Characterization of ISXax1, a Novel Insertion Sequence Restricted to Xanthomonas axonopodis pv. phaseoli (Variants fuscans and non-fuscans) and Xanthomonas axonopodis pv. vesicatoria[∇]

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ISXax1 is a novel insertion sequence belonging to the IS256 and Mutator families. Dot blot, Southern blot, and PCR analyses revealed that ISXax1 is restricted to Xanthomonas axonopodis pv. phaseoli (variants fuscans and non-fuscans) and X. axonopodis pv. vesicatoria strains. Directed AFLP also showed that a high degree of polymorphism is associated with ISXax1 insertion in these strains.

Insertion sequences (ISs) are small, mobile, genetic elements that are ubiquitously distributed within bacterial genomes (6, 19). For prokaryotes, IS elements have been classified into about 20 families based on (i) open reading frame organization, (ii) length and similarity of terminal inverted repeat (IR) sequences, (iii) length of target site duplication (direct target repeat [DR]), and (iv) features of the DDE motif, a conserved amino acid motif called the catalytic triad of bacterial transposases (Tpases) (http://www-is.biotoul.fr/is.html) (19, 29). ISs mediate various DNA rearrangements which can lead to diverse phenotypic effects (11). The association between ISs and pathogenic or virulence functions has been frequently observed in plant as well as animal pathogens (9, 13, 14, 25, 36). ISs play an important role in evolution by facilitating horizontal gene transfer between bacterial populations. They contribute significantly to the diversity of bacteria, enhancing the organisms' adaptive and evolutionary capacities (9, 25, 36). ISs can also be used as markers for bacterial species typing and for epidemiological investigations (2, 15, 30, 34).

Here, we report ISXax1, a newly identified insertion sequence in *Xanthomonas axonopodis* pv. phaseoli and its fuscans variant, the causative agents of common bacterial blight of bean (4). Both pathogens have the same host range and similar biochemical phenotypes except that the fuscans variant can produce a melanin-like pigment in culture (8). We also show that ISXax1 is carried by *X. axonopodis* pv. vesicatoria but not by *Xanthomonas vesicatoria*, the two causal agents of the bacterial spot disease of tomato and pepper (26, 32, 33).

Isolation and characterization of ISXax1. A subtractive hybridization performed between the *X. axonopodis* pv. phaseoli variant fuscans CFBP4834 strain (data not shown) and four strains from closely related species (*Xanthomonas campestris* pv. campestris CFBP2350, *Xanthomonas hortorum* pv. pelargonii CFBP2533, and *X. vesicatoria* CFBP1941 and CFBP2537) led to the isolation of a 166-bp DNA fragment (data not shown). A BLAST search (http://www.ncbi.nih.gov/BLAST/) (1) revealed that this subtracted DNA fragment has high sim-

ilarities with IS-like elements. We then performed inverse PCR (24) and directed-AFLP experiments to isolate the flanking sequences of this DNA fragment. To perform directed-AFLP experiments, we digested genomic DNA by EcoRI; the restricted DNA fragments were then ligated to Ecoadapt (an EcoRI-specific, double-stranded adapter resulting from hybridization between Ecoadapt 1 and 2 oligonucleotides [Table 1]) by using T4 DNA ligase and amplified with an either Xpha5-Ecocore or Xpha6-Ecocore pair of primers (Table 1). Both methods allowed us to determine the complete nucleotide sequence of this putative transposase gene. This novel IS element was named ISXax1 according to the IS Finder database (http://www-is.biotoul.fr/is.html).

Based on data from the IS Finder database, several features (size, IRs, and DRs) (Fig. 1) suggest that ISXax1 is a new member of the IS256 family (Table 2). BLAST searches also revealed that ISXax1 shares high similarities with putative Tpase genes classified within the IS256 family. Interestingly, 100% identity was found with a putative Tpase gene (locus XCVc0009; accession no. NC_007506) from *X. axonopodis* pv. vesicatoria strain 85-10 (31). In *X. axonopodis* pv. vesicatoria strain 85-10, this ISXax1 copy is unique and resides in plasmid pXCV38 (31). Additionally, the putative ISXax1 Tpase harbors a DDE motif at its C-terminal region (Fig. 2), matching the consensus sequence determined for the IS256 family (19). Fi-

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TABLE 1. Oligonucleotides used as primers and adapters to amplify ISXax1 sequences and ISXax1 flanking regions

