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Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper

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Abstract

Background: Bacterial spot of tomato and pepper is caused by four *Xanthomonas* species and is a major plant disease in warm humid climates. The four species are distinct from each other based on physiological and molecular characteristics. The genome sequence of strain 85-10, a member of one of the species, *Xanthomonas euvesicatoria* (*Xcv*) has been previously reported. To determine the relationship of the four species at the genome level and to investigate the molecular basis of their virulence and differing host ranges, draft genomic sequences of members of the other three species were determined and compared to strain 85-10.

Results: We sequenced the genomes of *X. vesicatoria* (*Xv*) strain 1111 (ATCC 35937), *X. perforans* (*Xp*) strain 91-118 and *X. gardneri* (*Xg*) strain 101 (ATCC 19865). The genomes were compared with each other and with the previously sequenced *Xcv* strain 85-10. In addition, the molecular features were predicted that may be required for pathogenicity including the type III secretion apparatus, type III effectors, other secretion systems, quorum sensing systems, adhesins, extracellular polysaccharide, and lipopolysaccharide determinants. Several novel type III effectors from *Xg* strain 101 and *Xv* strain 1111 genomes were computationally identified and their translocation was validated using a reporter gene assay. A homolog to Ax21, the elicitor of XA21-mediated resistance in rice, and a functional Ax21 sulfation system were identified in *Xcv*. Genes encoding proteins with functions mediated by type II and type IV secretion systems have also been compared, including enzymes involved in cell wall deconstruction, as contributors to pathogenicity.

Conclusions: Comparative genomic analyses revealed considerable diversity among bacterial spot pathogens, providing new insights into differences and similarities that may explain the diverse nature of these strains. Genes specific to pepper pathogens, such as the O-antigen of the lipopolysaccharide cluster, and genes unique to individual strains, such as novel type III effectors and bacteriocin genes, have been identified providing new clues for our understanding of pathogen virulence, aggressiveness, and host preference. These analyses will aid in efforts towards breeding for broad and durable resistance in economically important tomato and pepper cultivars.

Background

Bacterial spot disease of tomato and pepper presents a serious agricultural problem worldwide, leading to significant crop losses especially in regions with warm and humid climate. The disease is characterized by necrotic lesions on leaves, sepals and fruits, reducing yield and fruit quality [1]. The disease is caused by a relatively diverse set of bacterial strains within the genus *Xanthomonas*; strain nomenclature and classification for the strains that infect pepper and tomato have gone through considerable taxonomic revision in recent years. Currently, the pathogens are classified into four distinct pathogen groups (A, B, C, and D) within the genus *Xanthomonas*. Strains belonging to groups A, B and D infect both tomato and pepper. Group C strains are pathogenic only on tomato [2,3]. These phenotypically and genotypically distinct strains have different geographic distributions. Strains of group A and B are found worldwide. C strains have been increasingly found in the U.S., Mexico, Brazil, Korea and regions bordering

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the Indian Ocean, and D group strains are found in the former Yugoslavia, Canada, Costa Rica, U.S, Brazil and regions of the Indian Ocean [4-8]. Three of the four groups except for D were originally described as a single pathovar within *Xanthomonas campestris* and referred to as *X. campestris* pv. *vesicatoria*. The D group consisted of a strain isolated from tomato that had been designated '*Pseudomonas gardneri*' for many years [9] although De Ley provided evidence for placement in the genus *Xanthomonas* [10]. Subsequently all four groups were classified as separate species on the basis of physiological and molecular characteristics as follows: *Xanthomonas euvesicatoria* (group A), *Xanthomonas vesicatoria* (group B), *Xanthomonas perforans* (group C), and *Xanthomonas gardneri* (group D) [11].

Based on 16S rRNA analysis, *X. euvesicatoria* strain 85-10 (A group) and *X. perforans* (C group) together form a monophyletic group, whereas *X. vesicatoria* (B group) and *X. gardneri* (D group) cluster together with *X. campestris* pv. *campestris* (*Xcc*) *Xcc* strain 33913 [11]. Recently, a phylogenetic tree was constructed based on MLST (multi-locus sequence typing) data for A, B, C and D group strains and other xanthomonads [12]. The MLST approach revealed that *X. euvesicatoria* and *X. perforans* form a group along with *X. citri* strain 306 (*Xac*). *X. gardneri* is most closely related to *X. campestris* pv. *campestris* strains while *X. vesicatoria* forms a distinct clade [12]. This diversity among the four groups makes the *Xanthomonas*-tomato/pepper system an excellent example to study pathogen co-evolution, as distinct species have converged on a common host.

While integrated management approaches for control of bacterial spot disease are available, the development of host resistance is more economical and environmentally benign for the control of the disease [13,14]. Host resistance may also be required to replace the loss of some integrated management tools. Use of copper and streptomycin sprays over the years, for example, has led to the development of resistant strains [5]. At the same time, genetic resistance has been lost due to race shifts in pathogen populations [15-17]. Designing new and possibly durable resistance requires knowledge of pathogenicity factors possessed by the four groups.

Many candidate pathogenicity factors have been identified in strains of *Xanthomonas*. A number of virulence factors are employed by xanthomonads to gain entry into leaf or fruit tissue, and gain access to nutrients, while simultaneously overcoming or suppressing plant defenses. Different secretion systems and their effectors have been shown to contribute to the virulence of plant pathogens. The type III secretion system (T3SS) encoded by the *hrp* (Hypersensitive Response and Pathogenicity) gene cluster [18,19] and type III secreted effectors have been widely studied for their role in hypersensitivity and pathogenicity. Effectors common between strains are believed to be responsible for conserved virulence function and avoidance of host defense. Differences in effector suites have evolved in closely related strains of plant pathogens and strain-specific effectors may help to escape recognition by host-specific defenses [20-25]. Important insights into pathogenicity mechanisms of *X. euvesicatoria* strain 85-10 (hereafter, *Xcv*) have been obtained with its genome sequence [26]. Here we report draft genome sequences of type strains of the other three bacterial spot pathogen species: *X. vesicatoria* strain 1111 (*Xv* 1111) (ATCC 35937), *X. perforans* strain 91-118 (*Xp* 91-118), and *X. gardneri* strain 101 (*Xg* 101) (ATCC 19865). We have annotated and analyzed predicted pathogenicity factors in the draft genomes. Additionally, we have investigated differentiation between xanthomonads that might explain differences in disease phenotypes and in host range.

Results and Discussion

Draft genome sequences of *Xv* strain 1111, *Xp* strain 91-118 and *Xg* strain 101 were obtained by combining Roche-454 (pyrosequencing) and Illumina GA2 (Solexa) sequencing data

Initially, we sequenced *Xv* strain 1111 (ATCC 35937) (hereafter *Xv*), *Xp* strain 91-118 (hereafter *Xp*) and *Xg* strain 101 (ATCC 19865) (hereafter *Xg*) by 454 pyrosequencing [27]. *De novo* assembly using Newbler assembler resulted in 4181, 2360 and 4540 contigs, respectively, for *Xv*, *Xp* and *Xg*, with approximately 10-fold coverage for each strain (Additional file 1: Table S1). Many pathogenicity genes, including type III effectors, existed in the form of fragments given the relatively low coverage of the 454-based assembly. More complete assemblies were obtained using Illumina sequencing [28]. *De novo* assemblies of around 100-fold coverage were constructed from the Illumina data alone or combined with pre-assembled 454 long reads using CLC Genomic Workbench [29]. Combined 454 and Illumina sequencing produced a much better assembly than either technology alone (Table 1). Therefore, combined assemblies were chosen for all subsequent analyses. The average contig size in the combined 454 and Illumina assemblies was around 18 kb for *Xv* and *Xp*, and 10 kb for *Xg*. The N50 (minimum number of contigs needed to cover 50% of the assembly) values were 37 and 40 for *Xv* and *Xp*, respectively, and 83 for *Xg* indicating that final assemblies consist of a few large contigs allowing reasonably accurate whole genome comparisons.

The three strains were deduced to contain plasmids as evidenced by the presence of genes that are known to be involved in plasmid maintenance (e.g. *parB/F* genes). We have used adjacency to such genes to infer occurrence of certain other genes on plasmids.

Table 1 General sequencing and combined (454 and solexa) *de novo* assembly features of draft genomes of *Xv*, *Xp* and *Xg*

	Xanthomonas vesicatoria (*Xv*)	*Xanthomonas perforans* (*Xp*)	*Xanthomonas gardneri* (Xg)
Number of contigs	296	291	552
N50*	37	40	83
Mean contig length	18,686	18,082	10,014
Longest contig	153,834	133,836	88,536
Total length of contigs	5,531,090	5,262,127	5,528,125

*N50 - number of contigs that cover 50% of the genome assembly.

Relationships of the strains to other xanthomonads using whole genome comparisons

16S rRNA analysis and MLST-based phylogenetic analysis showed the diversity among the four bacterial spot species. We carried out phylogenetic analysis based on orthologous protein-coding genes from draft genomes and reference xanthomonads (Figure 1). Whole genome comparisons were performed using the MUMi index [30] to assess pairwise distance between the draft genomes and available reference *Xanthomonas* genomes as shown in the phylogenetic tree and the distance matrix (Additional file 2: Fig. S2). Another program, dnadiff, based on nucmer [31] showed the extent of homologies among the shared regions of the genomes by pairwise comparisons (Additional file 3: Table S3). All of the methods yielded consistent results: we were able to ascertain that among the three newly sequenced strains in relationship to the previously sequenced strains, *Xp* and *Xcv* form the closest pair, which is in turn closest to *Xac*. Next, *Xg* is closest to *Xcc*, with *Xv* forming a clade with *Xg* and the *Xcc* species group (Figure 1, Additional files S2 and S3).

