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Comparative Genomic Analysis of *Xanthomonas axonopodis* pv. citrumelo F1, Which Causes Citrus Bacterial Spot Disease, and Related Strains Provides Insights into Virulence and Host Specificity▽#

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Xanthomonas axonopodis* pv. citrumelo is a citrus pathogen causing citrus bacterial spot disease that is geographically restricted within the state of Florida. Illumina, 454 sequencing, and optical mapping were used to obtain a complete genome sequence of *X. axonopodis* pv. citrumelo strain F1, 4.9 Mb in size. The strain lacks plasmids, in contrast to other citrus *Xanthomonas* pathogens. Phylogenetic analysis revealed that this pathogen is very close to the tomato bacterial spot pathogen *X. campestris* pv. vesicatoria 85-10, with a completely different host range. We also compared *X. axonopodis* pv. citrumelo to the genome of citrus canker pathogen *X. axonopodis* pv. citri 306. Comparative genomic analysis showed differences in several gene clusters, like those for type III effectors, the type IV secretion system, lipopolysaccharide synthesis, and others. In addition to *pthA*, effectors such as *xopE3, xopAI*, and *hrpW* were absent from *X. axonopodis* pv. citrumelo while present in *X. axonopodis* pv. citri. These effectors might be responsible for survival and the low virulence of this pathogen on citrus compared to that of *X. axonopodis* pv. citri. We also identified unique effectors in *X. axonopodis* pv. citrumelo that may be related to the different host range as compared to that of *X. axonopodis* pv. citri. *X. axonopodis* pv. citrumelo also lacks various genes, such as *syrE1, syrE2*, and RTX toxin family genes, which were present in *X. axonopodis* pv. citri. These may be associated with the distinct virulences of *X. axonopodis* pv. citrumelo and *X. axonopodis* pv. citri. Comparison of the complete genome sequence of *X. axonopodis* pv. citrumelo to those of *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria provides valuable insights into the mechanism of bacterial virulence and host specificity.

Xanthomonas is an important genus of plant pathogenic bacteria (87). These Gram-negative rod-shaped pathogens belong to the class *Gammaproteobacteria* and can infect over 350 species of plants (12). Among the diseases on citrus, citrus bacterial canker (CBC) and citrus bacterial spot (CBS) are caused by distinct pathovars of *Xanthomonas* species. Citrus canker is caused by several pathogenic variants of *X. axonopodis* pv. citri (synonyms *Xanthomonas citri*, *Xanthomonas campestris* pv. citri, or *Xanthomonas citri* subsp. citri) (90, 109), whereas CBS is caused by *X. axonopodis* pv. citrumelo. *X. axonopodis* pv. citri strain 306 with a suspected origin in southeastern Asia causes Asiatic type A canker and is the most widespread and virulent form of CBC. It produces hyperplasic and hypertrophic (corky) lesions surrounded by oily or water-soaked margins and a yellow halo on leaves, stems, and fruits.

In 1984, a disease similar to citrus canker was discovered in citrus nurseries in central Florida, leading to the destruction of millions of seedlings (98). This new form of citrus canker was described as the "E-strain" group, also known as nursery strain canker. Leaf spots of this strain are irregular to round, 3 to 5 mm in diameter, flat, water soaked, often necrotic in the center, and usually surrounded by a chlorotic halo. Water-soaked elongate lesions with necrotic centers are also observed on twigs but not on fruits (16). Extensive efforts were put forth to eradicate this outbreak, resulting in the destruction of 20 million citrus plants at the cost of $94 million (91).

Strains in this group do not cause hyperplasia and the lesions continue to be flat with time, unlike CBC, which results in raised callus-like lesions. Further research revealed that the CBS pathogen is variable but widely distributed in the state and does not have the same host range as the Asiatic strain (34). CBS bacteria are most aggressive on trifoliate orange hybrids, including Swingle citrumelo (36). Populations of CBS bacteria decreased or varied in leaves of grapefruit (92). The origin of the strain remains unknown as it is not found outside Florida, and it is speculated to have moved to citrus from existing populations of *Xanthomonas* in Florida (33). Further research showed that these strains are serologically, geneti-

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cally, and physiologically distinct from the previously known citrus canker pathogenic groups (35) and are not susceptible to any of the phages commonly used to differentiate these groups (37). Because of the canker-like symptoms it caused, the disease was wrongly termed as E-strain citrus canker (32). The disease is now recognized as distinct from citrus canker and was named citrus bacterial spot, caused by *Xanthomonas axonopodis* pv. citrumelo (25). Other names associated with the bacteria include *X. campestris* pv. citrumelo and *X. alfalfae* pv. citrumelonis (31, 90). The nomenclature and classification for the strains of *Xanthomonas* that infect citrus have undergone extensive taxonomic revision in recent years and are still under debate (90, 110). Hence, in this report we chose to use classical nomenclature and address the CBS pathogen as *Xanthomonas axonopodis* pv. citrumelo, as approved by *Bergey's Manual of Systematic Bacteriology* (88).

Compared to *X. axonopodis* pv. citri, *X. axonopodis* pv. citrumelo has much reduced pathogenicity with limited host range. The host range of *X. axonopodis* pv. citri is broad, including most commercial citrus varieties, while *X. axonopodis* pv. citrumelo does not infect any commercial citrus varieties and is limited primarily to trifoliate orange, its hybrids, and a few other individual species (37). Citrus canker is endemic in Florida in most citrus-producing areas, while CBS occurs almost exclusively in nurseries, where young, susceptible tissue is abundant and irrigation is frequent (107). In field, greenhouse, and growth chamber, *X. axonopodis* pv. citrumelo generally does not cause disease when applied as a spray. Bacterial populations of CBS strains within lesions on most hosts except Swingle citrumelo decline rapidly with time (21).

In comparison with *X. axonopodis* pv. citri, the *X. axonopodis* pv. citrumelo strains isolated from Florida nurseries were found to vary widely in aggressiveness from each other (36). Restriction fragment length polymorphism analysis by Hartung and Civerolo (45) showed that *X. axonopodis* pv. citrumelo strains are not very closely related to *X. axonopodis* pv. citri and that they are not a form of canker. This was further corroborated by comparison of *X. axonopodis* pv. citrumelo strains with other xanthomonads with DNA-DNA hybridization, which showed that *X. axonopodis* pv. citrumelo is only about 60% similar to *X. axonopodis* pv. citri (21). Later, Cubero and Graham deduced that *X. axonopodis* pv. citrumelo is much more closely related to *X. campestris* pv. vesicatoria than to *X. axonopodis* pv. citri by using 16S rRNA gene analysis (16) as well as leucine-responsive regulatory protein gene analysis (15).

The mechanism accounting for the reduced pathogenicity and limited host range of *X. axonopodis* pv. citrumelo compared to *X. axonopodis* pv. citri remains unknown. To address this question, comparative genomic study was conducted in this present research by completing the genome sequence of *X. axonopodis* pv. citrumelo strain F1 (36). In comparison with *X. axonopodis* pv. citri, *X. axonopodis* pv. citrumelo is a genetically, pathogenically, and serologically diverse pathogen (4, 29, 36, 45). We decided to sequence *X. axonopodis* pv. citrumelo F1, which is a highly aggressive citrus bacterial spot bacterial strain and thus is more likely to infect citrus nurseries as a pathogen. To gain a better understanding of the ecological and evolutionary relationships between strains and species of *Xanthomonas*, we also compared *X. axonopodis* pv. citrumelo with the closely related *X. campestris* pv. vesicatoria strain 85-10 (syn. *X. euvesicatoria* or *X. axonopodis* pv. vesicatoria), which causes bacterial spot on tomato and pepper (64, 87, 109).

MATERIALS AND METHODS

Bacterial strain and DNA sequencing. The *X. axonopodis* pv. citrumelo strain F1 sequenced in this study was isolated from Avon Park, FL, in 1984. Genomic DNA was extracted from bacterial culture grown overnight at 28°C in nutrient broth medium by using a Wizard DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Quantity and quality of the DNA were measured spectrophotometrically (Nanodrop ND-1000; NanoDrop Tech. Inc., Wilmington, DE). Whole-genome sequencing was performed using two high-throughput sequencing techniques, 454 pyrosequencing and Illumina Solexa GA sequencing. Single and paired-end reads were generated on a 454 GS-FLX Titanium sequencer (454 Life Sciences, Branford, CT) in accordance with the manufacturer's protocol at the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida. Paired-end Illumina sequence reads were obtained using Illumina genome analyzer IIx (Illumina, Hayward, CA) at the Yale University Center for Genomics and Proteomics.

A total of 367,109 high-quality sequences with an average read length of 332 bp, representing more than 21-fold genome coverage, were obtained by 454 FLX sequencing. These sequences were assembled into contigs and scaffolds using the 454 *de novo* assembler Newbler 2.0. In total, 72 contigs were generated, of which 61 contigs were larger than 500 bp. The average size of the large contigs was 81 kb. These contigs were further grouped into five scaffolds based on paired-end reads. The maximum size of the scaffolds was 2,559,303 bases with an average of 990,948 bp. Solexa sequencing generated a total of 37,695,118 high-quality filtered sequence reads with an average read length of 74 bp. Average coverage was more than 400-fold. All reads were *de novo* assembled using CLCbio Genomics Workbench version 4.0, length fraction and similarity were set at 0.9, and all the other parameters were set as default values. This yielded 1,350 contigs (length weighted median N_{50} = 8,322; maximum length = 36,202; minimum length = 102).

Gap closure and assembly validation. The 1,350 contigs obtained from Illumina were used to confirm the assembly of 454 scaffolds. The Illumina contigs were aligned against the 454 scaffolds by using BLASTn to confirm the orientations and integrity of the assembled sequences and to close gaps and link contigs together within the scaffold. A *de novo* BamHI optical map of the genome of *X. axonopodis* pv. citrumelo was generated by OpGen technologies (Madison, WI). *In silico* BamHI restriction maps of the 5 scaffolds were constructed and aligned to the optical map according to their restriction fragment pattern by using MapSolver v.3.1 software (OpGen Technologies, Inc.). PCR primers (available upon request) were designed and Sanger sequences of these PCR products were used to close the gaps between the scaffolds. Final assembly was correlated with the optical map for further validation.