Oligonucleotide	Sequence (5′–3′)	Position on the ISXax1 sequence (5′–3′)
Xpha1	ACC CGC TGG GCC GGC TTC	382–399
Xpha2	CCT GCC ACG CCT TGA CCT C	547–529
Xpha5	GTG TAG TTG ACC AAC AGG CT	128–109
Xpha6	TGG CTG CAA GTC GTG ACG GA	718–736
Xpha10	GGC CAC AGG CAG GAG ACT GCC	28–48
Xpha11	CCT CAG CGG TAA CCC AAA CCA	1302–1282
Ecocore	GAC TGC GTA CCA ATT C	
Ecoadapt1	CTC GTA GAC TGC GTA CC	
Ecoadapt2	P-AAT TGG TAC GCA GTC[a]	

[a] Ecoadapt2 is phosphorylated (P) at the 5′ end.

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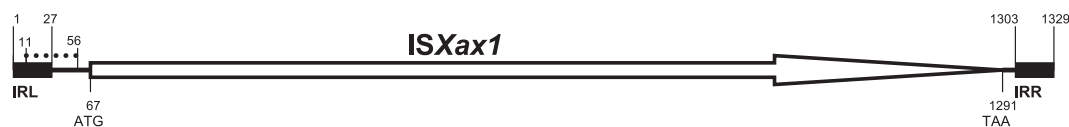
FIG. 1. Schematic representation of the main features of the IS*Xax1* nucleotide sequences in *X. axonopodis* pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria strains. The orientation of the IS*Xax1* gene is shown by an open arrow with the location of the ATG start codon (at position 67) and the TAA stop codon (at position 1291). The dotted line of the IS*Xax1* sequence (nucleotides 11 to 56) indicates the location of a putative promoter. The black boxes represent the positions of the two imperfect, 27-bp, terminal inverted repeats (the left IR [IRL], nucleotides 1 to 27, and the right IR [IRR], nucleotides 1303 to 1329) with five mismatches.

nally, IS*Xax1* can also be classified within the *Mutator* family, consisting of Tpases from eukaryotes and prokaryotes. Indeed, by analyzing the PROSITE database (http://www.expasy.org /prosite/), the signature of the *Mutator* family of Tpases (accession no. PS01007) was found within the IS*Xax1* Tpase sequence (Fig. 3).

The IS*256* family is widely distributed in bacteria since members of this family have been disclosed in *Actinobacteria*, *Firmicutes* (*Clostridia*, *Bacillales*, and *Lactobacillales*), and *Proteobacteria* (*Alpha-*, *Beta-*, and *Gammaproteobacteria*) (29). However, the description of IS*Xax1* is original as it appears that this IS element is the first characterized member of the IS*256* family in *Xanthomonas* strains. Do genomes of *X. axonopodis* pv. phaseoli and its variant fuscans harbor other IS elements belonging to the IS*256* family? Strikingly, among the plant-pathogenic bacteria whose genomes have been sequenced completely, *Pseudomonas savastanoi* pv. phaseolicola, a pathogen of bean, as are *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans, is the unique bacterium harboring many IS elements belonging to the IS*256* family (12). The forthcoming sequencing of the genome of our model strain, *Xapf*CFBP4834, will help us to speculate on the role played by this IS family in the evolution of bacteria and, in particular, of bean pathogens.

IS*Xax1* distribution in *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans strains. We studied the distribution of IS*Xax1* within a worldwide collection of both *X. axonopodis* pv. phaseoli (23 strains) and *X. axonopodis* pv. phaseoli variant fuscans (64 strains) by directed AFLP. IS*Xax1* was present in all tested strains (Fig. 4; data not shown), suggesting that IS*Xax1* has evolved in genomes of both *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans for a long time. This result is supported by the fact that the G+C content of IS*Xax1* (62.3 mol%) is similar to the average value of total DNA for *X. axonopodis* pv. phaseoli (~65 mol%) (32) and that the codon usage in IS*Xax1* and its

hosts (*X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans) does not differ (our unpublished data). A high polymorphism was revealed since the copy number ranged from 1 to 7 (confirmed by Southern blot hybridization) (Fig. 4), and 26 different IS*Xax1* insertional profiles were observed. No DNA fragment was common to all tested *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans strains, and bands shared among different patterns were rare. This high polymorphism of insertion might reflect the ability of IS*Xax1* to insert into many different sites in genomes of both *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans. Fifteen IS*Xax1* insertion profiles were generated by *X. axonopodis* pv. phaseoli strains, whereas only 11 profiles were obtained with *X. axonopodis* pv. phaseoli variant fuscans strains. This result confirms that the non-fuscans variant is more heterogeneous than the fuscans variant is (3, 21). Moreover, as identical IS*Xax1* profiles can result from strains isolated in distant countries, our approach confirms that the diversity observed in both *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans was not geographically structured for either of the two pathogens (20, 21). This observation is not surprising since *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans are both seedborne pathogens, and their dissemination through contaminated seeds is well known (27, 35). The lack of geographical differentiation has important practical implications, as available host resistance genes are likely to be effective for managing the disease in diverse geographical areas (20). Interestingly, no common IS*Xax1* insertion profile was shared by both pathogens. Thus, our study provides further data to show that *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans are genetically distinct. Indeed, our results are in complete agreement with the following previously used