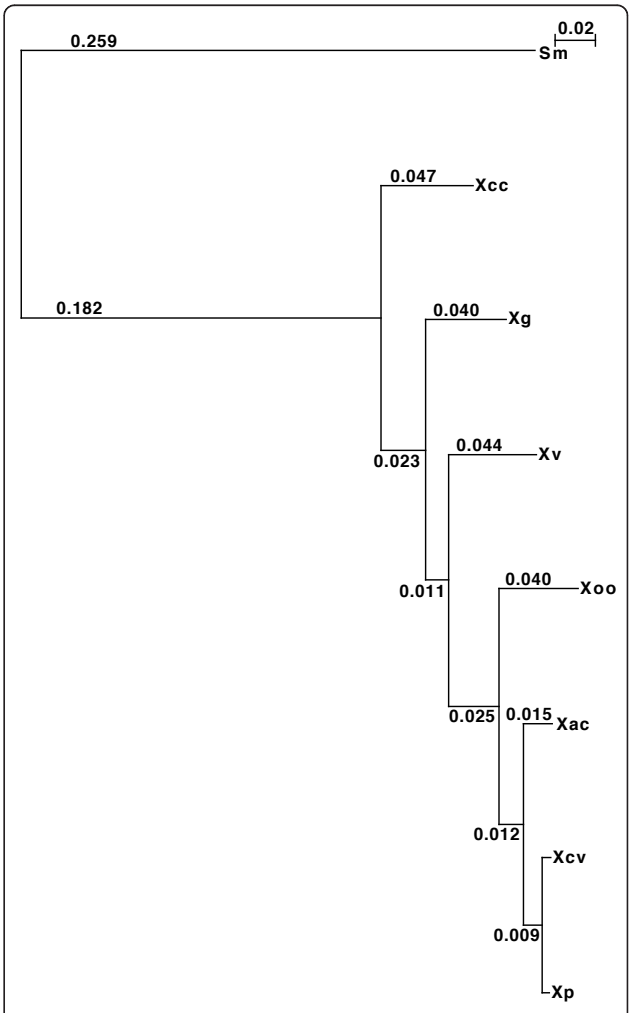

Figure 1 Maximum likelihood tree based on orthologous genes from xanthomonads and *Stenotrophomonas*. Concatenated amino acid sequences of the orthologous genes from four bacterial spot pathogen strains along with other sequenced xanthomonads were considered in the analysis. *Stenotrophomonas maltophilia* (*Sm*) was used as an outgroup. The evolutionary history was inferred using the Maximum likelihood method. The tree is drawn to scale, with branch lengths corresponding to the evolutionary distances. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

Four xanthomonads show variation in the organization of the type III secretion gene clusters

Annotation of the respective type III secretion gene clusters, or *hrp* genes showed that *Xp* has an almost identical and syntenic *hrp* cluster to that of *Xcv* (Figure 2). The most notable difference is that *hpaG* and *hpaF* encode the fusion protein XopAE in *Xp*, while they are present as separate genes in *Xcv*. Adjacent hypothetical protein XCV0410 (126 amino acid protein) is absent from *Xp*. *Xv* and *Xg* show greater similarity to the core *hrp* cluster genes of *Xcc* than to that of *Xcv*. *Xv* and *Xg* contain *hrpW* associated with the *hrp* cluster as in *Xcc*. Additionally, *xopD* in *Xv* and *Xg* is not associated with the *hrp* cluster as in *Xcc* (referred to as *psv* in *Xcc*). *PsvA* shows 74% and 84% sequence identity to the respective homologs from *Xv* and *Xg*. *XopA* (*hpa1*) from *Xcv* seems to be absent from *Xv* and *Xg*. Interestingly, we found a novel candidate effector gene (named *xopZ2*) upstream of *hrpW* in *Xv* and *Xg* (See below, Additional file 4: Fig. S4).

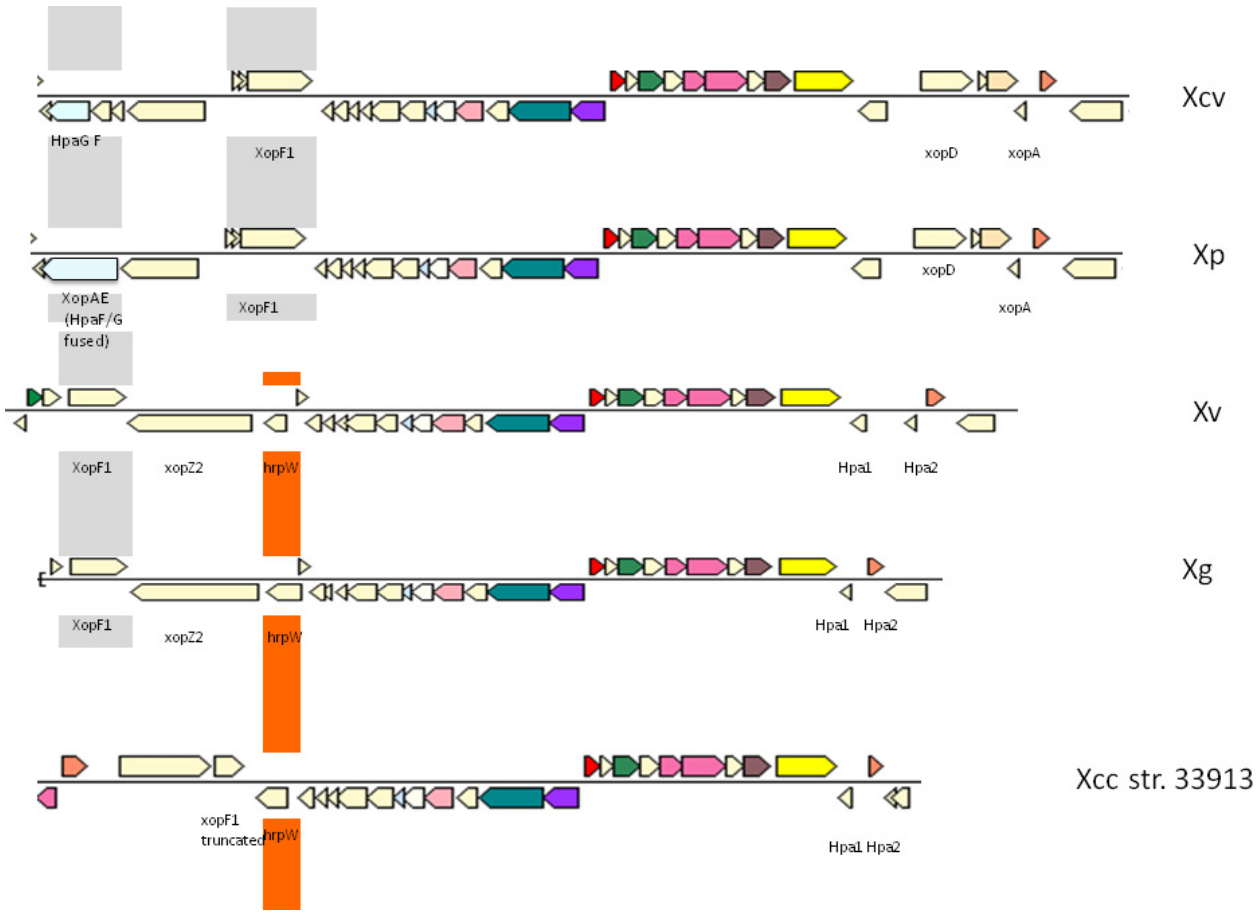

Figure 2 Comparison of type III secretion system cluster, its associated type III effector genes and helper genes of three draft genomes with already sequenced xanthomonads. Type III secretion gene clusters in five strains are shown. Boxes of the same colour indicate orthologous genes. Genes of special interest discussed in the paper are labeled. *Xp* has near identical *hrp* cluster as *Xcv*; *Xv* and *Xg* contain mosaic *hrp* cluster with organization and gene content similar to *Xcc*, but associated effectors are similar to *Xcv* along with novel effector gene associated with the cluster.

Finally, the *hrp*-associated effector *xopF1* is conserved and intact in all four tomato and pepper pathogens.

A reporter gene assay confirms translocation of novel type III effectors

We identified and annotated T3SS effectors from the three newly sequenced xanthomonads (See Methods). Several candidate effectors, which had not yet been experimentally confirmed in xanthomonads, and candidate effectors with plausible translocation motifs were identified (Tables 2, 3, and 4). Corroborative evidence for T3SS-mediated translocation of the candidate effectors was assessed by constructing fusion genes with the C-terminal end of AvrBs2 coding sequence ($avrBs2_{62-574aa}$) in a race 6 strain of *X. euvesicatoria*. Translocation was measured in pepper cv. ECW 20R, containing the resistance gene *Bs2* (Additional file 4: Fig. S4). Genes *xopAO*, *xopG*, *xopAM*, and XGA_0724 (belonging to the *avrBs1* class of effectors), of which homologs were previously found in *Pseudomonas* species, were demonstrated to direct AvrBs2-specific hypersensitive reactions in ECW 20R (Tables 3, Table 4, Additional file 4: Fig. S4). Another candidate effector gene *xopZ2*, associated with the *hrp* clusters in *Xv* and *Xg* (Figure 2), was also functional in the AvrBs2-based assay. Thus, we identified five effectors (*xopAO*, *xopG*, *xopAM*, *xopZ2*, XGA_0724) that have not been previously recognized in *Xanthomonas* and showed their functionality.

Core effectors among four xanthomonads give insight into infection strategies of the pathogen

Comparing the draft genome sequences of the three xanthomonads with that of *Xcv* allowed us to identify the core effectors conserved in all four strains as well as strain-specific effectors (Tables 2, 3, and 4).