Annotation and curation. Coding genes were identified using Softberry's FgenesB suite of bacterial operon- and gene-finding programs, based on the Markov chain model prediction algorithm at ICBR, UF (108). Predicted proteins were annotated by similarity searches against the NCBI nonredundant (nr) protein database (http://ncbi.nlm.nih.gov) and the clusters of orthologous groups (COG) database. A function was assigned to a predicted gene if it met the criteria of a minimum cutoff of 50% identity and 80% coverage of the gene length. In a few cases, additional putative protein-coding genes were annotated by direct homology search at the nr protein database using BLASTp. Each gene was also functionally classified by assigning a COG number. The rRNA genes were annotated by the FgenesB tool based on sequence conservation, while tRNA genes were detected with the tRNAscan-SE program (63). Insertion sequences were identified by submitting the whole genome to the IS Finder website (95). The CGView Server was used to generate graphical views of the genome (38). The results of the automated annotation were examined and curated manually using the JGI GenePRIMP pipeline (77).

Phylogenetic analysis. To determine the position of *X. axonopodis* pv. citrumelo within the evolutionary precinct of *Xanthomonas* pathovars, we used protein sequences of nine housekeeping genes, *uvrD, secA, carA, recA, groEL, dnaK, atpD, gyrB*, and *infB*, from 10 completely sequenced *Xanthomonas* spp. We also added sequences from three *Xylella fastidiosa* strains and three *Pseudomonas* spp. as well as from two *Stenotrophomonas maltophilia* strains. The sequences of *Ralstonia solanacearum* strains GMI1000 and PSI07 and *Burkholderia cenocepacia* strain NCTC 10247 were used as the outgroup species. Amino acid sequences of nine proteins from the above genomes were aligned using Clustal W (59), and the resulting alignments were concatenated. A phylogenic tree from concatenated genes was constructed using PAUP 4.0 (100) by the maximum likelihood method. The percentages of replicate trees in which the associated taxa clustered

together in the bootstrap test (1,000 replicates) are shown next to the branches in the tree.

Comparative analysis. For comparative analyses, the sequences of *X. campestris* pv. vesicatoria strain 85-10 (GenBank accession no. NC_007508) and *X. axonopodis* pv. citri strain 306 (GenBank accession no. NC_003919), which were determined as the closest relatives to *X. campestris* pv. citrumelo F1 in BLAST analyses as well as phylogenetic analysis, were retrieved from GenBank. Complete genome sequences of all the three *Xanthomonas* spp. and also specific regions were aligned and visualized in progressive mode using MAUVE (17). Pangenome analysis that included the core genome shared by all the three strains was done by an "all-against-all" BLAST of the protein sequences of the above genomes. The genes aligned based on amino acid sequence were considered orthologous if reciprocal BLASTp hits were found between two genes with *e* values of $\leq 10^{-20}$ and alignments exceeding 80% sequence identity and 80% query gene length. A gene was considered a singleton or unique to each strain if it had no hits with an *e* value of $\leq 10^{-5}$.

Additional sequence analysis. Candidate type III secretion system (T3SS) effectors were identified using both nucleotide and protein blasts by comparison to the *Xanthomonas* effector database (http://www.xanthomonas.org). Putative perfect and imperfect PIP box sequences TTCGC-N_{15}-TTCGC and TTCGC-N_{16}-TTCG, respectively, were identified using custom scripts (23). Genomic regions with atypical G+C content were identified using the web-based software GC-profile. It uses a suite of segmentation programs to identify regions with differential G+C content in the genome (26).

Pectate lyase assay. Cultures were grown on nutrient agar at 28°C and then suspended in sterile deionized water to an optical density at 540 nm of 0.3. Hildebrand's media A, B, and C were used to test for pectolytic activity (47). In short, the medium contained bromothymol blue dye, calcium chloride, 2% sodium polypectate, and 0.4% agar. The pH was adjusted to 4.5, 7.0, and 8.5 for media A, B, and C. One-microliter portions of the cultures were inoculated onto the plates and incubated at 28°C for 6 days before pitting was confirmed due to pectate lyase production.

Nucleotide sequence accession number. The complete genome of *Xanthomonas axonopodis* pv. citrumelo strain F1 has been deposited at EMBL/DDBJ/GenBank under the accession number XACM_F1.sqn XACM CP002914.

RESULTS AND DISCUSSION

Sequencing and general features of the genome. *X. axonopodis* pv. citrumelo was sequenced using 454 GS-FLX pyrosequencing (both unpaired and paired end) (65) and paired-end Illumina/Solexa sequencing (8). The reads from 454 and Illumina were assembled into contigs separately, an overview of which is presented in Table S1 in the supplemental material. Although the genome coverage obtained through Illumina was much higher than that obtained through 454, the longer GS-FLX reads resulted in a much better assembly of contigs. The 72 contigs obtained were aligned in the right order to obtain 5 scaffolds by using the paired-end reads. Aligning 1,350 Illumina contigs and using the ones overlapping the 454 contigs solved most gaps within scaffolds. Pyrosequencing has a higher error rate around homopolymers (48), resulting in insertion-deletion errors in assembly and thus in-frame stop codons in genes. Illumina data, on the other hand, have errors mainly due to mismatches (19). Hence, they were used to correct the errors in the scaffold sequences by mapping the Illumina reads against the 454 consensus sequences using CLCbio Genomics Workbench version 4.0 (6).

After all these intensive and time-consuming efforts, the assembly still contained 5 scaffolds with internal gaps, which were difficult to resolve due to repeat regions. Thus, optical mapping was used to obtain a *de novo* BamHI restriction map with no requirement for previous sequence information (60). The *in silico* restriction maps of scaffolds were aligned to this structural map to reveal the correct alignment and orientation of all the contigs as shown in Fig. 1A. The genome was completely closed by primer walking and validated by manually inspecting all areas of imperfect match between the optical map and the sequence assembly (Fig. 1B). The genome sequence was further corroborated either by high coverage with the 454 and Illumina data or by resequencing of the region (data not shown).

X. axonopodis pv. citrumelo strain F1 has a single, circular chromosome of 4,967,469 bp (Fig. 2) with no plasmids. Details of the general features of the genome are shown in Table 1. The G+C content of the chromosome averages 64.92%, which is similar to that of other *Xanthomonas* genomes. The chromosome displays a clear GC skew transition typical of prokaryotic genomes, indicative of a bidirectional replication mechanism (82). GC skew analysis and blast comparison were used to locate the origin of replication at the point with an excess of G over C corresponding to the beginning of the leading strand, and *dnaA* was the first of the coding sequences (CDSs) of the genome.

The *X. axonopodis* pv. citrumelo genome contains 4,202 putative CDSs and 60 structural RNAs (Table 1). The genome shows a coding density of 86.53%, characteristic of most xanthomonads. There is no asymmetry in the distribution of the CDSs on the chromosome between the leading-strand 2,131 (50%) and the lagging-strand 2,131 (50%). After FgenesB annotation and manual curation, 3,481 CDSs (82.42%) could be assigned to one or more COG functional classes (see Table S2 in the supplemental material), whereas there was not enough evidence for 721 CDSs to be assigned to any COG category. Two sets of 5S-16S-23S rRNA, clustered in operons, were found located in a region of approximately 500 kb (between bp 4379256 and bp 4847563) on the left replichore. A total of 54 tRNA genes with specificities for all 20 amino acids were also identified.

Phylogenetic relatedness of *X. axonopodis* pv. citrumelo to other xanthomonads. To establish the phylogenetic relationship of *X. axonopodis* pv. citrumelo strain F1 with respect to other selected members of completely sequenced *Xanthomonas*, we compared a set of nine housekeeping genes. These genes are highly conserved and show no evidence of horizontal transfer among the 10 xanthomonads as well as the other plant pathogens sequenced. These genes have provided robust analysis and resolved evolutionary relationships reliably in other studies (14). For this analysis, we focused on bacteria with complete genomes and excluded draft genomes due to their limitations (75). We created an alignment of the nine proteins, concatenated the sequences, and reconstructed the phylogenetic tree by using the maximum likelihood method (Fig. 3). The phylogenetic tree indicates that *X. axonopodis* pv. citrumelo strain F1 groups most closely with *X. campestris* pv. vesicatoria and *Xanthomonas axonopodis* pv. citri, forming a clade distinct from other xanthomonads. The closest relative of *X. axonopodis* pv. citrumelo is *X. campestris* pv. vesicatoria 85-10, which causes bacterial spot disease in tomato and pepper (105). This is consistent with the results obtained by comparison of optical maps of the three chromosomes (Fig. 1C). *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria appear to be separated from *X. axonopodis* pv. citri, which is included in this cluster; this is supported by a good bootstrap value of 100 at this node. However, the relationship with *X. axonopodis* pv. citri is sufficiently close that they share nucle-