TABLE 2. Main IS*Xax1* features compared to those of IS*256* family members[a]

Feature	Value for:	
	IS*Xax1*	IS*256* family
Size	1,329 bp	1,300 to 1,500 bp
No. of ORFs	1 (408 aa)	1
Size of inverted terminal repeats (IRs)	27 bp	24 to 41 bp
Size of direct target repeats (DRs)	8 bp	8 or 9 bp
Conserved terminal base pair 5′ to 3′[b]	GG	G(g/a)

[a] Data are derived from IS Finder database (http://www-is.biotoul.fr/is.html).
[b] Uppercase letters refer to mostly (and often) conserved bases. Lowercase letters separated by slashes indicate alternative conservation at that position.
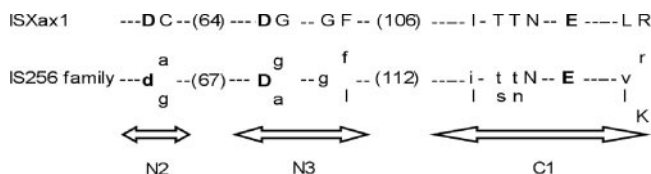

FIG. 2. Alignment of the IS*Xax1* DDE motif and the corresponding consensus sequence of the IS*256* family (IS Finder database [http://www-is.biotoul.fr/is.html]). Highly conserved amino acids are shown in bold letters. For the IS*256* family, uppercase letters indicate conservation within the family and lowercase letters indicate predominant amino acids. The numbers of amino acids between the motif regions are shown in parentheses. The N2, N3, and C1 domains containing the conserved DDE amino acids are also shown (19). The spacing between the three amino acids forming the DDE catalytic triad is variable depending upon IS*256* family members (19).

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ISXax1   (aa 239-263)   DCLKGFEAIEAVFPQTVQLCIVH
IS6120   (aa 131-155)   DCALGFKAVREVFPAKEQRCWFH
IS1201   (aa 228-252)   DCVVGMTAIAKTYPQHFQRCLVH
IS1081   (aa 233-257)   DAHAGLAAIGATLPAAWQRCRTH
IST2     (aa 116-140)   DCAMGFAAIDEAYPEGQQRCWVH
ISRm5    (aa 231-255)   DCLKGFEAITAVFPQIVQTCIVH
IS905    (aa 228-252)   DCFKGLQITSQAYPLKQQRCLIH
ISRm3    (aa 230-254)   DDHAGLAAIGEVIPEAWQRCYVH
IS406    (aa 232-256)   DDHAGLAARRAVLPSPWQRCQFH
IS256    (aa 233-257)   DAHKGLSAIRKSFTNSWQRCQVH
consensus                Dg G  ai  fp  QrC vH
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FIG. 3. Alignment of protein sequences of 10 IS elements (belonging to the IS256 family according to the IS Finder database [http://www-is.biotoul.fr/is.html]) which shows the signature of the *Mutator* family of transposases (PROSITE database [http://www.expasy.ch/cgi-bin/nicesite.pl?PS01007]). The bacterial origin and the accession number for each insertion sequence are as follows: for ISXax1 (*Xanthomonas axonopodis* pv. phaseoli and variant fuscans), AY935340; for IS6120 (*Mycobacterium smegmatis*), P35883; for IS1201 (*Lactobacillus helveticus*), P35880; for IS1081 (*Mycobacterium bovis*), P60231; for IST2 (*Acidithiobacillus ferrooxidans*), P35884; for ISRm5 (*Sinorhizobium meliloti*), Q52873; for IS905 (*Lactococcus lactis*), P35881; for ISRm3 (*Sinorhizobium meliloti*), P80011; for IS406 (*Burkholderia cepacia*), P24575; and for IS256 (*Staphylococcus aureus*), P19775. The location of the signature within each protein sequence is shown in parentheses. Black and gray backgrounds indicate identical and similar amino acids, respectively. The consensus protein sequence is shown below the alignment. This alignment was made by BOXSHADE software, version 1.6.6 (http://www.ch.embnet.org/software/BOX_form.html).

methods: DNA-DNA hybridization (10), random amplified polymorphic DNA (3, 18), repetitive-element PCR (18, 20, 21), pulsed-field gel electrophoresis (5), restriction fragment length polymorphism analyses using *hrp* and pectate lyase genes as probes (5), and PCR-restriction fragment length polymorphism of the ribosomal genes (20).

