXc4*) whose transposase is 40% identical to TnpA of *TnXax1*, and others appear to have lost the plasmid during laboratory culture (35).

Moreover, a second related plasmid, pXCV183 from *X. camp-*

estris pv. *vesicatoria*, carries a potential transposon, *TnXca1* (see Table S2 in the supplemental material), which shares the same IR tips and carries TnpA, TnpS, and TnpT with 59, 98, and 91% identity, respectively, to those from *TnXax1* (Table S4). Although the Asian *X. oryzae* group strains do not carry *TnXax1* relatives, *X. oryzae* PXO carries structures similar to *TnXca1*, which carries only 29 instead of 92 bp *TnXax1* IRs. The *X. oryzae* PXO *TnXca1* derivative carries *tnpA* and *tnpS* genes with 59 and 80% identity, respectively, to those in *TnXax1* (Table S4). An additional and partial copy without IRs is also found in *X. citri* subsp. *citri* strain B100 (Table S4). Since the *TnXca1* IRs and transposition-related genes share considerable similarity to those of *TnXax1*, *TnXca1* may be able to provide a functional transposase for the MICs found in *X. oryzae* PXO. It is noteworthy that a partial *TnXca1*-related structure is also found in the genome of *Pseudoxanthomonas spadix* strain BD-a59, thus indicating that this class of transposon is not exclusive to the *Xanthomonas* genus (Table S4).

DISCUSSION

We have identified a novel class of Tn3 family-derived transposable elements in *Xanthomonas* species which could potentially mediate combinatorial spread and diversification of plant virulence factors. The group includes *TnXax1*, an apparently intact transposon from *Xanthomonas citri*, and we have also identified a number of related structures in a variety of xanthomonads. These exhibit several different genetic organizations and carry different types of passenger genes, mostly T3SEs. Although the right sides of these structures fall into relatively homogenous groups, the left sides are highly variable: each derivative appears to have a unique left side, indicating the occurrence of different recombination and duplication events. The IRs of these structures are commonly associated with passenger genes, mostly TALE genes, forming MIC elements. Target site duplications of 5 bp, commonly generated by elements from the Tn3 family, were also identified in some of the *TnXax1*-related structures and MICs, strongly supporting the idea that these are products of true transposition events.

Studies with *Bacillus cereus* strains showed that their MICs (which carry genes encoding products such as endopeptidase, antibiotic resistance proteins, or regulatory factors) can be mobilized *in trans* by a cognate transposase (47). Different MIC structures carrying TALE genes are often found in *Xanthomonas* strains, and their role in the emergence of pathogenicity may be considerable.

Relationship of MIC clusters to the emergence of *Xanthomonas* pathogenicity. The transposition of TALE-carrying MICs in *Xanthomonas* strains presents an intriguing puzzle, since they appear to be grouped in clusters. This may indicate that the MICs in each cluster were amplified and diverged from a single insertion event. Moreover, in several citrus pathogens and related species, the MIC-associated TALE genes are often found in plasmids, whereas in the *X. oryzae* and *X. oryzae* pv. *oryzicola* species, these occur in the chromosome. In addition, the presence of plasmids may be essential to the development of disease caused by *X. fuscans* subsp. *aurantifolii* strains. For instance, the plasmid pXcB from *X. fuscans* subsp. *aurantifolii* can be fully transferred *in planta* and is central to the development of citrus canker (37). Since some *X. oryzae* group members from North America carry plasmids similar to pXCV38, it is possible that a plasmid-borne *TnXax1* relative and TALE-carrying MICs transiently invaded members of the *X. oryzae* group and subsequently spread by transposition driven by