Table 2 Core effectors present in all four tomato and pepper xanthomonads

Effector class	*Xcv*	*Xv*	*Xp*	*Xg*	Pfam domains	References
AvrBs2	XCV0052	XVE_4395	XPE_2126	XGA_3805	Glycerophosphoryl diester phosphodiesterase	[104]
XopD	XCV0437	XVE_2372	XPE_2945	XGA_3151	C48-family SUMO cysteine protease (Ulp1 protease family); EAR motif	[105]
XopF1	XCV0414	XVE_3220	XPE_2922	XGA_2763	-	[105]
XopK	XCV3215	XVE_0354	XPE_1077	XGA_3563	-	[106]
XopL	XCV3220	XVE_0359	XPE_1073	XGA_0320	LRR protein	[107]
XopN	XCV2944	XVE_0564	XPE_1640	XGA_0350	ARM/HEAT repeat	[108]
XopQ	XCV4438	-	XPE_0810	XGA_0949	Inosine uridine nucleoside N-ribohydrolase	[105]
XopR	XCV0285	XVE_0593	XPE_1215, XPE_3295	XGA_1761	-	[106]
XopX	XCV0572	XVE_ 3610 XVE_3609 (partial)	XPE_1488 XPE_1553	XGA_3272 (second copy with frameshift)	-	[109]
XopZ1	XCV2059	+ (*)	XPE_2869	+(*)	-	[106]
XopAD	XCV4315/ 4314/4313	XVE_4177	XPE_4269	XGA_0755	SKWP repeat protein	[110]

*Xv and Xg contain effector *xopZ2* belonging to the same family *xopZ*.

Table 3 Type III effectors specific to each species

Effector	Locus tags	Effector homolog	Pfam domains/biochemical motifs	Comments/Reference
Effectors specific to *Xv*				
XopJ2	XVE_4840 (partial); XVE_3769 (partial)	AvrBsT	C55-family cysteine protease or Ser/Thr acetyltransferase	[40]
XopAG	XVE_2415	AvrGf1	-	[39]
XopAI	XVE_4756	XAC3230	-	[25]
Effectors specific to *Xg*				
class avrBs1	XGA_0724	AvrA (84% identity)	-	This study
AvrHah1 (Fragmented in assembly)	XGA_4845/XGA_3187	AvrBs3	Transcriptional activator, nuclear localization	AvrBs3 present in few euvesicatoria strains [41].
XopAO	XGA_1250	AvrRpm1 (61% identity)	-	This study
XopAQ	XGA_2091	Rip6/rip11	No known domains	[46]
XopAS	XGA_0764/0765	HopAS1	No known domains	This study
Effectors specific to *Xp*				
XopC2	XPE_3585	Rsp1239	Haloacid dehalogenase-like hydrolase	[24]
XopJ4	XPE_1427	AvrXv4	SUMO protease (experimental); YopJ-like serine threonine acetyl transferase domain (predicted)	[38,105]
XopAF	XPE_4185	AvrXv3	Transcriptional activator domain	[37]
XopAE	XPE_2919	HpaF/G	LRR protein	[24]
Effectors specific to *Xcv*				
AvrBs1	XCVd0104	AvrBs1	-	[26]
XopC1	XCV2435	XopC	Phosphoribosyl transferase domain and haloacid dehalogenase-like hydrolase	[105]
XopJ1	XCV2156	XopJ	C55-family cysteine protease or Ser/Thr acetyltransferase	[105]
XopJ3	XCV0471	AvrRxv	C55-family cysteine protease or Ser/Thr acetyltransferase	[26]
XopO	XCV1055		Unknown	[26]
XopAA	XCV3785	ECF	Early chlorosis factor, proteasome/cyclosome repeat	[26]
XopAI	XCV4428	AvrRxo1	-	[26]

Table 4 Effectors specific to particular groups of species

Effector class	Locus tags	Pfam domains	Comments/References
Effectors common to all pepper pathogens *Xv*, *Xcv* and *Xg*			
XopE2	XCV2280, XVE_1190, XGA_2887	Putative transglutaminase	[114]
XopG	XCV1298, XVE_4501, XGA_4777	M27 family peptidase clostridium toxin	This study
Effectors common to *Xv*, *Xg* but absent from *Xp* and *Xcv*			
XopAM	XVE_4676, XGA_3942	-	This study
HrpW	XVE_3222, XGA_2761	Pectate lyase	HrpW associated with hrp cluster, May not be T3SE [111]
AvrXccA1	XVE_5046, XGA_0679	LbH domain containing hexapeptide repeats (X-[STAV]-X-[LIV]-[GAED]-X)- acyltransferase enzyme activity	May not be T3SE [112]
XopZ2	XGA_2762, XVE_3221	Not known	This study; Associated with hrp cluster.
Effectors common to *Xg* and *Xcv* but absent from *Xp* and *Xv*			
XopB	XGA_4392, XCV0581	-	[113]
Effectors common to *Xp* and *Xcv* but absent from *Xg* and *Xv*			
XopE1	XPE_1224, XCV0294	Putative transglutaminase	[114]
XopF2	XPE_1639, XCV2942	-	[105]
XopI	XPE_3711, XCV0806	F-box domain	[115]
XopP	XPE_3586, XPE_4695 (Partial), XCV1236		[105]
XopV	XPE_4158, XCV0657	-	[106]
XopAK	XPE_4569, XCV3786	-	Not confirmed to be effector in Xanthomonas; Homolog of effector in *Pseudomonas*.
XopAP	XPE_1567, XCV3138	Lipase class III	45% identity to homolog in *Xp*; Homolog of rip38 from R. solanacearum RS1000 [46]
Effectors present in *Xv* and *Xp* but absent from *Xg* and *Xcv*			
XopAR	XVE_3216, XPE_2975	-	[46]

At least 11 effector genes form a core set of common effectors for xanthomonads infecting tomato and pepper (Table 2). Of these 11, eight effector genes (*avrBs2, xopK, xopL, xopN, xopQ, xopR, xopX* and *xopZ*) were found to be conserved in all sequenced xanthomonads including the three draft genomes presented here with the exceptions of *X. albilineans* and *X. campestris* pv. *armoraciae*. These genes might be necessary for maintaining pathogenicity of these xanthomonads in a wide range of host plants. XopN has been reported to suppress PAMP (pathogen-associated molecular pattern)-triggered immunity by interacting with tomato TARK1 and TFT1 [32]. *XopF1* is conserved in tomato and pepper xanthomonads. Although a homolog of *xopF1* is found in *Xcc*, the respective gene is truncated [34]. Hence, *xopF1* is a potential pathogenicity determinant in tomato. A *xopF1* deletion mutant of *Xcv* did not show any difference in virulence when compared to wild type *Xcv* on the susceptible cultivar of pepper ECW, suggesting XopF1 is not the lone factor for pathogenicity of *Xcv* on pepper [33]. Another effector gene, *xopD*, is associated with the *hrp* gene cluster in *Xcv* and *Xp*. However, *xopD* appears to have translocated to another location in the genome in case of *Xg, Xv* and *Xcc* strains. XopD is annotated as "Psv virulence protein" in *Xcc* genome [34] and has been shown to be a chimeric protein sharing a C terminus with XopD from *Xcv* [35]. Although *xopD* homologs from *Xv* and *Xg* are syntenic with the *psv* gene in *Xcc*, *Xv* and *Xg* have intact full-length copies of *xopD* as in *Xcv*, indicating that the x*opD* could be another effector exclusive to the tomato pathogens and a possible pathogenicity determinant in tomato. *XopD* has been shown to enhance pathogen survival in tomato leaves by delaying symptom development [36]. Two tandem copies of *xopX* are found in *Xg*. However, one gene in *Xg* appears to be inactive due to a frameshift mutation. In *Xp*, the two copies of *xopX* are found in different locations in the genome with neighboring genes, including chaperone gene *groEL*, which is also duplicated. Orthologs of *xopZ* are also found in all four xanthomonads, with 82% identity for *Xcv* and *Xp* and 35% identity for *Xg* and *Xv*. Apart from low sequence identity in *Xv* and *Xg*, gene-specific rearrangements appear to have occurred within each ortholog. We propose that the overall low amino acid relatedness (pairwise sequence identities below 50%) of this effector

in *Xv* and *Xg* warrants assigning the proteins to a new family within the *xopZ* class, named x*opZ2*, while the orthologs from *Xcv* and *Xp* belong to family of *xopZ1* as originally described in *Xoo* and as supported by pairwise sequence identities of at least 60% (see above, Figure 2, Table 4).

Effectors unique to *Xp* might be responsible for restricting growth on pepper

Xp is pathogenic only on tomato. The avirulence gene, *avrXv3*, present in *Xp*, was previously shown to elicit an hypersensitive response (HR) in pepper cv. ECW [37]. An *avrXv3* knockout mutant of *Xp* is not virulent in pepper cv. ECW indicating that other factors are associated with host specificity. Comparing effector repertoires of the pepper pathogens *Xg*, *Xcv*, and *Xv* with *Xp* may provide clues to the factors that are responsible for reduced virulence (Table 4). Besides *avrXv3*, the only effectors present in *Xp* and absent or inactive in *Xg*, *Xv* and *Xcv* are *xopC2*, *xopAE* and *xopJ4* (*avrXv4*) (Table 3). The gene *avrXv4* is absent from other sequenced xanthomonads and shows gene-for-gene interaction with the *Xv4* resistance gene from the wild tomato relative *Solanum pennellii* but does not contribute to restricted growth of *Xp* on pepper [38]. The effector *xopC2* is a homolog of the effector *rsp1239* from *Ralstonia solanacearum* GMI1000 and *xopAE* encodes an LRR protein with homology to the *R. solanacearum* effector PopC. Both genes, *xopC2* and *xopAE*, are truncated in *Xcv*. Therefore, these two effectors may trigger immunity in pepper. Interestingly, *Xp* contains a paralog of *xopP*. The two copies are found next to each other in the genome and share 75% identity at the amino acid level. The second copy is next to the candidate effector *xopC2*, which is unique to *Xp* among tomato and pepper pathogens. Effectors *xopC2* and *xopP* may both act to restrict growth in pepper. Moreover, there are at least two effectors, *xopE2* and *xopG*, present in the pepper pathogens *Xcv*, *Xv* and *Xg* but absent from *Xp*. These effectors may be essential pathogenicity factors in pepper.