ISXax1 is restricted to *X. axonopodis* pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria strains. To investigate whether ISXax1 is widespread in bacteria, further dot blot and Southern blot hybridizations (28) were performed on a large collection of strains belonging to *X. axonopodis* pathovars and other bacterial species (data not shown). No fragments homologous to ISXax1 were detected in any of the strains tested except *X. axonopodis* pv. vesicatoria strains (Fig. 4; data not shown). This result was confirmed by PCR with primer pairs Xpha1-Xpha2 and Xpha10-Xpha11 (Table 1), allowing the amplification of internal ISXax1 DNA fragments from all 13 *X. axonopodis* pv. vesicatoria strains tested, originating from different countries (data not shown). A polymorphism of the ISXax1 insertion within *X. axonopodis* pv. vesicatoria strains was shown by directed AFLP (Fig. 4).

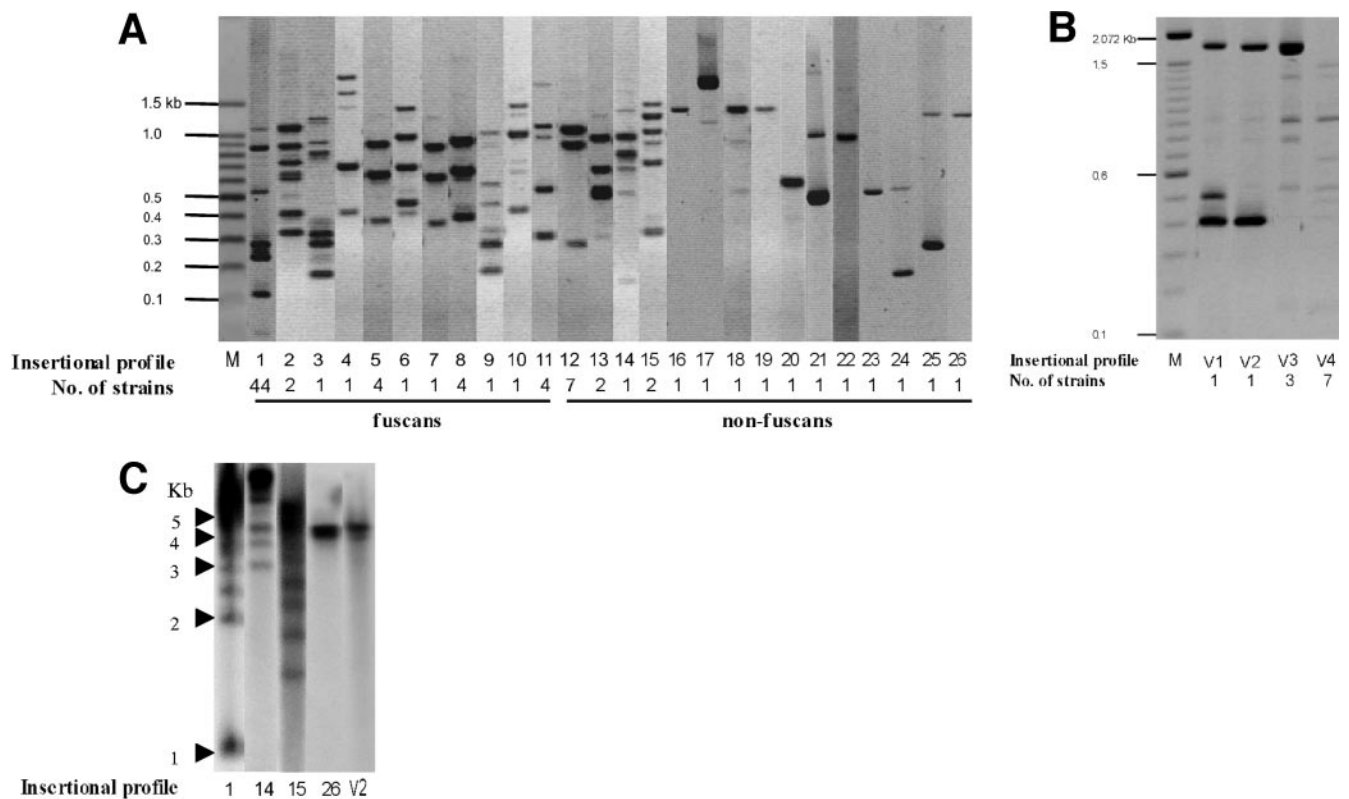Finally, the fact that ISXax1 is carried by only three taxa (*X.*