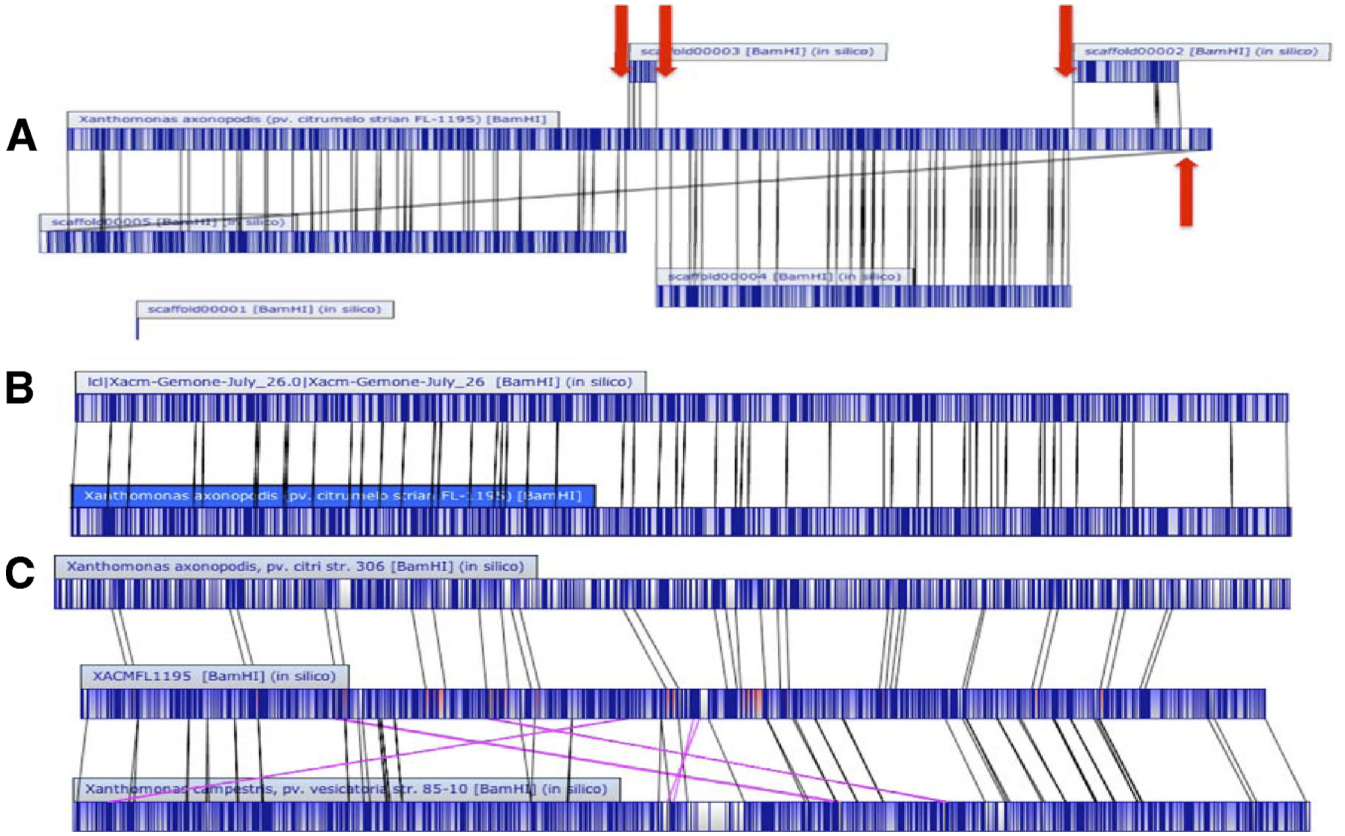

FIG. 1. Alignments between the whole-genome optical maps and the *in silico* genome sequence assemblies at various stages of the project. Dark blue represents cut sites, light blue regions indicate alignment, and white regions indicate no alignment. (A) Early comparison of an optical map derived from BamHI digestion of the *X. axonopodis* pv. citrumelo F1 genome to the assembled scaffolds generated by sequencing. The *X. axonopodis* pv. citrumelo optical map derived from BamHI digestion of the chromosome is presented as a single contig in the center. The sequenced genome contains five scaffolds that have a corresponding match to the optical map. Scaffold 1 is too small to be mapped using current optical map technology. However, during gap closure it was placed between contigs 3 and 4. The finishing strategy including gap closure was simplified using the optical map as an assembly model. Red arrows indicate where PCR gap closure was done. (B) Comparison of the final assembly of the *X. axonopodis* pv. citrumelo genome (top) to the optical map (bottom) for the BamHI digestion. (C) Comparison of the finished sequence of *X. axonopodis* pv. citrumelo (center) to the BamHI optical map of *X. axonopodis* pv. citri strain 306 (top) and *X. campestris* pv. vesicatoria strain 85-10 (bottom). Dark blue represents cut sites, light blue represents aligned regions, red represents regions aligning to both sequences, and white represents unaligned regions. Alignment lines for inversions and translocations are highlighted in pink. Inverted and translocated regions are highlighted in yellow.

otide sequence identity of over 98% in most conserved regions. Interestingly, *X. axonopodis* pv. citrumelo F1 has been shown to be 56% and 58% similar to *X. axonopodis* pv. citri A strain 9771 and *X. campestris* pv. vesicatoria strain 58, respectively, by DNA-DNA hybridization analysis (21). It is noteworthy that other strains of *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria were used in that comparison rather than the sequenced *X. axonopodis* pv. citri A strain 306 and *X. campestris* pv. vesicatoria strain 85-10. Interestingly, Gent and colleagues (29) had shown that the pathovars of citrumelo are indistinguishable from a few other *X. axonopodis* pathovars and that they do not form a monophyletic cluster by repetitive element PCR. However, *X. axonopodis* pv. citrumelo F1 sequenced here is an aggressive strain which was not included in the previous study.

Comparison of chromosome organization of *X. axonopodis* pv. citrumelo to that of *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. The chromosome organization of *X. axonopodis* pv. citrumelo was compared with that of two closely related strains, *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria, by using MAUVE in progressive mode. Though most of the genome is colinear, *X. axonopodis* pv. citrumelo harbors some translocations and inversions around the replication terminus of the chromosome (Fig. 4). The unequal replichores might have been due to this reorganization. The *X. axonopodis* pv. citrumelo genome has one major inversion with a translocation and two major deletions compared to *X. axonopodis* pv. citri, whereas there are three inversions with translocations and two major deletions compared to *X. campestris* pv. vesicatoria. Many of the rearranged and deleted blocks were flanked by transposons and/or integrases, indicating that this rearrangement may be a result of horizontal gene transfer (HGT).

The genome of *X. axonopodis* pv. citrumelo does not harbor any plasmid. In contrast, *X. axonopodis* pv. citri contains two (pXAC33 and pXAC64) and *X. campestris* pv. vesicatoria contains four (pXCV2, pXCV19, pXCV38, and pXCV183) plasmids. Plasmids of xanthomonads have been reported to play important roles in pathogenicity. Plasmid pXAC64 of *X. axonopodis* pv. citri carries the *pthA4* gene, a homolog of *pthA*,

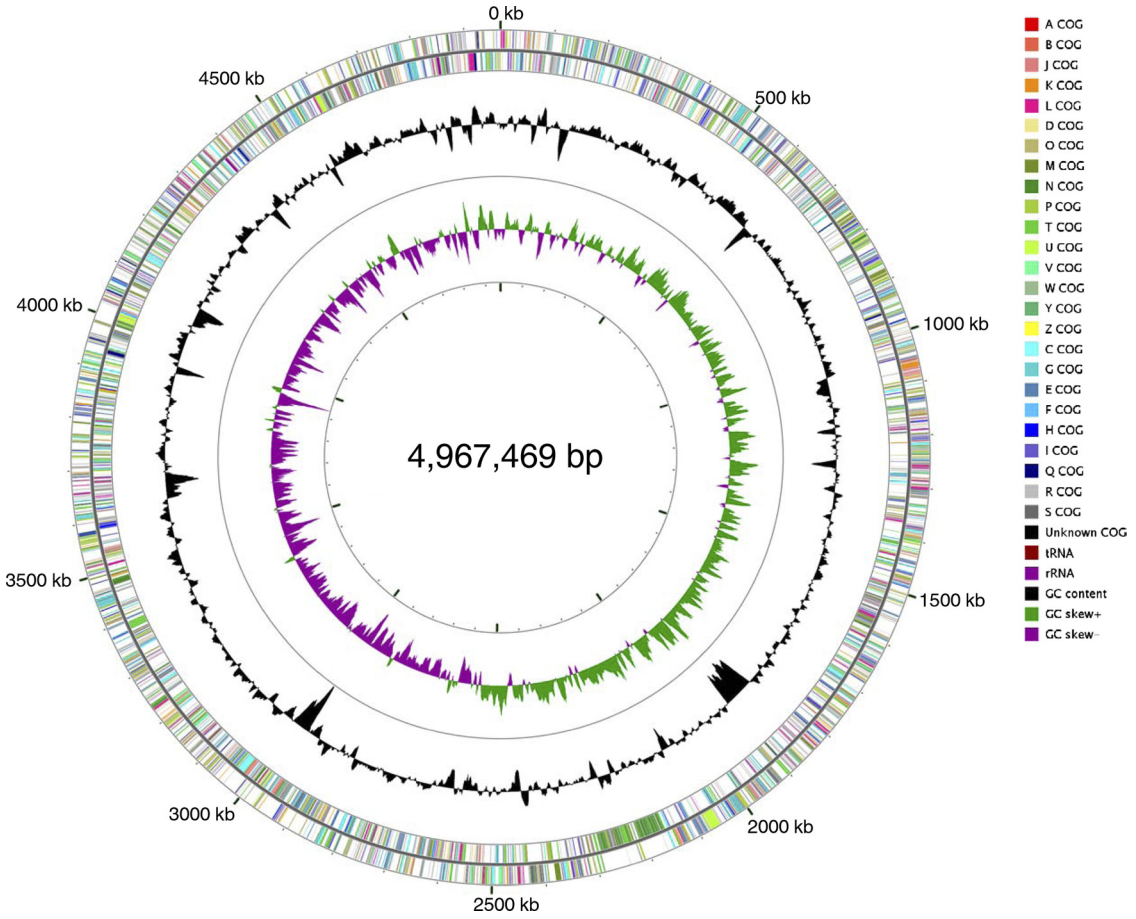

FIG. 2. Circular representation of *X. axonopodis* pv. citrumelo F1. Circles from outside to inside: first, scale bar in kilobases; second and third, predicted coding sequences of chromosome on leading and lagging strands, respectively (colors according to COGs); fourth, G+C content; fifth, G+C skew.

which is capable of conferring the ability to cause canker-like symptoms to strains of *X. axonopodis* pv. citrumelo (99). On the other hand, plasmids pXCV38 and pXCV183 carry putative *vir*/*tra*- and *icm*/*dot*-like type IV secretion systems, respectively (105).