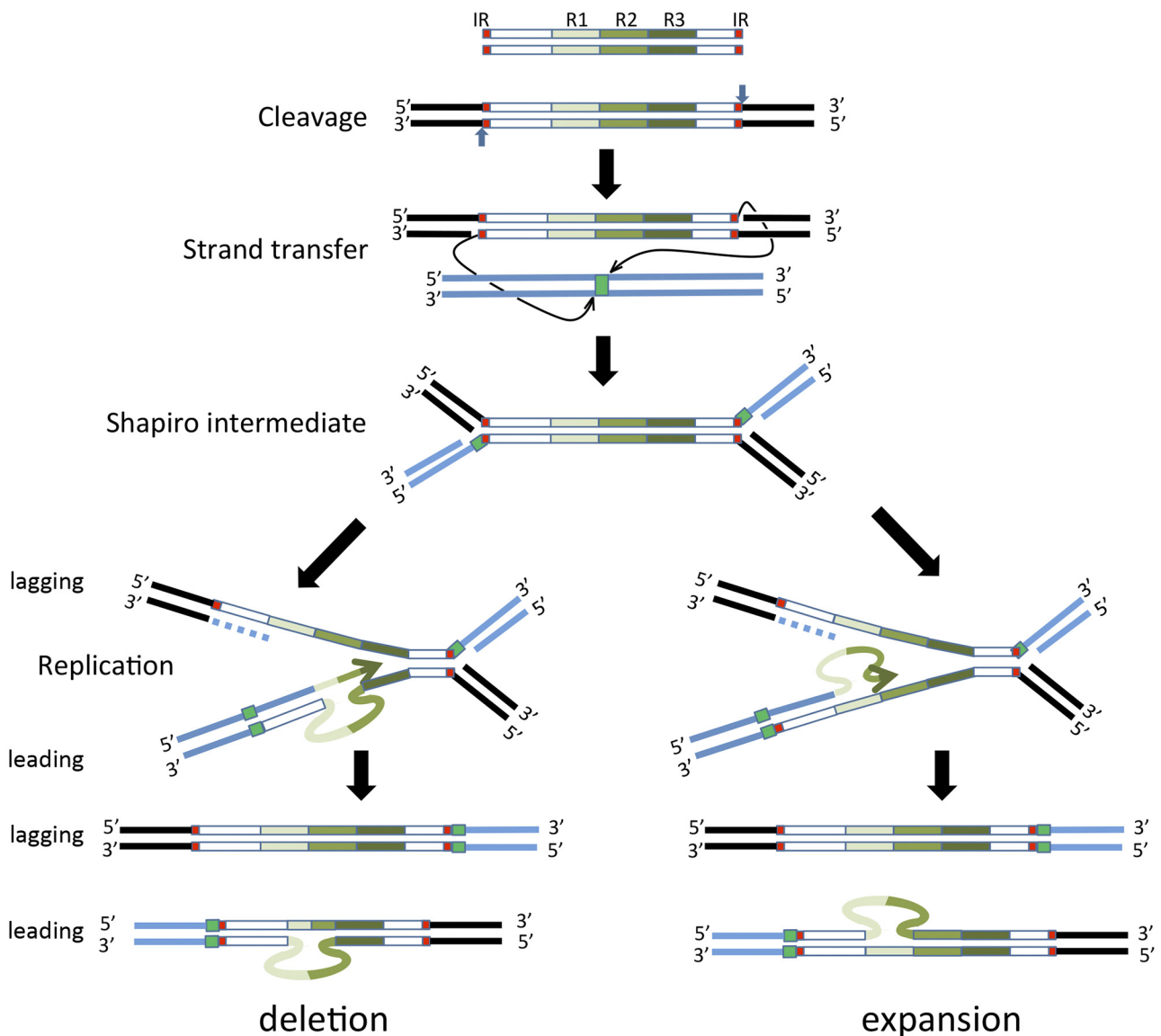


FIG 5 A model for generating variability in the number of TALE gene repeats. The proposed steps of Tn3 family replicative transposition are shown. Top to bottom: double-strand transposon showing the terminal inverted repeats (IRs, red boxes) and three repeat sequences (green scale boxes); the transposon *in situ* in the transposon donor molecule (flanking black bars), indicating the polarity of the two DNA strands and the polarity of cleavage (vertical blue arrows); transfer of the 3' OH strand at each end of the transposon (thin arrows) at staggered positions (green square) into the target molecule (blue bars); formation of the branched Shapiro intermediate with both donor (black bars) and target (blue bars) DNA attached; replication of the intermediate structure showing leading strand synthesis (bottom branch of the fork) and lagging strand synthesis (top branch of the fork); replication fork slippage on the leading strand template (left) resulting in removal of repeat sequences or slippage on the nascent strand (right) resulting in expansion of repeat sequences, which are each resolved in a second round of replication.

Tn*Xax1* or by a cognate transposase from a closely related element (such as IS*Xc4* or Tn*Xca1*). This mechanism may have been responsible for delivering the *pthA4*- and *pthAw*-carrying MICs to *X. citri* subsp. *citri* pathotype A and *X. citri* subsp. *citri* strain A^w 12879, respectively. These effectors are considered central for the development of the citrus canker (43). The *X. oryzae* pv. *oryzicola* strain BLS256 does not have TALE-carrying MICs, and the majority of its TALEs group in a monophyletic clade. Moreover, the lines of evidence presented and the tree topology, shown in Fig. 3, strongly suggest that, after acquisition by transposition, the *X. oryzae* pv. *oryzicola* BSL256 TALEs evolved principally by dupli-

cation rather than by transposition. Although gene duplication is considered one of the major mechanisms through which new genetic material is generated, transposition can promote bacterial evolution in quantum leaps. Since *X. oryzae* pv. *oryzicola* BSL256 is less widespread and causes less severe disease than members of the *X. oryzae* group (3), the absence of MIC-carrying TALEs observed in this strain may have affected its pathogenicity.