Species-specific effectors

Xv possesses two unique effector genes, *xopAG* (*avrGf1*) and *xopAI* (Table 3). A phylogenetic analysis of *xopAG* showed that *xopAG* from *Xv* is closely related to *xopAG* from *X. citri* A^w, which has been shown to be responsible for causing an HR on grapefruit [39]. XopAI is a chimeric protein, which contains a conserved myristoylation motif at its N terminus, like XopJ1. This effector class also includes the homolog *XAC3230* from *Xac* as well as XAUB_26830 and XAUC_23780 from *X. fuscans* subsp. *aurantifolii* strains B and C, respectively [25]. The presence of transposons and phage elements in close proximity helps to explain the evolution of this novel effector in *Xac* by terminal reassortment [35]. *Xv* also contains effector gene *avrBsT*, which is responsible for the hypersensitive response on pepper. Loss of the plasmid containing *avrBsT* in *Xcv* strain 75-3 allows the strain to cause disease on pepper [40].

Xg contains at least two effectors, *avrHah1* (an *avrBs3*-like effector gene) and *xopB as* does *Xcv*, and share sequence identity of 82% and 86% respectively to the corresponding effectors of *Xcv*. However, AvrHah1 appears to specify a different phenotype when compared to avrBs3 from *Xcv*. *AvrHah1* was shown to be responsible for increased watersoaking on pepper ECW-50R and 60R, whereas *Xcv* strains carrying *avrBs3* show a phenotype that consists of small raised fleck lesions on pepper [41]. Another effector gene, *xopB*, has a PIP box at the 5' end in *Xcv*, whereas the homolog in *Xg* does not contain a PIP box. Neighboring genes to *xopB* in the respective strains are completely different between genomes, suggesting lack of synteny between the two species in this region (Table 4). *XopB* from *Xg* is 92% identical at the amino acid level to the homolog in *Xcv*. Deletion mutants of *xopB* from *Xcv* did not show any difference in virulence, indicating it does not contribute significantly to virulence [42]. However, *xopB* may contribute to virulence in *Xg*. We also identified eight effector genes that are unique to *Xcv* (Table 3). With the exception of *xopAA* (early chlorosis factor), all of these genes belong to regions of low GC content compared to average genome GC content (64.75%): *avrBs1* (42%), *xopC1* (48%), *xopJ1* (*xopJ*) (57%), *xopJ3* (*avrRxv*) (52%), *xopO* (52%), *xopAJ* (*avrRxo1*) (51%).

Few effectors are shared among phylogenetically related group strains

Although *Xp* and *Xcv*, and *Xv* and *Xg* form distinct phylogenetic groups (Figure 1), relatively few effectors are shared between these species. For *Xp* and *Xcv*, they share at least six effectors - *xopE1, xopF2, xopP, xopV, xopAK, xopAP*, which are absent from the other two genomes (Table 4). *Xv* and *Xg* appear to be most closely related to strains of *X. campestris* pv. *campestris*, and this relationship is reflected in the suite of effector genes. In fact, *Xg* and *Xv* share four effector genes with *Xcc*, namely, *xopAM, avrXccA1, hrpW* and *xopZ2*, with the caveat that *hrpW* and *avrXccA1* may not function as intracellular effectors (Table 4). Furthermore, the genomic regions containing these genes are syntenic in *Xg*, *Xv* and *Xcc*.

X. gardneri shows evidence of effector acquisition by horizontal gene transfer

Effector homologs of *avrA, hopAS1* and *avrRpm1* from *P. syringae* pv. *tomato* T1 and *P. syringae* pv. *syringae*

B728a are found in *Xg* with 79%, 41% and 61% identity at the amino acid level, respectively (Table 3, Additional file 4: Fig. S4). Other *X. gardneri* strains also contain these effectors based on PCR screening (data not shown). These three effectors, XGA_0724 (belonging to *avrBs1* class), XGA_0764/XGA_0765 (*xopAS*) and XGA_1250 (*xopAO*), are unique to *X. gardneri*. The C terminal region of XGA_0724 shows 53% identity to *avrBs1* from *Xcv*. Hence according to the *Xanthomonas* effector nomenclature [24], XGA_0724 from *Xg* was placed under the class *avrBs1*. XGA_0764/XGA_0765 and XGA_1250 have not yet been reported to be found in xanthomonads and were assigned to new classes *xopAS* and *xopAO*. *X. gardneri* strains have been found to be associated with tomato and have a lower optimum temperature for disease development similar to that of pathovars of *Pseudomonas syringae* [43]. A high score by Alien_hunter analysis [44], along with very low GC content (45% for XGA_0724 and 48% for XGA_01250, 59% for XGA_0764/XGA_0765) and the proximity of mobile genetic elements provides evidence for horizontal gene transfer (Additional file 5: Table S5). Effector *xopAS* appears to be separated into two ORFs XGA_0764 and XGA_0765 by internal stop codon. The functionality of effector *xopAS* needs to be confirmed by *in planta* reporter gene assay. *AvrA* of *P. syringae* pv. *tomato* PT23 was shown to contribute to virulence on tomato plants [45]. Acquisition of XGA_0724 by *Xg* might have conferred increased virulence on tomato. *AvrRpm1* from *P. syringae* pv. *syringae* possesses a myristoylation motif, which is absent from homologs in *Xg*. This modification in *Xg* might have been acquired to escape host recognition. Another candidate effector gene, *xopAQ*, in *Xg* is found 68 bps downstream of a perfect PIP box. The gene shows 65% identity at the amino acid level to *rip6/11*, a novel effector from *R. solanacearum* RS1000 [46].

All four xanthomonads contain Ax21 coding gene but only *Xcv* contains a functional sulfation gene

The *ax21* (activator of XA21-mediated immunity) gene is conserved among *Xanthomonas* species and is predicted to encode a type I-secreted protein that may serve as a quorum sensing signaling molecule [47]. A 17-amino acid sulfated peptide from the N-terminal region of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Ax21 (axY^S22) was shown to bind and activate the XA21 receptor kinase from rice, demonstrating that Ax21 is a conserved PAMP that can activate plant immune signaling [48]. The *ax21* gene is present in *Xcv* (93% identity with *Xoo* PXO99 protein), *Xp* (94%), *Xv* (91%), and *Xg* (88%). The axY^S22 peptide is 100% conserved in *Xcv*, *Xp* and *Xv*, while in *Xg* there is a change from leucine to isoleucine at residue 20; this is unlikely to alter the activity of the peptide, since changing this residue to alanine had no effect on recognition by XA21 [48].

Recognition of axY^S22 by the XA21 receptor requires sulfation of tyrosine 22, which requires the putative sulfotransferase RaxST. In contrast to *ax21*, the *raxST* gene is more variable in these genomes, which is consistent with a report of sequence differences in this gene among *Xoo* strains [49]. Furthermore, in *Xp*, there is a single-nucleotide insertion at position 65, causing a frameshift mutation. The *Xv* and *Xg* genomes do not contain *raxST*; therefore, the *ax21* gene products may be nonfunctional in these strains. These findings have implications for the further study of the role of Ax21 in quorum sensing and virulence, as well as for the usefulness of the XA21 receptor to confer resistance to xanthomonads in crop plants.

Two type II secretion systems are conserved in all four *Xanthomonas* genomes

Most cell-wall degrading enzymes, such as cellulases, polygalacturonases, xylanases, and proteases, are secreted by a type II secretion system (T2SS). The Xps T2SS, present in all xanthomonads, has been studied for its contribution to virulence in *Xcc* and *Xoo* [50,51]. Another T2SS cluster, known as the Xcs system, is found only in certain species of *Xanthomonas*, e.g. *Xcc*, *Xac*, and *Xcv*. The Xps system secretes xylanases and proteases and is under control of *hrpG* and *hrpX* [52], indicating differential regulation. Both Xps and Xcs systems are present in all three draft genomes.

Xanthomonads possess diverse repertoires of cell-wall degrading enzymes, which are present in diverse genomic arrangement patterns

Each species of *Xanthomonas* has its own collection of genes encoding endoxylanases, endoglucanases, and pectate lyases which contribute to cell wall deconstruction during pathogenesis. We have compared these repertoires from the three draft genomes and other xanthomonads as detailed in Table 5. The genes are designated for different families of glycosyl hydrolases (GH) and polysaccharide lyases (PL) that include the enzymes that cleave glycosidic bonds in the structural polysaccharides of plant cell walls.