TABLE 1. General features of *X. axonopodis* pv. citrumelo strain F1 genome

Chromosome feature	Value
Genome size (bp)	4,967,469
GC content (%)	64.92
Plasmids	0
Protein-coding region (%)	86.53
Predicted CDSs	
Protein-coding genes	4,202
With COGs	3,087
With Pfam	3,293
With TIGRfam	1,314
Connected to KEGG pathways	1,189
rRNA	6
rRNA operons	2
tRNA	54

The absence of plasmids from *X. axonopodis* pv. citrumelo may have contributed to the reduced virulence of the CBS strain.

HGT and genome plasticity. Horizontal gene transfer (HGT) is recognized as one of the major mechanisms for genome plasticity leading to diversification and speciation of the bacteria (74). A simple method to identify potential horizontally transferred genes is to look for regions having atypical G+C content in the genome. The G+C content of *X. axonopodis* pv. citrumelo genome ranged from 48.90% to 68.41%, with an average of 64.92%. The segmentation results predicted eight regions of low GC content, which are recognized as genomic islands (see Table S3 in the supplemental material). The negative cumulative GC profile of these regions is also different in comparison to the whole genome (see Fig. S1A). A sharp drop in the G+C content of these regions distinctly separates them from the rest of the genome (see Fig. S1B).

These regions vary in size from approximately 3 kb to 64 kb. It was noteworthy that one of the genomic islands, from bp 2970113 to bp 3004362, codes for *virB4-*, *virB11-*, *virB9-*, and *virD4*-expressed proteins, which are part of the type IV secretion system, and components of type IV pilus like *fimT, pilE*, and pilus tip-associated proteins. The presence of such genes

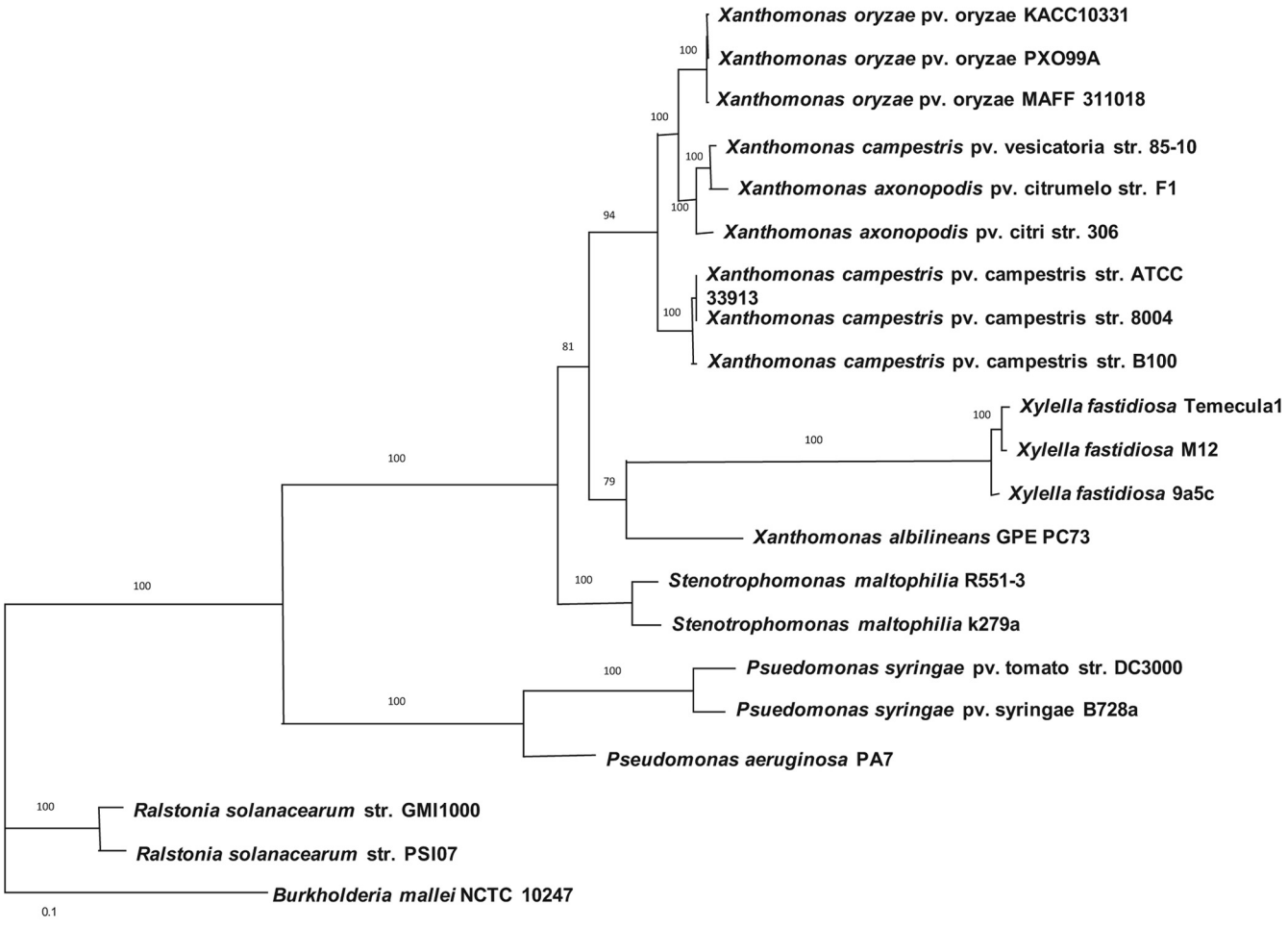

FIG. 3. Maximum likelihood tree of the genome of *X. axonopodis* pv. citrumelo F1 showing the relationship to other fully sequenced xanthomonads and related species. The tree was constructed using concatenated protein sequences of nine housekeeping genes (*uvrD, secA, carA, recA, groEL, dnaK, atpD, gyrB*, and *infB*) aligned using Clustal W. A phylogenic tree was constructed in PAUP (version 4.0) from concatenated sequences by using the maximum likelihood method. The sequences of *Ralstonia solanacearum* strains GMI1000 and PSI07 and *Burkholderia cenocepacia* strain NCTC 10247 were used as outgroup species. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The bar (0.1) at the bottom represents the number of amino acid substitutions per site.

in genomic regions indicative of horizontal gene transfer is in agreement with earlier reports (105). It was also observed that about 50% of open reading frames (ORFs) in the two biggest regions, from bp 1827507 to bp 1891340 and from bp 3664590 to bp 3686175, were determined to be orphan genes. Orphan genes have a very limited phylogenetic distribution and have no recognizable homologs. A recent study in *Escherichia coli* demonstrated that most orphan genes encode functional proteins (18). Thus, orphan genes may encode functional proteins in *X. axonopodis* pv. citrumelo and might contribute to virulence or differential host range of the strain.

In addition, these regions have a high number of integrase and transposase genes, which is a conserved feature of genomic islands. The genome of *X. axonopodis* pv. citrumelo includes 41 insertion sequence (IS) elements. A majority of these transposases belong to the IS*3* family, including Ixac2 and IS*1404*. We also found 2 IS elements each from the IS*5* and IS*1595* families and 3 elements belonging to the Tn3 family.

Comparison of proteins encoded by *X. axonopodis* pv. citrumelo to those encoded by *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. We compared the proteomes of the above three *Xanthomonas* spp. by using reciprocal BLASTp. A Venn diagram representing the pangenome of all three genomes is shown in Fig. 5. The comparison of the predicted protein sequences revealed that 3,292 CDSs are shared by all three genomes. These genes represent about three-quarters of the genome, forming the core set that includes conserved housekeeping and virulence genes essential for plant infection. Of the remaining 910 predicted genes in *X. axonopodis* pv. citrumelo, 119 have homologs only in *X. axonopodis* pv. citri and are absent from *X. campestris* pv. vesicatoria. These genes may include virulence factors necessary for infecting the common citrus host. The number of homologs in *X. campestris* pv. vesicatoria, at 385, is much higher than that in *X. axonopodis* pv. citri, further confirming that the CBS strain is much closer to *X. campestris* pv. vesicatoria than it is to *X. axonopodis* pv. citri.

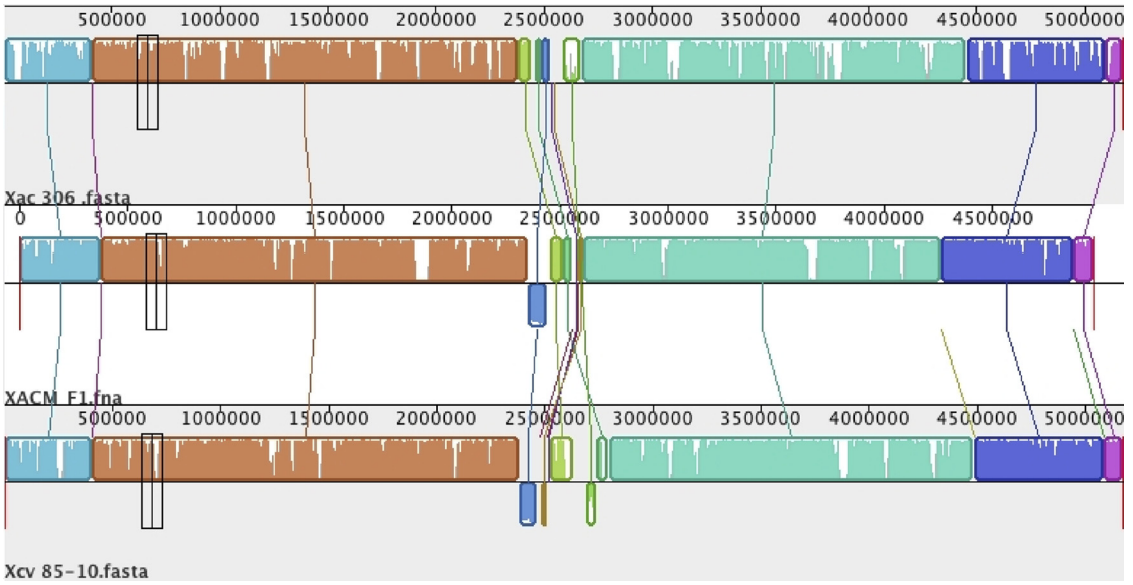

FIG. 4. MAUVE alignment of the genome sequences of *X. axonopodis* pv. citri (Xac) strain 306, *X. axonopodis* pv. citrumelo (XACM) strain F1 and *X. campestris* pv. vesicatoria (Xcv) strain 85-10. Conserved and highly related regions are colored, and low-identity unique regions are in white (colorless).