Generating T3SE and TALE diversity by transposition. In addition to insertion and genome expansion, the Tn3 family replicative transposition mechanism could itself generate sequence variation in several ways. Reassortment of passenger genes leading

to changes in the T3SE repertoire could occur by “resolution” (site-specific recombination) between alternative *rst* sites from different elements, as shown for Tn4651-related transposons (33). Replicative Tn3 transposition would itself provide an exquisite mechanism for generating TALE diversity. TALE genes include a number of conserved nearly identical tandem repeat sequences that encode the 34-amino-acid repeats, including the RVD site. Changes in the repeat numbers, RVDs, and sequences in the TALE genes might occur by replication slippage between direct repeats and errors during the replicative stage of transposition (Fig. 5) or by unequal crossing-over, as has been proposed for amplification of other repeated DNA segments (48, 49). The example presented in Fig. 5 shows that replication slippage on the template strand (bottom left) would give rise to a reduction in the number of repeat sequences, while slippage on the nascent strand (bottom right) would result in repeat expansion following a second round of replication. Similar activities might occur on the lagging strand and lagging strand template. This mechanism may remodel the bacterial T3SE arsenal, producing novel effectors capable of binding to new DNA motifs to regulate new sets of host genes. Alternatively, such rearrangements may be generated similarly during replication of the host replicons or during conjugative transfer of the plasmid vectors. Experiments are under way to test these hypotheses.

Tn3 family elements from closely related genera. The involvement of Tn3 family elements with virulence and pathogenicity is not an exclusive feature of the *Xanthomonas* genus. Additional Tn3 family transposons identified in other *Gammaproteobacteria*, such as *Pseudomonas aeruginosa* (TnPa43) (50, 51) and *Stenotrophomonas maltophilia* (TnStma1) (52, 53), share TnXax1 IR sequences (GAGGG tips). These are also involved in assisting the bacterial host-related lifestyle, since they also carry genes directly or indirectly involved in pathogenicity and virulence or tolerance of high levels of various toxic metals. *Xanthomonas*, *Pseudomonas*, and *Stenotrophomonas* species are gammaproteobacteria and share a close common ancestor. Lateral gene transfer mediated by these Tn3-like transposable elements may have had an important role in speciation, shaping the life styles of their bacterial hosts. The *Xanthomonas* and *Pseudomonas* genera are composed of several species capable of infecting different plant hosts. Even though more than 200 plant species are hosts for *Xanthomonadales* and *Pseudomonadales* species (54), most *Xanthomonas* and *Pseudomonas* species exhibit a strict host range, resulting in specific combinations of plant-pathogen interactions that result in pathogen infection (or disease). The mobilization of TnXax1 and its relatives and the potential recombination and shuffling of the T3SEs and TALEs in MIC structures are probably major sources of pathogenicity variation in these plant pathogens. However, we would like to point out that, in this study, we have only addressed transposons of a subclass of the Tn3 family, those with IRs whose sequences end in GAGGG. It is possible that some of the many other transposable elements present in some of these xanthomonad genomes are also involved in transmitting pathogenic determinants. Work is under way to determine this.

MATERIALS AND METHODS

Bioinformatics analysis. Insertion sequences were annotated using the ISfinder database (55) and ISSaga tool (56). Global comparisons using sequenced *Xanthomonadales* genomes deposited in the public GenBank repository were made using BLAST (57) with standard parameters, except

that the low-complexity-region filter was deactivated and a word size of 7 was used.

The molecular phylogenetic analysis of the TALE genes was performed using the maximum-likelihood method based on the general time-reversible model (58). The full-length nucleotide sequences of the TALE genes were aligned and edited to maintain the order of the conserved tandem repeats, with no gap inclusion in order to respect the targeted nucleotide sequence in the plant and, therefore, the TALE specificity. The bootstrap consensus tree was inferred with 500 replicates (59). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. Initial trees for the heuristic search were obtained automatically by applying the Neighbor Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (3 categories [+G, parameter = 1.0586]). The analysis involved 122 TALE sequences, including a total of 8,174 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (60).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02505-14/-/DCSupplemental>.

- Figure S1, PDF file, 0.8 MB.
- Table S1, PDF file, 0.1 MB.
- Table S2, PDF file, 0.1 MB.
- Table S3, PDF file, 0.1 MB.
- Table S4, PDF file, 0.1 MB.

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