Genes encoding secreted endoxylanases regulated by the x*ps* genes have been described for their contributions to virulence, including XCV0965 [52] encoding GH30 endoxyalanase. The GH30 family catalyses the cleavage of methylglucuronoxylans in the cell walls of monocots and dicots at a β-1,4-xylosidic bond penultimate to one linking the xylose residue that is substituted by an α-1,2-linked 4-O-methylglucuronate residue [53,54]. Such an enzyme secreted by *Erwinia chrysanthemi* generates oligosaccharides that are not

Table 5 Repertoire of cell wall degrading enzymes in xanthomonads

Gene name	Family	Enzymatic function	*Xp*	*Xac*	*Xcv*	*Xv*	*Xg*	*Xcc* strain 33913	*Xoo* strain KACC
Xylanases									
xyn10A	GH10	Endo-β-1,4-xylanase EC:3.2.1.8	2014	4254	4360	2337	1172	4118	4429
xyn10B			2016	4252	4358	-	-	-	4428
xyn10C			2020	4249	4355	2333	0341	4115	-
aguA	GH67	α-glucuronidase EC:3.2.1.139	4318	4227	4333	4712	2473	4102	4419
xyn51A	GH51	β-D-Arabino-furanosidase EC:3.2.1.55	0180	1286	1335	1029/1030	2303	1191	1317
xyn5A	GH5	Endo-β-1,4-xylanase EC:3.2.1.8	4682	0933/34 partial	0965	-	-	0857	3618
Glucanases									
cel8A	GH8	Endo-1,4-β-D glucanase	1965	3516	3641	0432	-	-	-
cel9A	GH9		2345	2522	2704	1327	0588	2387	-
Pectate lyases									
pel1A	PL1	Pectate lyase EC:4.2.2.2	3841	3562	3687	1933	4024	0645	0821
pel1B			1563	2986	3132	3512	0893	2815	-
pel1C			-	2373	2569	-	-	-	-
pel3A	PL3	Pectate lyase EC:4.2.2.2	-	2922	-	3222	2761	1219	-
pel4A	PL4	Rhamno-galacturonan lyase EC:4.2.2.-	1975	3505	3632	2592	4531	3377/78/79	1078
pel9A	PL9	Pectate lyase EC:4.2.2.2	-	-	2278	1927	1853	-	2265
pel10A	PL10	Pectate lyase EC:4.2.2.2	-	-	-	4069	5124	0122	-

Different cell wall degrading enzymes, such as xylanase, pectate lyase, glucanases, were compared for their repertoires among already sequenced xanthomonads including our draft genomes. Genes identified by their locus tags in the respective genomes.

assimilated for growth, suggesting a function in which it contributes to cell wall deconstruction for access to pectates for growth substrate [53]. It is interesting to note the orthologous genes encoding GH30 enzymes are absent in *Xg* and *Xv*, with a truncated *xyn30* gene in *Xac*. On the basis of sequence homology, *xyn30* genes may also contribute to virulence in *Xoo, Xcc and Xp*.

The more common GH10 endoxylanases, which occur in several bacterial and fungal phyla, have been implicated in the virulence of plant pathogenic bacteria and fungi [55,56]. In *Xoo*, deletion of the gene encoding a GH10 *xyn10B* resulted in diminished virulence [57]. All sequenced *Xanthomonas* genomes contain either two or three copies of *xyn10* genes, all of which are within a gene cluster that may comprise a single operon (Figure 3). The GH10 endoxylanases are the best studied of all of the xylanases, and structure/function relationships may be inferred on the basis of gene sequence. The action of these enzymes on glucuronoxylans generates xylotriose, xylobiose, and small amounts of xylose that generally serve as substrates for growth. Also generated is methylglucuronoxylotriose, that is formed to the extent that xylose residues in the β-1,4 xylan backbone are substituted with α-1,2-linked 4-O-methylglucuronate residues [58].

An adjacent gene cluster in an opposite orientation contains *agu67* gene encoding a GH67 α-glucuronidase that serves to catayze the removal of 4-O-methylglucuronate from the reducing terminus of methylglucuronoxylotriose. This activity provides a synergistic function to the overall xylanolytic process to generate xylotriose, which is converted to xylose by xylanases and xylosidases for complete metabolism [59]. The coregulation of operons encoding XynB and Agu67 enzymes occurs as a logical condition to coordinate expression of genes that encode these and additional enzymes that collectively process glucuronxylans and glucuronoarabinoxylans for complete metabolism. The accessory enzymes and transporters necessary for the function of these enzymes are embedded within these operons in Gram positive bacteria [60-62] and share similarities noted here with *Xanthomonas* spp.. These include the genes encoding two glycohydrolases, a β-xylosidase and an α-L-arabinofuranosidase. Also included in this cluster are genes encoding enzymes for intracellular metabolism of

glucuronate and xylose, including glucuronate isomerase; xylulose isomerase; D-mannonate dehydratase; and D-mannonate oxidoreductase. Genes encoding mannitol dehydrogenase and the hexuronate transporter, as well as the TonB-dependent receptor and LacI transcriptional regulator, flank these two operons.

The arrangement and content of xylanolytic enzymes differentiate *Xanthomonas* species into three groups (Figure 3). Here, we propose a common nomenclature for xylanases, the genes for which have been annotated in the sequenced genomes. Members of the first group are *Xac*, *Xcv* and *Xp* in which all three genes encoding GH10 endoxylanases (*xyn10A*, *xyn10B* and *xyn10C*) are present, and with additional genes further downstream in this cluster. Members of the second group are *Xcc*, *Xv* and *Xg* in which genes encoding two of the three endoxylanases are present (*xyn10A* and *xyn10C*) and where one or more of the the downstream genes are absent. *Xoo* strains represent a third group in which a different set of two endoxylanase encoding genes are present (*xyn10A* and *xyn10B*) and where the β-galactosidase and gluconolactonase genes flanking *xyn10C* are absent. It is noteworthy that the organization of genes in the cluster encoding the α-glucuronidase is conserved across *Xanthomonas* species.

Genes involved in several Type IV secretion systems are present in genomes and plasmids

Like *Xcv*, the tomato pathogens, *Xg*, *Xv* and *Xp*, also appear to contain more than one copy of a type IV secretion system (T4SS) cluster (Figure 4A, B). Two T4SS clusters (Vir and Dot/Icm type) are present in *Xcv*, and genes belonging to both of these systems are found on plasmids [26]. The Dot/Icm type system is absent from *Xv*, *Xp* and *Xg*.

In *Xv* and *Xp*, genes for one T4SS are on a plasmid and the second one on the chromosome while in *Xg*, two T4SS gene clusters are on a plasmid and one is on the chromosome. The two T4SS clusters on plasmids of *Xg* do not show any similarity to the genes for T4SS in *Xac*, *Xcv*, *Xcc* and *Xoo*. Of the two T4SS clusters in *Xg*,

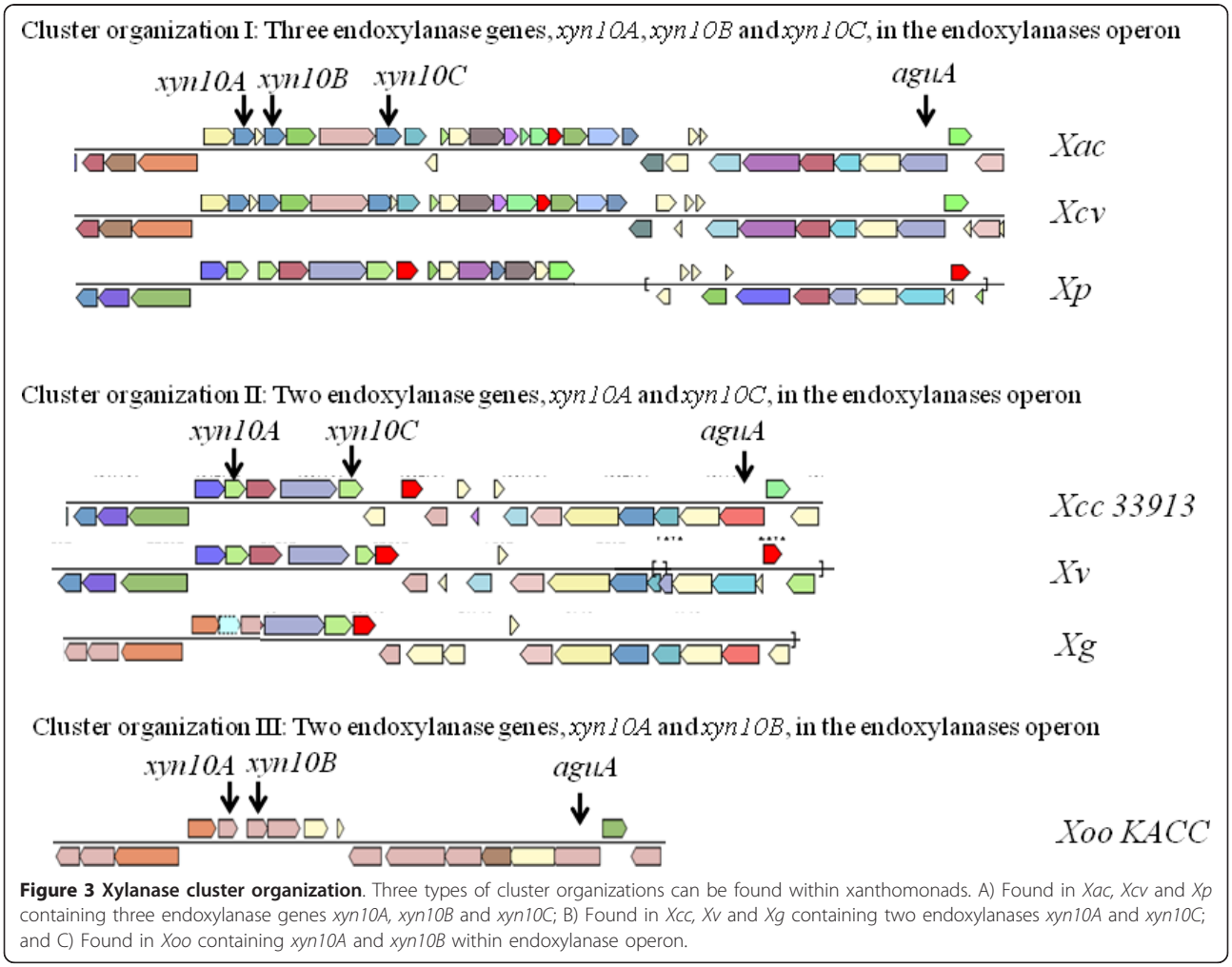

Figure 3 Xylanase cluster organization. Three types of cluster organizations can be found within xanthomonads. A) Found in *Xac, Xcv* and *Xp* containing three endoxylanase genes *xyn10A, xyn10B* and *xyn10C*; B) Found in *Xcc, Xv* and *Xg* containing two endoxylanases *xyn10A* and *xyn10C*; and C) Found in *Xoo* containing *xyn10A* and *xyn10B* within endoxylanase operon.