A total of 406 protein coding genes are unique to *X. axonopodis* pv. citrumelo, of which 298 are hypothetical genes, 26 are mobile genetic elements, and 82 are singletons with predicted functions. Moreover, 174 genes show homologs in distinctly related *Xanthomonas* or other highly related bacteria, suggesting their acquisition by horizontal gene transfer. The significant features shared between the genomes as well as the differences between the genomes are discussed in detail below.

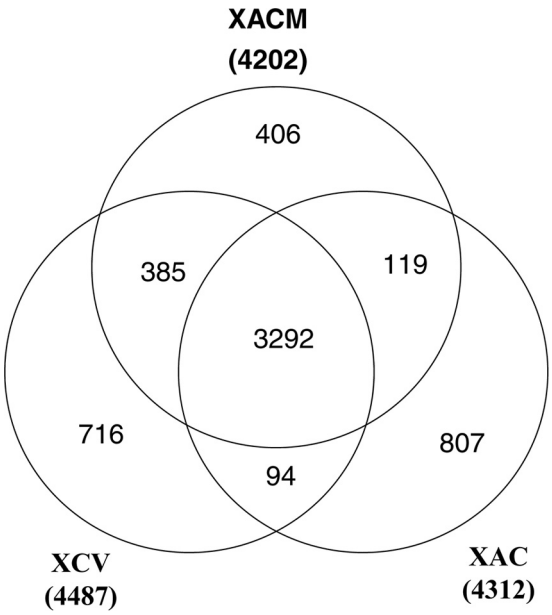

FIG. 5. Venn diagram representing the pangenome of *X. axonopodis* pv. citrumelo F1 (XACM), *X. campestris* pv. vesicatoria strain 85-10 (XCV), and *X. axonopodis* pv. citri strain 306 (XAC). Numbers in parentheses represent the protein coding genes on the chromosome in each species; numbers of shared genes are also shown within the diagram.

T3SS gene clusters. Gram-negative bacteria use T3SS to translocate virulence factors into the host cell. In *X. axonopodis* pv. citrumelo, the T3SS is encoded by 27 genes, the organization of which is in close synteny with the *hrp* cluster of *X. campestris* pv. vesicatoria and *X. axonopodis* pv. citri (see Fig. S2 in the supplemental material). The cluster includes all nine *hrc* (hypersensitive response conserved) genes, which encode T3SS structural components, and all 9 *hrp* genes, some of which encode components of the *hrp* pilus. These genes, present only in phytopathogenic bacteria, are associated with the rapid programmed death of plant cells at the site of infection in most nonhost or resistant host plants and for pathogenesis in susceptible hosts (10). The major difference from *X. axonopodis* pv. citri is that the *X. axonopodis* pv. citrumelo cluster lacks the hypothetical gene upstream of *hrpF* and instead has three additional genes in the same locus. These 3 genes consist of XACM_0383, which encodes a hypothetical protein with 97% amino acid identity to a hypothetical protein XPE_2921 in *X. perforans*, a pathogen of tomato causing bacterial spot (51); XACM_0384, which is 99% identical to outer membrane protein F1 (XopF1) from *X. campestris* pv. vesicatoria; and XACM_0385, with no obvious homologs. The final gene in this cluster, XACM_0407, shares 98% similarity to putative transglycosylase gene *hpaH* from *X. perforans*. As compared to that of *X. campestris* pv. vesicatoria, the T3SS of *X. axonopodis* pv. citrumelo lacks 2 *hrp*-associated genes, *hpaG* and *hpaE*; 2 outer protein genes, *xopD* and *xopA*; and 5 hypothetical genes, XCV_0410, XCV_0412, XCV_0436, XCV_0438, and XCV_0439, with no known functions. The loss of these genes from *X. axonopodis* pv. citrumelo might have contributed to host range and virulence variation.

Repertoire of T3SS effectors of *X. axonopodis* pv. citrumelo in comparison to those of *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. T3SS effectors were identified in the *X. axonopodis* pv. citrumelo genome and compared with those of *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. Considerable differences were observed in the effector repertoires present in these three strains (Table 2). Twenty-two effectors were identified in *X. axonopodis* pv. citrumelo, whereas 25 and 30 effectors were identified in *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria, respectively. We subdivided them into three groups—core, partially shared, and species specific—depending on their presence in the three strains. The core effectors shared by all the three strains consist of 17 effector genes. Of this core set, 9 effector genes (*avrBs2, xopK, xopL, xopN, xopP, xopQ, xopR, xopX*, and *xopZ*) are present in the genomes of all sequenced *Xanthomonas* spp. with the exception of *X. albilineans* and *X. campestris* pv. armoraciae, the latter of which has only *xopP* and *xopR*. These genes might be essential effector genes required for the pathogenicity of xanthomonads in plant hosts. The effector *avrBs2* belongs to a family known for effector-triggered immunity in plants (101). It elicits hypersensitive reaction (HR) in plants carrying the *Bs2* resistance gene (67) and is needed for the full virulence of the pathogen on susceptible hosts. Effector *xopQ*, which belongs to the *hopQ1* family from *Pseudomonas*, has also been known as an avirulence determinant in *Nicotiana benthamiana*, since the *Pseudomonas syringae* pv. tomato DC3000 deletion mutant (deletion of *hopQ1-1*) acquired the ability to grow to high levels and produce bacterial speck lesions in nonhost *N. benthamiana* (111). XopN has been shown to interact with TARK1 and TFT1 proteins from tomato, thus repressing pathogen-associated molecular pattern-triggered immunity (55). The homologs of effectors *xopL, xopP*, and *xopQ* have been shown to contribute to pathogenicity in *X. campestris* pv. campestris (49). Both *xopX* and *xopZ* potentially interfere with host innate immunity, thus making the plant more susceptible. The remaining 8 core effectors (*xopA, xopE1, xopF2, xopI, xopV, xopAD, xopAE*, and *xopAK*) are not present in all xanthomonads and might be responsible for pathogenicity in some plant hosts while inducing resistance in others (113). It is likely that none of the effectors belonging to the core group are responsible for the difference in virulence and host range of *X. axonopodis* pv. citrumelo, *X. axonopodis* pv. citri, and *X. campestris* pv. vesicatoria.

The partially shared effectors are present in only two of the three strains. This group consists of *xopC1, xopF1*, and *xopAJ*, shared by *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria only, as well as *xopE2*, shared by *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria but absent from *X. axonopodis* pv. citrumelo. *xopF1* is found in all *Xanthomonas* species except *X. axonopodis* pv. citri, which encodes a truncated version of the same. In *X. axonopodis* pv. citrumelo, a homolog of *xopF1* is present, which shares 99% similarity to that gene in *X. campestris* pv. vesicatoria. The *xopAJ* homolog of *X. axonopodis* pv. citrumelo shares 99% similarity with *xopAJ-avrRxo1* of *X. campestris* pv. vesicatoria. This gene is truncated in *X. axonopodis* pv. citrumelo due to a deletion mutation at bp 1056 in the gene that resulted in early termination of the protein at 379 amino acids as opposed to its homolog of 450 amino acids in *X. campestris* pv. vesicatoria. The *xopC1* effector gene encodes a haloacid dehalogenase-like hydrolase with several phosphoribosyltransferase domains. This gene is present in *X. axonopodis* pv. citrumelo but is fragmented across the genome (XACM_2129, XACM_2132, and XACM_2248) due to genome rearrangement and transposon insertion and thus is likely to be nonfunctional. *xopE2*, which has been identified in various xanthomonads, has recently been shown to be involved in the virulence of *X. campestris* pv. vesicatoria group B strains on tomato but not in that of group A strains (62). It has also been related to suppression of HR, indicating that it plays a dual role in different host plants (62).

Two species-specific effectors, *xopC2* and *xopW*, were found in *X. axonopodis* pv. citrumelo. Though homologs of *xopC2* are found in both *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria, they might be nonfunctional. *X. axonopodis* pv. citrumelo consists of a *xopC2* gene with its closest homolog in *X. perforans*, which causes bacterial spot only on tomato (80). *X. axonopodis* pv. citrumelo also has a truncated gene, XACM_0435, which is a homolog of *xopW* from *X. oryzae* pv. oryzicola and might be nonfunctional. *X. campestris* pv. vesicatoria has at least 9 unique effectors, as listed in Table 2. Some effectors, such as *avrBs1, avrBs1.1*, and *xopJ3*, are known avirulence factors. These effectors might be important for pathogenicity in tomato and pepper. *X. axonopodis* pv. citri possesses four unique effectors, *avrBs3-pthA, xopE3, xopAI*, and *hrpW*, which are absent from *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria (Table 2).