FIG. 4. Representation of the different insertional profiles obtained by directed AFLP (A and B) or by Southern blot hybridization (C) (28). (A and B) Genomic DNA was first digested by EcoRI, which does not cut within ISXax1, and the restricted fragments were then ligated to Ecoadapt (an EcoRI-specific, double-stranded adapter resulting from hybridization between Ecoadapt 1 and 2 oligonucleotides [Table 1]) by using T4 DNA ligase and amplified with the Xpha-Ecocore pair of primers (Table 1). (C) Genomic DNA was digested by EcoRI, which does not cut within ISXax1, and the restricted fragments were hybridized with the ISXax1 probe (generated by using Xpha10 and Xpha11 primers [Table 1]). DNAs are from (A) 64 *X. axonopodis* pv. phaseoli variant fuscans strains and 23 *X. axonopodis* pv. phaseoli strains and from (B) 12 *X. axonopodis* pv. vesicatoria strains. Profiles 1 to 11 belong to the fuscans strains of *X. axonopodis* pv. phaseoli, and those from 12 to 26 belong to the non-fuscans strains. Profiles V1 to V4 were generated by *X. axonopodis* pv. vesicatoria strains. The number of strains sharing the same insertional profile is indicated below each profile number. M corresponds to the molecular mass marker.

axonopodis pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria) belonging to the same species (*X. axonopodis*) may suggest that IS*Xax1* was already present in *X. axonopodis* strains before host specialization occurred. However, IS*Xax1* is not present in *X. axonopodis* pathovars, such as *X. axonopodis* pv. aurantifolii, *X. axonopodis* pv. citrumelo, *X. axonopodis* pv. dieffenbachiae, or *X. axonopodis* pv. manihotis, which belong to the same DNA-DNA homology, AFLP, or repetitive-element PCR groups (26, 33). So, IS*Xax1* would have been lost in certain *X. axonopodis* pathovars during evolution. Another explanation could be that *X. axonopodis* pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria acquired IS*Xax1* from an unrelated bacterial species through a horizontal gene transfer event, and then IS*Xax1* was transmitted vertically within *X. axonopodis* pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria. This last hypothesis is supported by recent observations in *Gammaproteobacteria* (including *Xanthomonas* strains). Analyses of *Xanthomonas* genomes clearly show that these bacteria have been subjected to numerous horizontal gene transfers during evolution, sometimes from phylogenetically distant organisms (7, 17). Moreover, gene acquisition is considered to be a major factor contributing to the genomic diversity of these bacteria but, paradoxically, once acquired, these genes are rarely transferred among lineages (16).

What could be the contribution of IS*Xax1* in *X. axonopodis* pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria genomes? We tried to answer this question by analyzing the flanking sequences of eight independent IS*Xax1* insertions in strains *Xapf*CFBP4834, *Xap*CFBP6991, and *Xap*CFBP6994. We showed that IS*Xax1* is inserted into noncoding regions (except for one copy in strain *Xapf*CFBP4834 inserted into the *aceK* homolog, encoding an isocitrate dehydrogenase kinase/phosphatase, *X. axonopodis* pv. vesicatoria [accession no. CAJ25689]). We also observed that the locations of these IS*Xax1* insertions seem variable from strain to strain (Fig. 4; data not shown). These results suggest that this IS element is likely not involved in the host specificity or pathogenicity of *X. axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli variant fuscans. It has been shown that *Xanthomonas* genomes were invaded by numerous IS elements since more than 100 transposase genes were disclosed in each genome except those in *X. axonopodis* pv. vesicatoria (31). An interesting feature is that these genomes carry distinctive sets of transposable elements; some of these IS elements are shared by all *Xanthomonas* genomes, whereas others exhibit more restricted distributions (22, 23). Therefore, we suggest that IS*Xax1* might be, as are other IS elements in *Xanthomonas* (22, 23), an important driver of the differentiations and evolutions of *X. axonopodis* pv. phaseoli, *X. axonopodis* pv. phaseoli variant fuscans, and *X. axonopodis* pv. vesicatoria genomes.

Nucleotide sequence accession number. The sequence for the novel IS element was deposited in GenBank under accession no. AY935340.

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