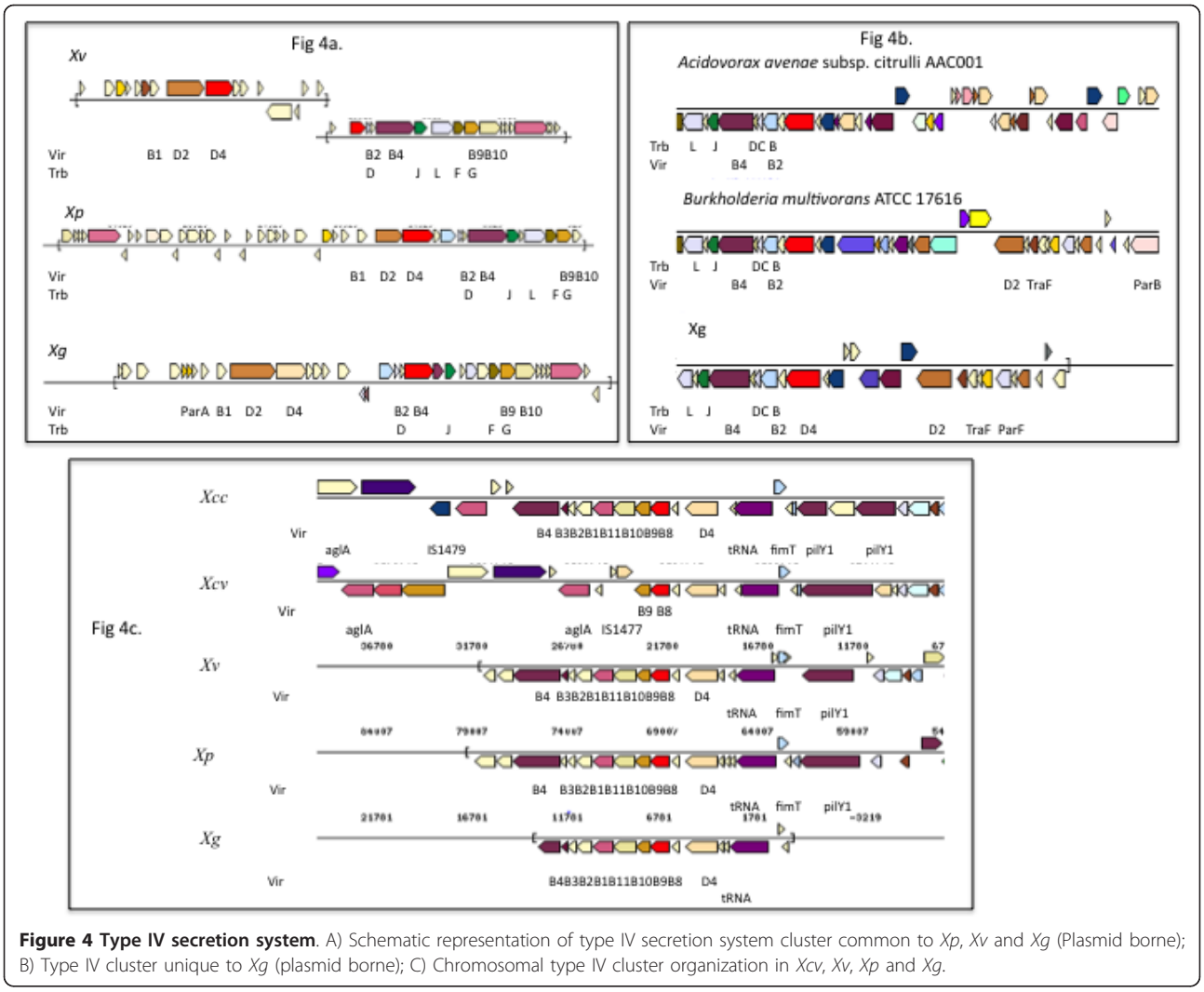

Figure 4 Type IV secretion system. A) Schematic representation of type IV secretion system cluster common to *Xp*, *Xv* and *Xg* (Plasmid borne); B) Type IV cluster unique to *Xg* (plasmid borne); C) Chromosomal type IV cluster organization in *Xcv*, *Xv*, *Xp* and *Xg*.

one is also found in *Xv* and *Xp*. This cluster appears to be exclusive to these three tomato pathogens (Figure 4A). The genes belonging to this cluster show low (30-45%) identity to the T4SS clusters from *Ralstonia, Burkholderia, Bradyrhizobium*, and *Stenotrophomonas maltophilia*. The other cluster from *Xg*, which is absent from *Xv* and *Xp*, shows very high identity (98%) and synteny to the T4SS cluster of *Burkholderia multivorans* and around 89% identity to a T4SS cluster of *Acidovorax avenae* subsp. *citrulli* (Figure 4B).

Apart from the plasmid borne T4SS genes, *Xcv* also contains a portion of a type IV system cluster on the chromosome and consists of *VirB6*, *VirB8, VirB9, VirD4* genes. This chromosomal cluster is flanked by a transposon element (IS1477) that might indicate its horizontal gene transfer. *Xp*, *Xg* and *Xv* genomes contain a complete chromosomal T4SS cluster showing high identity to the T4SS chromosomal clusters from *Xcc* (Figure 4C).

Type V secreted adhesins function in synergism during pathogenesis

Different adhesins have been shown to function at different stages of the infection process starting with attachment, entry, later survival inside host tissue and colonization by promoting virulence [63,64]. FhaB hemagglutinin, important for leaf attachment, survival inside plant tissue and biofilm formation, is present in all four tomato pathogens. In *Xcv*, *fhaB* is divided into two separate open reading frames, XCV1860 and XCV1861, with the two-partner secretion domains being present in XCV1860. Sequence alignment indicates that *fhaB* is possibly inactivated in *Xcv* by the internal stop codon that separates XCV1860 from XCV1861. In the case of *Xoo* PXO99A, the *Xanthomonas* adhesin-like proteins XadA and XadB promote virulence by enhancing colonization of the leaf surface and leaf entry through hydathode [64]. As in *Xcv* and *Xac*, *Xp* encodes

two copies of *xadA*, while *Xv* and *Xg* possess a single ortholog of *xadA* as does *Xcc*. YapH and the type IV pilus protein PilQ were shown to be involved in virulence in *Xoo* during later stages of growth and migration in xylem vessels. In *Xcv*, *Xc*, and *Xoo*KACC, two copies of *yapH* are present. There are two *pilQ* orthologs in *Xcv* and only one in other sequenced xanthomonads. Next to the *fhaB* and *fhaC* adhesin genes, *hms* operon is present in the genomes of xanthomonads, the homologs of which are *pga* operon genes in *E. coli* involved in biofilm formation [65].

Type VI secretion system is present in *Xcv*, *Xv* and *Xp*

Type VI secretion system (T6SS) has been shown recently to contribute to host pathogen interactions during pathogenesis in *Vibrio cholerae*, *Burkholderia pseudomallei* and *Pseudomonas aeruginosa*. Hcp (Haemolysin-coregulated protein) and Vgr (valine-glycine repeats) proteins are exported by the T6SS [66]. T6SS clusters can be assigned to three different types in xanthomonads (Table 6). *Xcv* and *Xp* possess two types of T6SSs (type 1 and 3); whereas *Xv* contains only a single type of T6SS, type 3. As in *Xcc*, there is no T6SS cluster in *Xg* (Table 6, Additional file 6: Table S6).

LPS locus displays remarkable variation in sequence and number of coding genes and shows host specific variation

The lipopolysaccharide (LPS) biosynthesis cluster has been studied in detail in *Xcc* [67], which comprises three regions; region 1 from *wxcA* to *wxcE* involved in biosynthesis of water soluble LPS antigen; region 2 (*gmd, rmd*) coding for LPS core genes; and region 3 from *wxcK* to *wxcO* coding for enzymes for modification of nucleotide sugars and sugar translocation systems. This LPS biosynthesis locus is positioned between highly conserved housekeeping genes, namely cystathionine gamma lyase (*metB*) and electron transport flavoprotein (*etfA*), as reported in other xanthomonads [68]. Comparison of this cluster from draft genomes to the already sequenced xanthomonads revealed high variability in the number of genes and their sequences. *Xv* and *Xg* have an identical type of LPS gene cluster of 17.7 kb encoding 14 open reading frames (Figure 5A) which is similar in organization and sequence identity to the LPS locus from *Xcc* strains. Interestingly, *Xg* and *Xv* also contain two glycosyl transferases involved in synthesis of xylosylated polyrhamnan as seen in *Xcc* [69], in contrast to glycosyl transferases (*wbdA1, wbdA2*) involved in synthesis of polymannan in *Xcv* [26]. This suggests that basic structure of O-antigen in *Xg* and *Xv* is similar to *Xcc*. The three tomato/pepper pathogens *Xcv*, *Xv* and *Xg* have retained an ancestral type of LPS gene cluster (Figure 5A and 5B). On the other hand, *Xp* has acquired a novel LPS gene cluster during the course of evolution and is completely different in sequence and number of genes that are encoded. In *Xp*, this LPS locus is 17.3 kb long and encodes 12 ORFs, all of which are absent in the corresponding genomic region of *Xcv*, *Xv* or *Xg*. Also the first five ORFs flanking the *metB* side of the LPS locus in *Xp* (Figure 5A, ORFs colored in red) showed very low or no identity to region 1 of the LPS