The differences in the repertoires of T3SS effectors in *X. axonopodis* pv. citrumelo and *X. axonopodis* pv. citri might contribute to their differences in virulence and host range. T3SS effectors have been known to contribute to pathogenicity and multiplication of pathogens *in planta* (42). T3SS effectors benefit the pathogens by altering the physiology of the host cell and suppressing plant defenses (39). T3SS effectors might contribute to host range by suppressing host defenses as virulence factors or narrowing the host range when certain effectors are specifically recognized by the plant as avirulence factors (43). Importantly, *avrBs3-pthA* is present in *X. axonopodis* pv. citri A strain 306 while absent from *X. axonopodis* pv. citrumelo F1 and *X. campestris* pv. vesicatoria 85-10. However, it is noteworthy that many other *X. campestris* pv. vesicatoria strains contain *avrBs3* and homologs (103). In *X. axonopodis* pv. citri strain 306, there are 4 copies of *pthA, pthA1, pthA2, pthA3*, and *pthA4*, on two plasmids, which are all absent from *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria. PthA4 with 17.5 repeats, which is same as PthA, is known to play an important role in citrus canker as knockout of *pthA4* abolished the development of citrus canker symptom development (115). PthA is responsible for the development of hypertrophic and hyperplasic symptoms and cell death, and its mutation leads to a reduction in the ability of bacteria to disseminate from infected lesions (116). PthA also contributes to epidermal rupture and necrosis, which promotes the exudation and dissemination of *X. axonopodis* pv. citri. Interestingly, the *pthA* gene from the *X. axonopodis* pv. citri strain, when introduced to *X. axonopodis* pv. citrumelo, conferred the ability to cause raised pustules (99). PthA and its homologs do not determine host range according to a previous study (3), indicating that neither of the complementing homologs nor any of the noncomple-

TABLE 2. Effector repertoire of *X. axonopodis* pv. citrumelo strain F1, *X. axonopodis* pv. citri strain 306, and *X. campestris* pv. vesicatoria strain 85-10

Effector class	Designation(s) in:			Pfam domains	Reference(s)
	X. axonopodis pv. citrumelo strain F1	*X. axonopodis* pv. citri strain 306	*X. campestris* pv. vesicatoria strain 85-10		
Core effectors present in all three strains					
AvrBs2	XACM_0049	XAC0076	XCV0052	Glycerophosphoryl diester phosphodiesterase	53
XopA (HpaI/HpaG)	XACM_0406	XAC0416	XCV0440		73
XopE1 (AvrXacE1)	XACM_0271	XAC0286	XCV0294	Putative transglutaminase	106
XopF2	XACM_2726	XAC2785[a]	XCV2942		86
XopI	XACM_0750	XAC0754	XCV0806	F-box protein	104
XopK	XACM_3001	XAC3085	XCV3215		24
XopL	XACM_3007	XAC3090	XCV3220	LRR protein	50
XopN	XACM_2728	XAC2786	XCV2944	ARM/HEAT repeat	55
XopP	XACM_1178	XAC1208	XCV1236		86
XopQ	XACM_4215	XAC4333	XCV4438	Inosine uridine nucleoside *N*-ribohydrolase	86
XopR	XACM_0263	XAC0277	XCV0285		24
XopV	XACM_0604	XAC0601	XCV0657		24
XopX	XACM_0532	XAC0543	XCV0572		66
XopZ	XACM_2036	XAC2009	XCV2059		24
XopAD	XACM_4086	XAC4213	XCV4315[a] XCV4314[a] XCV4313[a]	SKWP repeat protein	40, 79
XopAE (HpaF/HpaG)	XACM_0381	XAC0393	XCV0409[a] XCV0408[a]	LRR protein	113
XopAK	XACM_3563	XAC3666	XCV3786		79
Effectors shared by *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria but not present in *X. axonopodis* pv. citri					
XopC1	XACM_2129[b] XACM_2132[b] XACM_2248[b]		XCV2435	Phosphoribosyltransferase domain and haloacid dehalogenase-like hydrolase	86
XopF1 (Hpa4)	XACM_0384		XCV0414		86
XopAJ (AvrRxo1)	XACM_4204		XCV4428	Zeta toxin	117
Effectors shared by *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria but not present in *X. axonopodis* pv. citrumelo					
XopE2 (AvrXacE3, AvrXccE1)		XACb0011	XCV2280	Putative transglutaminase	106
Effectors unique to *X. axonopodis* pv. citri					
PthA (AvrBs3, TAL)		XACa0022(PthA1) XACa0039(PthA2) XACb0015(PthA4) XACb0065(PthA4)		Transcriptional activator, nuclear localization	2
XopE3 (AvrXacE2)		XAC3224		Putative transglutaminase	71
XopAI		XAC3230		Putative ADP-ribosyltransferase	105
HrpW (PopW)		XAC2922		Pectate lyase	76
Effectors unique to *X. campestris* pv. vesicatoria					
AvrBs1			XCVd0104		105
XopB			XCV0581		72
XopD			XCV0437	C48-family SUMO cysteine protease (Ulp1 protease family), EAR motif	86
XopG			XCV1298	M27 family peptidase clostridium toxin	105
AvrBs1.1 (XopH)			XCVd0105	Putative tyrosine phosphatase	105

Continued on following page

TABLE 2—*Continued*

Effector class	Designation(s) in:			Pfam domains	Reference(s)
	X. axonopodis pv. citrumelo strain F1	*X. axonopodis* pv. citri strain 306	*X. campestris* pv. vesicatoria strain 85-10		
XopJ1			XCV2156	C55-family cysteine protease or Ser/Thr acetyltransferase	86
XopJ3 (AvrRxv)			XCV0471	C55-family cysteine	105
XopO			XCV1055	protease or Ser/Thr acetyltransferase	105
XopAA			XCV3785	Early chlorosis factor, proteasome/cyclosome repeat	105
Effectors unique to *X. axonopodis* pv. citrumelo					
XopC2	XACM_1180	XAC1209[a] XAC1210[a]	XCV1238[a] XCV1237[a]	Haloacid dehalogenase-like hydrolase	113
XopW	XACM_0435				24

[a] Inactive/pseudogene.
[b] Partial sequences due to interruption by IS elements during HGT.

menting paralogs of *pthA* suppresses the avirulence of the *X. axonopodis* pv. citri A* strain on grapefruit. However, *hssB3.0*, a homolog of *pthA*, was shown to be responsible for the host-specific suppression of virulence of *X. axonopodis* pv. citri A strain KC21 on *Citrus grandis* cultivars but not on other *Citrus* species, such as *Citrus sinensis* (93). This suppression led to reduced aggressiveness rather than a change in host range, since *X. axonopodis* pv. citri A strain KC21 still causes citrus canker symptoms on *Citrus grandis*.

Other *X. axonopodis* pv. citrumelo-specific effectors might contribute to the broad host range of *X. axonopodis* pv. citri compared to that of *X. axonopodis* pv. citrumelo. *xopE3* (*avrXacA2*) is a putative transglutaminase enzyme gene that belongs to the *hopX* (*avrPphE*) family and is widespread among phytopathogenic bacteria (71). XopAI is putative effector protein reported only in the three canker-causing strains *X. axonopodis* pv. citri 306, *X. aurantifolii* strain B, and *X. aurantifolii* strain C as well as in one *X. vesicatoria* strain, namely, 1111 (68, 80). The role of XopAI in the virulence of *Xanthomonas* remains to be characterized. HrpW is not known to be associated with virulence, although it contains domains resembling hairpins and pectate lyases. It may not function as an intracellular effector but is secreted by the T3SS. HrpW in several other phytopathogens is known to elicit an HR in nonhost plants (54). Alternately, the limited host range of *X. axonopodis* pv. citrumelo might result from the presence of the *X. axonopodis* pv. citrumelo-specific *xopC2* and *xopW* serving as avirulence factors. The presence of all the species-specific effectors in *X. axonopodis* pv. citri and *X. axonopodis* pv. citrumelo may be the main factors determining the host range of the pathogens. Further study is needed to understand their contribution to *X. axonopodis* pv. citri and *X. axonopodis* pv. citrumelo for infecting different hosts.

The difference in effector repertoires of *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria might contribute to their different host specificities, with *X. axonopodis* pv. citrumelo infecting citrus seedlings and *X. campestris* pv. vesicatoria 85-10 causing bacterial spot disease on both pepper and tomato plants (80, 87). It has been suggested that the specific effector set of a given bacterial strain is the potential determinant of host range (105). Compared to *X. campestris* pv. vesicatoria, *X. axonopodis* pv. citrumelo contains *xopC2* and *xopW*, (which are absent from *X. campestris* pv. vesicatoria), while *X. axonopodis* pv. citrumelo lacks *avrBs1, xopB, xopD, xopG, xopH* (*avrBs1*), *xopJ1, xopJ3* (*avrRxv*), *xopO*, and *xopAA* (which are present in *X. campestris* pv. vesicatoria) (Table 2). *avrBs1* is known to encode a 50-kDa protein with homology to AvrA of *Pseudomonas syringae* pv. glycinea. This protein specifies avirulence on pepper cultivars containing the resistance gene *Bs1* (69). XopD is known to alter host transcription, promote pathogen growth, and delay the development of disease symptoms (56). XopB attenuated cell proliferation when expressed in yeast and also caused cell death in *N. benthamiana* leaves but not in tomato (89). XopJ homologs are known to inhibit host protein secretion and interfere with defense responses (7). Other effectors including XopG, XopO, and XopAA in *X. campestris* pv. vesicatoria have not been studied in detail. How these effectors contribute to the different host ranges and different virulences of *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria needs further characterization.

Other secretion systems associated with virulence. Xanthomonads have at least five more protein secretion systems other than the T3SS, including types I, II, IV, V, and VI. Genes involved in all the secretion systems were identified in the *X. axonopodis* pv. citrumelo F1 genome. Secretion systems are of fundamental importance for the translocation of proteins and other molecules. They play important roles in the virulence of different bacterial pathogens. The relevant features of these virulence-associated secretion systems of *X. axonopodis* pv. citrumelo shared with *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria are presented below.

(i) T1SS. The type I secretion system (T1SS) has not been shown to contribute to virulence in *Xanthomonas* spp. (11). Instead, T1SS is required for Xa21-mediated immunity in rice against *X. oryzae* pv. oryzae PXO99. Ax21 (activator of Xa21-mediated immunity) is highly conserved in *Xanthomonas* spp. and secreted by T1SS. In *X. axonopodis* pv. citrumelo, the Ax21 protein (XACM_0208) is 100% identical with the *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria proteins and 93% identical with the *X. oryzae* pv. oryzae PXO99 protein. In

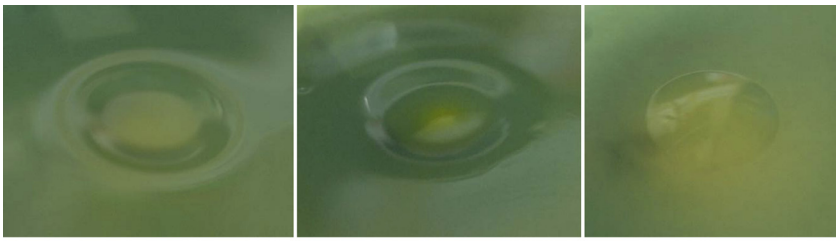

FIG. 6. Comparison of the pecate lyase production by X. *axonopodis* pv. citri strain 306 (XAC), *X. axonopodis* pv. citrumelo strain F1 (XACM), and *X. campestris* pv. vesicatoria strain 85-10 (XCV). Pitting can be seen in Hildebrand's agar medium at pH 8.5 when pectate lyase-positive strains X. *axonopodis* pv. citri 306 and *X. campestris* pv. vesicatoria 85-10 were inoculated. No pitting is seen for *X. axonopodis* pv. citrumelo strain F1. All strains were incubated in Hildebrand's agar medium at 28°C for 6 days.

addition, RaxST is required for sulfation, and three genes, *raxA, raxB*, and *raxC*, are required for the secretion of Ax21 (44). The gene *raxA* in *X. axonopodis* pv. citrumelo (XACM_1188) may be nonfunctional due to a frameshift mutation, whereas the proteins encoded by *raxB* (XACM_1189), *raxC* (XACM_3355), and *raxST* (XACM_1187) are 99 to 100% identical to *X. campestris* pv. vesicatoria proteins. *X. axonopodis* pv. citri, on the other hand, contains only the *raxC* gene.

(ii) T2SS. *X. axonopodis* pv. citrumelo is also equipped with the *xps* and *xcs* type II secretion system (T2SS) clusters, which secrete toxins and degradative enzymes. The T2SS clusters in *X. axonopodis* pv. citrumelo are very conserved compared to those identified in *X. campestris* pv. vesicatoria and *X. axonopodis* pv. citri, with *xps* being composed of 11 genes and *xcs* of 12 genes (see Fig S3 in the supplemental material). The *xps* T2SS, which is found in all the sequenced xanthomonads, is known to affect virulence in *X. campestris* pv. vesicatoria and also enhance translocation by T3SS. The *xcs* T2SS, on other hand, is restricted to only some *Xanthomonas* spp. and has no obvious virulence function (102). The T2SSs are known to secrete many plant cell wall-degrading enzymes like cellulases, xylanases, lipases, and proteases, among others. Each species has its unique set of enzymes, which helps degrade components of the plant cell wall, thus assisting in pathogenesis. The range of these enzymes in *X. axonopodis* pv. citrumelo was compared to the ones found in *X. campestris* pv. vesicatoria and *X. axonopodis* pv. citri (see Table S4 in the supplemental material). Enzymes such as cellulase, protease, and pectate lyase have been known to promote bacterial nutrition and also virulence (20, 83). XynC, an endoxylanase in *X. campestris* pv. vesicatoria, is known to be secreted by the *xps* T2SS under the control of *hrpG* and *hrpX* and contribute to virulence (102). *X. axonopodis* pv. citrumelo contains a homolog of this gene, XACM_0913, that may play a similar role. Most of the enzymes show functional redundancy and hence the loss of one gene might not affect virulence. Rajeshwari et al. (81) showed that double mutants, with mutations of both lipase and xylanase, show much reduced virulence as compared to the single mutants in *X. oryzae* pv. oryzae.

It is interesting that *X. axonopodis* pv. citrumelo is deficient in pectate lyase function. In contrast with the four genes in *X. campestris* pv. vesicatoria and the three in *X. axonopodis* pv. citri, *X. axonopodis* pv. citrumelo shows the presence of only two genes. However, both the genes, XACM_2919 and XACM_3456, are pseudogenes that have stop codons, resulting in truncated protein. Thus, these proteins may be nonfunctional. This was confirmed by inoculating the strains onto Hildebrand's medium. Both *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria produced pitting in the agar at pH 8.5, confirming pectate lyase activity. *X. axonopodis* pv. citrumelo did not produce any pitting, as seen in Fig. 6, supporting the hypothesis that it is pectate lyase deficient. A pectate lyase gene homolog, *xagP*, has been shown to induce an HR in tobacco and pepper in *X. axonopodis* pv. glycines (52). The role of pectate lyase in citrus pathogens remains to be determined. Several T2SS substrates have been shown not only to affect virulence but also to induce plant defense responses. T2SS and its substrates are also controlled by T3SS regulators (41).

(iii) T4SS. In bacteria, the type IV secretion system (T4SS) is known to contribute to virulence. Two T4SS clusters are present in both *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. Both the clusters are of the Vir type in *X. axonopodis* pv. citri (see Fig. S4 in the supplemental material), where one is located on the chromosome and the other is on plasmid (1). *X. campestris* pv. vesicatoria, on the other hand, codes for one Vir and the other Dot/Icm-type cluster (see Fig. S4), both on plasmids, and a partial Vir cluster on the chromosome (105). *X. axonopodis* pv. citrumelo codes for only one Vir-type T4SS cluster on the chromosome. The cluster in *X. axonopodis* pv. citrumelo does not show high similarity to Vir-type T4SS of either *X. axonopodis* pv. citri or *X. campestris* pv. vesicatoria. With the exception of *virD4*, which shows homologs in both *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria, most of the predicted T4SS genes in *X. axonopodis* pv. citrumelo share sequence similarity with genes in strains of *Stenotrophomonas maltophilia*, which is an aerobic Gram-negative environmental bacterium commonly found in soil, water, and animals (46). *X. axonopodis* pv. citrumelo also codes for a VirK and two VirJ-like proteins outside the T4SS cluster. The function of the *virK* protein is unknown and it has been linked to T2SS substrates instead of T4SS (41). VirJ is a perisplasmic chaperone believed to mediate the association between the T4S pilus and substrate proteins (13).

(iv) T5SS. Both *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria encode a two-partner secretion system, which belongs to the type V secretion system (T5SS). It translocates large proteins such as adhesins and has been identified in many bacterial pathogens. *X. axonopodis* pv. citrumelo codes for a filamentous hemagglutinin-like protein, FhaB. A complete homolog of the corresponding gene can be found in *X. axonopo-*

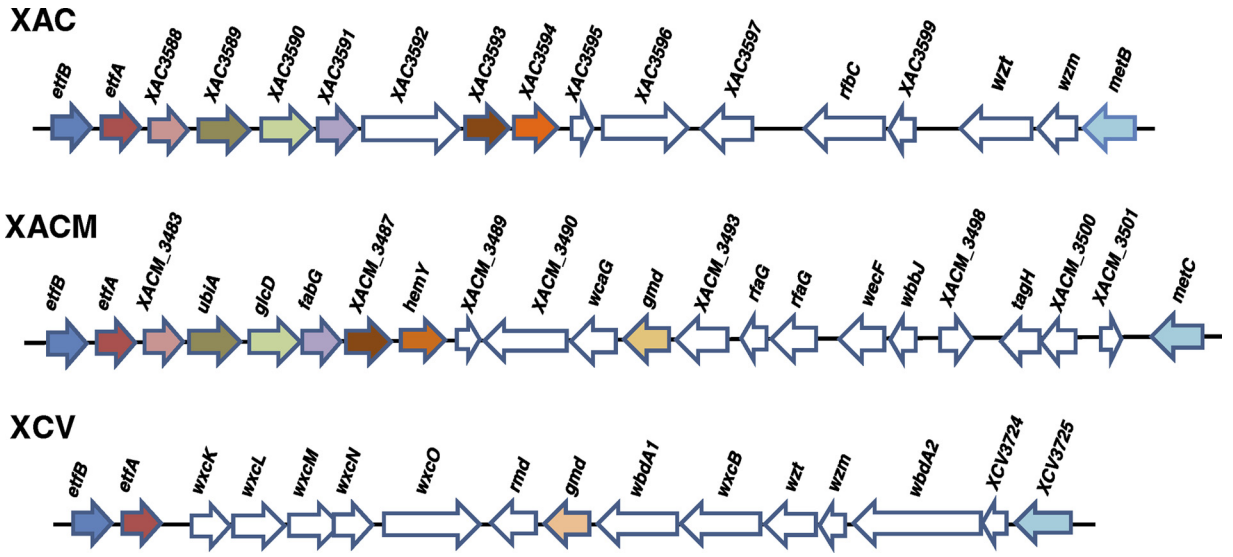

FIG. 7. Relative organization of the LPS gene cluster in the genomes of *X. axonopodis* pv. citrumelo strain F1 (XACM), X. *axonopodis* pv. citri strain 306 (XAC), and *X. campestris* pv. vesicatoria strain 85-10 (XCV). Not to scale. Conserved and highly related genes are colored and low-identity or unique genes are white.

dis pv. citri but is inactivated in *X. campestris* pv. vesicatoria by an internal stop codon-inducing mutation. In *X. axonopodis* pv. citrumelo, the gene is interrupted due to genomic rearrangements, which indicates that it might be inactive. The rearrangement has caused the gene to split, with insertion of 2 hypothetical genes, XACM_1838 and XACM_1839, between the two *fhaB* fragments. Transposon genes in the vicinity may have instigated this change. *fhaB* is involved in attachment and biofilm formation in *X. axonopodis* pv. citri, and its loss affects the virulence of the bacterium (30). The *fhaB* gene in *X. axonopodis* pv. citrumelo is likely to be inactive due to the insertion, and the lack of the functional gene could contribute to the low virulence of *X. axonopodis* pv. citrumelo compared to that of *X. axonopodis* pv. citri.

(v) T6SS. Recently, a new secretion system was identified in *Vibrio cholerae* and *Pseudomonas aeruginosa* and was named the type VI secretion system (T6SS). T6SS is evolutionarily related to bacteriophage, likely a remnant of phage injection machinery (84). T6SS has diverse roles in virulence, symbiosis, interbacterial interactions, and antipathogenesis in different bacteria (84). *X. campestris* pv. vesicatoria is found to have two clusters of T6SS: cluster type I and cluster type II, of which the latter is split into two locations. *X. axonopodis* pv. citri has only cluster type II T6SS, which like that of *X. campestris* pv. vesicatoria is split into two locations (9). The distribution of the T6SS in all three *Xanthomonas* is compiled in Table S5 in the supplemental material. *X. axonopodis* pv. citrumelo codes for two clusters, cluster type I from XACM_2098 to XACM_2121 and cluster type II from XACM_4015 to XACM_3979. Both of the clusters are homologs to the ones found in *X. campestris* pv. vesicatoria. *X. axonopodis* pv. citri, on the other hand, has only T6SS cluster type II, encoded from XAC4147 to XAC4112.