Table 6 Type VI secretion clusters in different xanthomonads

Strain	T6SS #1	T6SS #2	T6SS #3
Phosphorylation-type regulators:	Kinase/Phosphatase/Forkhead	-	Kinase/Phosphatase/Forkhead
AraC-type regulators:	-	-	AraC
Xvv NCPPB702	YES	/	/
Xcm NCPPB4381	YES	/	/
Xaub	/	/	YES
Xauc	/	/	YES
Xac	/	/	XAC4116 - XAC4148
Xv	/	/	YES
Xp	YES	/	YES
Xcv	XCV2120 - XCV2143	/	XCV4206 - XCV4244
Xoo KACC10331	XOO3034 - XOO3052	XOO3466 - XOO3517	/
Xoo MAFF 311018	XOO2886 - XOO2906	XOO3286 - XOO3319	/
Xoo PXO99A	XOO0245 - XOO0270	XOO2029 - XOO2060	/
Xoc BLS256	XOC2523 - XOC2545	XOC1309 - XOC1370	/
Xg	/	/	/
Xcc ATCC33913	/	/	/
Xcc 8004	/	/	/
Xca 756C	/	/	/
Xalb	/	/	/

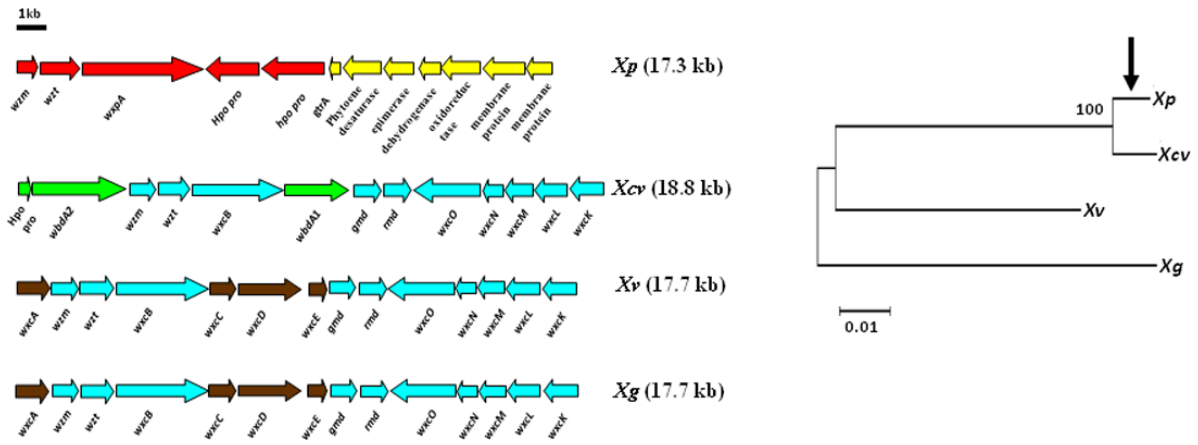

Figure 5 The Structure and phylogeny of the LPS cluster. A) Schematic comparison of LPS gene clusters described in the present study. Genes conserved in different strains are given identical color. Genes specific to individual strains are given unique color. "Hpo pro" indicates an ORF encoding for a hypothetical protein. The red color-coded genes in *Xp* genes are absent in any of the sequenced xanthomonads. B) Phylogenetic tree based on conserved *metB* and *etfA* genes that flank the variable LPS locus. Strains abbreviations are as in the main text. Arrow indicates the horizontal gene transfer event in the lineage that gave rise to *Xp*.

locus in the other xanthomonads. However, these ORFs still belong to the same Pfam families [70] that are usually present in this region, for example, ABC transporters and glycosyl transferases. The second half of the LPS cluster flanking *etfA* side encodes six ORFs, which are homologs of the LPS cluster genes from *Xac*, *Xcm* and *Xoo*. Phylogenetic insight based on conserved *metB* and *etfA* genes that flank the LPS locus suggest that the ancestor of all the *Xanthomonas* pathogens of pepper and tomato studied in this paper had the same LPS gene cluster, however putative horizontal gene transfer events at this locus have led to the acquisition of a novel LPS gene cluster in *Xp* (Figure 5B). Alien_hunter analysis also supports this acquisition with a high score showing this region to belong to an anomalous region (Additional file 5: Table S5). This event might have played a major role in changing the specificity of *Xp* towards tomato and its dominance over its relative(s) as reported previously [71], similar to variant epidemic strain of *Vibrio cholerae*, reported to be a major reason for its emergence and cholera outbreak during the 1990's in the Indian subcontinent [72]. Identity in terms of sequences and gene organization among pepper pathogens and absence of those genes from *X. perforans* and a novel LPS cluster in the tomato pathogen *X. perforans* suggest a role of this cluster in host specific variation.

Analysis of DSF cell-cell signaling system

RpfC/RpfG are two-component signaling factors and are involved in DSF (diffusible signal factor) cell-cell signaling [73-76], known to co-ordinate virulence and biofilm gene expression. The genomes of *Xv*, *Xp*, and *Xg* carry an *rpf* (*r*egulation of *p*athogenicity *f*actors) gene cluster (Table 7) that is found in all xanthomonads and which encodes components governing the synthesis and perception of the signal molecule DSF [74,75]. The Rpf of the DSF system regulates the synthesis of virulence factors and biofilm formation and is required for the full virulence of *Xcc*, *Xac*, *Xoc*, and *Xoo* [77-81]. RpfF is responsible for the synthesis of DSF, whereas, RpfC and RpfG are implicated in DSF perception and signal transduction [73-76]. RpfC is a complex sensor kinase, whereas RpfG is a response regulator with a CheY-like receiver domain that is attached to an HD-GYP domain. HD-GYP domains act in degradation of the second messenger cyclic di-GMP [82]. In addition to genes encoding these products, *Xg* and *Xp* have *rpfH*, which encodes a membrane protein related to the sensory input domain RpfC but whose function is unknown. *Xv* contains *rpfH* but with an internal stop codon, whereas functional *rpfH* is present in *Xcv* and *Xcc*, and totally absent in *Xac* and *Xoo*.

Cyclic di-GMP signaling

Cyclic di-GMP is a second messenger known to regulate a range of functions in diverse bacteria, including the virulence of animal and plant pathogens [83-85].

Table 7 A comparison of *rpf* cluster from *rpfB* to *rpfG* found across a range of *Xanthomonas* genomes

Gene Name	*Xcc*8004	*Xoo*	*Xcv*	*Xv*	*Xp*	*Xg*
rpfB	XC_2331	XOO2868	XCV1921	2934	0530	2948
rpfF	XC_2332	XOO2869	XCV1920	2932	0528	2950
rpfC	XC_2333	XOO2870	XCV1919	2930	0526	2952
rpfH	XC_2334	Absent	XCV1918	2928/2926*	0524	2954
rpfG	XC_2335	XOO2871	XCV1917	2924	0522	2956

The cellular level of cyclic di-GMP is controlled by a balance between synthesis by GGDEF domain diguanylate cyclases and degradation by HD-GYP or EAL domain phosphodiesterases. GGDEF, EAL and HD-GYP domains are largely found in combination with other signaling domains, suggesting that their activities in cyclic di-GMP turnover can be modulated by environmental cues. A number of proteins involved in cyclic di-GMP signaling have been implicated in virulence of *Xcc* [86,87]. The genome of *Xcv* encodes 3 proteins with an HD-GYP domain and 33 proteins with GGDEF and/or EAL domains. As in other *Xanthomonas* spp., the HD-GYP domain proteins are completely conserved in *Xcv*, *Xv*, *Xg* and *Xp*. There is also almost complete conservation of GGDEF/EAL domain proteins between *Xcv* and three draft genomes, although *Xv* has no ortholog of XCV1982 (Additional file 7: Table S7). In addition, the EAL domain protein (XCVd0150) encoded on a plasmid in *Xcv* is absent in the other strains.

Copper resistance (*cop*) genes are present in *Xv* and copper homeostasis (*coh*) genes are present in all strains

Among the *Xcv*, *Xv*, *Xp* and *Xg* strains sequenced, *Xv* is the only one resistant to copper and the only strain harboring a set of plasmid borne genes, namely *copL*, *copA, copB, copM, copG, copC, copD*, and *copF* that are also present in copper resistant strains of *Xac* (unpublished data/Behlau, F. personal communication) and *S. maltophilia* [88]. Genes *copA* and *copB* have been previously annotated as copper resistance related genes for many different xanthomonad genomes including *Xoo*, *Xoc*, *Xcv*, *Xac* and *Xcc*. Homologs of these genes are also present in *Xv, Xg* and *Xp* and are located on the chromosome. Additionally, upstream of *copA* on the chromosome of all strains, there is an ORF that shares homology with plasmid *copL*. In contrast to what has been published, chromosomal *copA* and *copB* are not responsible for copper resistance but likely for copper homeostasis and/or tolerance. While strains harboring the plasmid-borne *cop* genes, like in *Xv*, are resistant to copper and can grow on MGY agar (manitol-glutamate yeast agar) amended with up to 400 mg L^{-1} of copper sulfate pentahydrate, strains that have only the chromosomal *cop* genes as for *Xcv*, *Xp* and *Xg*, are sensitive to copper and can only grow on media amended up to 75 mg L^{-1} of copper. Nucleotide sequence of plasmid *cop* genes in *Xv* are 98% similar to the ones found in *Xac* and *Stenotrophomonas*, whereas chromosomal *copLAB* from *Xv* is 83% identical to homolog ORFs in *Xcv*, *Xg* and *Xp*. When *copL*, *copA* and *copB* genes from *Xv* located on the plasmid are compared to the homologs on the chromosome of the same strain, the identity of nucleotide sequences is 27, 73, and 65%, respectively. To avoid further confusion or misinterpretation, we suggest that the nomenclature of the chromosomal *copL*, *copA* and *copB* genes in xanthomonads should be changed to *cohL*, *cohA* and *cohB*, respectively, referring to copper homeostasis genes. New nomenclature has been adopted in the annotation of the draft genomes.