Bacterial surface structures. (i) LPS. Lipopolysaccharide (LPS) serves a dual role as a physical barrier by protecting bacteria from antibacterial substances produced by plants and also as an inducer of plant defense-related genes (70). Flanked by highly conserved housekeeping genes for cystathionine gamma lyase (*met*) at one end and electron transport flavoprotein (*etf*) at the other end, the genome of *X. axonopodis* pv. citrumelo contains a cluster of 22 genes involved in LPS biosynthesis. The LPS gene cluster in a 24.5-kb region in *X. axonopodis* pv. citrumelo is markedly different in both number and composition from the 17-gene cluster in *X. axonopodis* pv. citri and more so from the 16-gene cluster in *X. campestris* pv. vesicatoria (Fig. 7). The flanking genes of *etfB, etfA*, and *metB-metC* are conserved in all three genomes. The LPS locus of *X. axonopodis* pv. citrumelo has at least six homologs to *X. axonopodis* pv. citri and only one to *X. campestris* pv. vesicatoria (Fig. 7). The LPS cluster is involved in synthesis of *O*-antigen polysaccharide. It is known to be important for biofilm formation on the host and contributes to virulence. Two such loci, XAC3586 and *rfbC*, have been experimentally shown to contribute to the virulence of *X. axonopodis* pv. citri on grapefruit (*Citrus paradisi* cv. Duncan grapefruit) (61). *X. axonopodis* pv. citrumelo does not have homologs for either of these genes. This may contribute to the poor survival of *X. axonopodis* pv. citrumelo *in planta* and hence the low virulence compared to *X. axonopodis* pv. citri. Interestingly, the *wzt* mutants of *X. axonopodis* pv. citri showed more water soaking on citrus plants (58). XACM_3499 in *X. axonopodis* pv. citrumelo is the closest homolog to *wzt*, with a low protein identity of 34% and 41% to its orthologs in *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. Also, the *X. axonopodis* pv. citrumelo gene codes for a truncated protein with half missing from the C-terminal region as compared to its orthologs; thus, it might be nonfunctional. This may have led to variation in the symptoms of *X. axonopodis* pv. citrumelo, which shows pronounced water soaking compared to *X. axonopodis* pv. citri. It was also suggested that there is no obvious correlation of the content of the LPS gene cluster with host specificity (64). Thus, the variation in the LPS gene cluster among *X. axonopodis* pv. citrumelo, *X. axonopodis* pv. citri, and *X. campestris* pv. vesicatoria might

contribute to their differences in virulence or symptom development in plant hosts rather than serving as a determinant of their differential host range. In either case, these differences are consistent with the hypothesis of strong diversifying selection on this locus put forward by Patil et al. (78).

(ii) Extracellular polysaccharides. Extracellular polysaccharides (EPS, called xanthan gum in *Xanthomonas*) are an important component of a biofilm and contribute to the epiphytic fitness of *Xanthomonas* spp. (85). They are postulated to promote colonization of plant tissues by protecting the pathogens from harsh environmental conditions and to contribute to occlusion of vascular tissues in wilts and blights (57). *X. axonopodis* pv. citrumelo contains the complete gum gene cluster from *gumA* to *gumP*, which is syntenic to those found in *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. The identity of gum genes ranges from 88 to 100% among the three *Xanthomonas* species. Thus, it is unlikely that EPS plays any role in the differences in virulence and host range of *X. axonopodis* pv. citrumelo, *X. axonopodis* pv. citri, and *X. campestris* pv. vesicatoria.

(iii) Flagella. *Xanthomonas* is known to contain all the genes for flagellum synthesis and motility. Flagella are encoded by various genes located in 4 clusters, which are characteristically flanked by transposase genes. *X. axonopodis* pv. citrumelo contains complete flagellar structure and motility genes in four similar clusters. The flagellar genes in *X. axonopodis* pv. citrumelo are mostly organized in an order similar to those in *X. campestris* pv. vesicatoria and *X. axonopodis* pv. citri (see Fig. S5 in the supplemental material). Cluster 1 consists of *motA* (XACM_3590) and *motB* (XACM_3592) and cluster 2 of *motB* (XACM_1939) and *motC* (XACM_1940). These genes encode flagellar motor proteins required for the rotation of flagella. Cluster 3 in *X. axonopodis* pv. citrumelo consists of 24 genes from XACM_1954 to XACM_1977, involved in synthesis and regulation of flagella that is syntenic with *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria. The gene *fliK* in this cluster may be a pseudogene, which is nonfunctional due to frameshift mutations. Mutations in *fliK* affect flagellar hook length in animal pathogenic bacteria (114). However, no detectable difference was observed between the motilities of *X. axonopodis* pv. citri and *X. axonopodis* pv. citrumelo (data not shown). *X. axonopodis* pv. citrumelo has a cluster comprising 24 genes from XACM_1991 to XACM_2014, which is highly conserved as compared to both *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria.

Interestingly, the genes that lie between these clusters are different in *X. axonopodis* pv. citrumelo and *X. axonopodis* pv. citri. A notable difference includes the absence of a homolog of XAC1927 from *X. axonopodis* pv. citrumelo. This gene, encoding an Fe-S oxidoreductase and located on a probable pathogenicity island, has been linked to virulence in *X. axonopodis* pv. citri (58). The absence of this gene from *X. axonopodis* pv. citrumelo could possibly contribute to the low virulence of *X. axonopodis* pv. citrumelo compared to *X. axonopodis* pv. citri on citrus.

(iv) *rpf* cluster. The regulation of pathogenicity factors (*rpf*) genes control the synthesis of diffusible signal factor (DSF), which plays a major role in quorum sensing, thus controlling various virulence factors in *X. axonopodis* pv. citri (94). Three core genes, *rpfF, rpfC*, and *rpfG*, control the synthesis of the DSF molecule and signal transduction. *rpfF* is responsible for the production of DSF, whereas *rpfC-rpfG* are two-component signaling factors. RpfC is a sensor protein and RpfG is a response regulator. RpfG has a HD-GYP domain that regulates the amount of cyclic di-GMP. Furthermore, this is involved in the regulation of the DSF regulon, thus affecting the virulence of the pathogen *X. axonopodis* pv. citri (5). In addition to all the *rpf* genes found in *X. axonopodis* pv. citri, the CBS pathogen *X. axonopodis* pv. citrumelo contains a functional *rpfH*, which lies nestled between *rpfC* and *rpfG*. This gene encodes a protein which is structurally similar to the sensory domain of RpfC. *rpfH* is also present in *X. campestris* pv. vesicatoria and *X. campestris* pv. campestris but absent from *X. axonopodis* pv. citri. A study by Slater et al. (97) showed that mutation in the *rpfH* gene in *X. campestris* pv. campestris did not affect the DSF pathway, and thus its role in this operon is unclear. It would be interesting to study whether its presence affects the virulence of *X. axonopodis* pv. citrumelo on citrus.

Other strain-specific genes that might contribute to the distinct virulences of *X. axonopodis* pv. citri and *X. axonopodis* pv. citrumelo on citrus. Overall, 807 *X. axonopodis* pv. citri-specific genes were missing in *X. axonopodis* pv. citrumelo and *X. campestris* pv. vesicatoria. Besides the genes discussed above, *X. axonopodis* pv. citri also contains other genes missing from *X. axonopodis* pv. citrumelo that may be responsible for its higher virulence. A plant-like natriuretic peptide (PNP) gene (*XacPNP*), which is expressed specifically during the infection process in *X. axonopodis* pv. citri, is one such gene. XAC2654 encodes this plant-like hormone that induces changes in host photosynthetic efficiency, thereby weakening host defense (28). It has been shown that *X. axonopodis* pv. citri PNP mimics host PNP and results in improved host tissue health and consequently better pathogen survival in the lesions (27). The *X. axonopodis* pv. citrumelo genome was found to have neither a homolog of XAC2654 nor the surrounding region in its genome. Interestingly, *X. axonopodis* pv. citri also contains genes with putative toxin-producing functions. The genes *syrE1* and *syrE2* are similar to those found in *Pseudomonas syringae* that encode the phytotoxin syringomycin (22). These nonribosomal peptide synthetases, which might produce toxins, are absent from the *X. axonopodis* pv. citrumelo genome. *X. axonopodis* pv. citri also codes for hemolysin-type calcium-binding proteins XAC2197 and XAC2198 and also contains potential secretion genes *hlyB* and *hlyD*. These genes are also found in the citrus pathogen *Xylella fastidiosa*, and their products belong to the RTX toxin family (96). They are known to be pore-forming cytotoxins which act as virulence factors, and individual toxins often exhibit host specificity in eukaryotes (112). The region containing the toxin genes is absent from *X. axonopodis* pv. citrumelo. Another region of 20 kb, approximately from Mb 1.72 to 1.74, is specific to *X. axonopodis* pv. citri and is not found in *X. axonopodis* pv. citrumelo. It contains at least two genes, XAC1496 and XAC1507 (*mobL*), which are involved in the virulence of the canker pathogen (115). This region has a very low G+C content of 50% and is surrounded by integrase genes, suggesting that *X. axonopodis* pv. citri might have acquired it through recent HGT events. These might be potential genes contributing to the high aggression shown by *X. axonopodis* pv. citri on citrus as compared to *X. axonopodis* pv. citrumelo.

In conclusion, we completed the genome sequencing of *X. axonopodis* pv. citrumelo F1, combining 454 GS-FLX pyrosequencing (both unpaired and paired-end) and paired-end Illumina/Solexa sequencing and closing the gap using Sanger sequencing. Comparison of the finished genome sequence of *X. axonopodis* pv. citrumelo to those of *X. axonopodis* pv. citri and *X. campestris* pv. vesicatoria provides valuable insights into the emergence of new virulent strains with different host ranges and distinct virulences. Such knowledge contributes to our understanding of bacterial evolution and the role of various systems in virulence and host range of the pathogen. These strain-specific genes need to be functionally characterized to understand their roles in virulence and host specificity.

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