Genes unique to *X. perforans* as compared to pepper pathogens give clues to its predominance over *Xcv* in the field and host specificity

Thirteen gene clusters were found to be specific to the tomato pathogen *Xp* when compared to the other three strains (Additional file 8: Table S8). A part of the clusters are syntenic to the genomic regions specific to the three pepper pathogens, suggesting the replacement of these genomic regions from pepper pathogens in correspond to these region in *Xp*. These replaced regions in *Xp* might provide potential candidates for host range determinants. Most notable among these regions was the LPS cluster genes (See above). Other such regions include the avirulence genes *avrXv3* and *avrXv4*, a TIR-like domain containing protein, oxidoreductases, and bacteriocin-like proteins that were not found in any other sequenced xanthomonads. Importance of bacteriocin-like genes in *Xp* has already been studied for its predominance in the field over T1 strains [89,90]. Alien_hunter analysis showed that the bacteriocin BCN-A region belongs to an anomalous region indicating possible horizontal gene transfer of this region (Additional file 5: Table S5).

Pepper pathogenicity/aggressiveness factors increased *in planta* growth of *Xp*

Comparison of proteomes of *Xv, Xg, Xcv* against *Xp* showed 68 genes exclusive to pepper pathogens which might be candidate virulence factors on pepper (Additional file 9: Table S9). These include 16 genes with known function, 35 coding for mobile genetic elements, and 17 genes with unknown function/hypothetical proteins. Out of the 16 genes with known function, *xopG* was confirmed to be a type III effector using the *avrBs2* reporter gene assay and 6 genes belong to the LPS biosynthesis gene cluster. These 16 genes were searched against already sequenced genomes of *Xac*, *Xcc* and *Xoo*. The *wxcO* gene, which codes for O-antigen, has been identified to be a virulence factor in the *X. fuscans* - bean pathosystem by subtractive hybridization [91]. Three genes, XCV1298, XCV1839 and *wxcO*, were initially selected for the verification of their contribution to virulence in pepper. Individual genes along with their promoter regions were cloned into pLAFR3 and conjugated individually and in combination into *X. perforans*

ME24 (91-118Δ*avrXv3*), which no longer elicits an HR in pepper. However, *in planta* growth of ME24 is more similar to that of an avirulent strain than the virulent pepper strain TED3 race 6. ME24 transconjugants carrying *wxcO* and *XCV1839* in combination showed increased *in planta* growth and also comparatively increased number of lesions on pepper cv. ECW when compared to ME24 revealing that these two genes play in fact a role in pepper pathogenicity (Figure 6).

Genes specific to *Xg* as compared to other tomato/pepper pathogens may explain its aggressive nature on tomato and pepper

Comparison of genes from *Xg* against *Xcv*, *Xp* and *Xv* genes showed the presence of 625 genes specific to *Xg* (Additional file 10: Table S10). These include four type III effectors (*avrBs1* member, *xopAO*, *avrHah1*, *xopAQ*), twenty-one genes belonging to the unique type IV secretion system cluster and associated genes. These genes can be speculated to contribute to the aggressive nature of *Xg* strains on tomato and pepper. *Xg* also contains a unique beta xylosidase not present in any other xanthomonads. Type II secreted beta xylosidase has been studied for its role in plant cell wall digestion. Moreover, *Xg* contains XGA_3730 coding for a hemolysin-type calcium-binding repeat containing protein, a homolog of which is found in *Xylella* strains with 55% sequence identity. In *Xylella*, this gene is annotated as a member of a family of pore forming toxins/RTX toxins. Its homolog is also found in other plant pathogens (i.e. *P. syringae* pv. *syringae* B728a and *R. solanacearum* GMI1000). This protein has been described as a type I effector in *X. fastidiosa* strain temecula (PD1506) [92]. RTX toxin family members, especially of the hemolysin type, have been shown to be virulence factors in a variety of cell types in eukaryotes [93,94]. Finally, a gene XGA_0603 coding for lanthionine synthetase (lantibiotic biosynthesis) is found among these *Xg* specific genes, a homolog of which is found in *Xvm* NCPPB702. LanL enzymes in pathogenic bacteria contribute to virulence by modifying the host signaling pathways, in most cases by inactivating MAPKs [95].

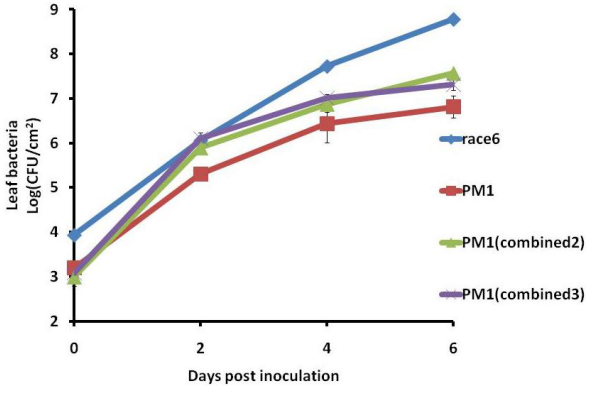

Figure 6 Pepper specificity genes increasing *in planta* growth of *Xp*. *In planta* growth of PM1 transconjugants (combined 2 [XCV1839+*wxcO*]; combined 3 [XCV1839+*wxcO*+*xopG*]); PM1 and pepper virulent strain pepper race 6 represented in log (CFU/cm^2 of leaf tissue) at 0, 2, 4, and 6 days post inoculation.

Genes common to all tomato pathogens but absent from other sequenced xanthomonads

In order to see what defines the tomato pathogens, we compared the four sequenced genomes (*Xv*, *Xp*, *Xg* and *Xcv*) to other sequenced xanthomonads. We found seven genes that were conserved in all four tomato pathogens and absent from most of other sequenced xanthomonads with the exception of *Xcm*, *Xvv*, *Xaub* and *Xauc*, which possess homologs for six out of these seven genes (Table 8). Only the hypothetical protein XCV2641 seems to be specific to the four tomato pathogens. This gene shows only 35% sequence identity to a gene from *Xvv* and *Xcm*. A homolog of the hypothetical protein, XCV4416 was found in *Xau*, but is absent from all other sequenced xanthomonads. Genes homologous in *Xcm* and *Xvv* include two transposase genes both belonging to the transposase 17 superfamily (XCV0615, XCV0623), XCV0041 (putative penicillin amidase fragment), XCV0111 (lignostilbene-alpha, beta dioxygenase), XCV0112 (uncharacterized protein conserved in bacteria) (Table 8). Interestingly, XCV0111 encodes a protein known to be involved in phenylpropanoid degradation. Phenylpropanoids are well known plant secondary metabolites induced during defense response upon pathogen attack [96]. It appears that the four tomato pathogens along with *Xvv* and *Xcm* have acquired this function to disarm the basal plant defense.

The evolution of pathogenicity clusters corresponds to the MLST-based phylogeny

The correlation between tree topology using MLST and phylogeny based on the sequences of pathogenicity clusters and the *avrBs2* effector gene, which is found in all xanthomonads, was tested. Based on MLST, *Xp* and *Xcv* group together along with *Xac* while *Xg* is more closely related to *Xcc*. *Xv* forms a different clade and is more closely related to the *Xcc* group. As can be seen in Figure 7, phylogeny based on MLST is congruent with phylogeny based on the pathogenicity clusters (*gum*, *hrp* cluster) and based on the *avrBs2* effector, suggesting that overall these clusters were vertically inherited from the most recent common ancestor of these strains.

Conclusions

The interaction of *Xanthomonas* strains with tomato and pepper represents a model system for studying plant-pathogen co-evolution because of the diversity present

Table 8 Genes present in all four tomato and pepper pathogens but absent from all other sequenced xanthomonads

Locus tag for *Xcv*85-10	Possible function	Homolog present in any other genera	GC content
XCV0623	Transposase 17 superfamily Hypo protein -COG belonging to transposase, inactive derivatives	In *Stenotrophomonas*, *Acidovorax Xanthomonas campestris* pv. musacearum NCPPB4381	0.59
XCV2641	Hypothetical protein	*X. c.* musacearum and *X. c.* vasculorum (identity 37, 31% respectively)	0.65
XCV4416	Hypothetical protein	*Pectobacterium carotovorum X. fuscans* pv. aurantifolii	0.57
XCV0615	Transposase 17 superfamily Hypothetical protein COG1943 (transposase, inactivated derivates)	*Acidovorax*, *X. c.* musacearum and *X. c.* vasculorum	0.62
XCV0112	COG4704 uncharacterized protein conserved in bacteria	*Stenotrophomonas*, *X. c.* musacearum and *X. c.* vasculorum	0.65
XCV0111	putative lignostilbene-alpha,beta-dioxygenase-phenylpropanoid compound degradation	*Stenotrophomonas*, *Ralstonia*, *X. c.* musacearum and *X. c.* vasculorum	0.66
XCV0041	putative penicillin amidase (fragment)	*Ralstonia*, *X. c.* musacearum and *X. c.* vasculorum	0.64

among the strains causing bacterial spot. Although the four *Xanthomonas* species infect the same host, tomato, and cause very similar disease, they are genetically diverse pathogens. The comparative genomic analysis has provided insights into the evolution of these strains. Whole genome comparisons revealed that *Xg* and *Xv* are more closely related to *Xcc* than *Xcv* and *Xp*. A few pathogenicity clusters, such as *hrp*, *xcs* and *xps* of *Xg* and *Xv*, were similar in terms of genetic organization and sequence identity to *Xcc* (Figure 8). However, a few pathogenicity clusters of the four strains belonging to four phylogenetic groups showed different evolutionary origins. While the

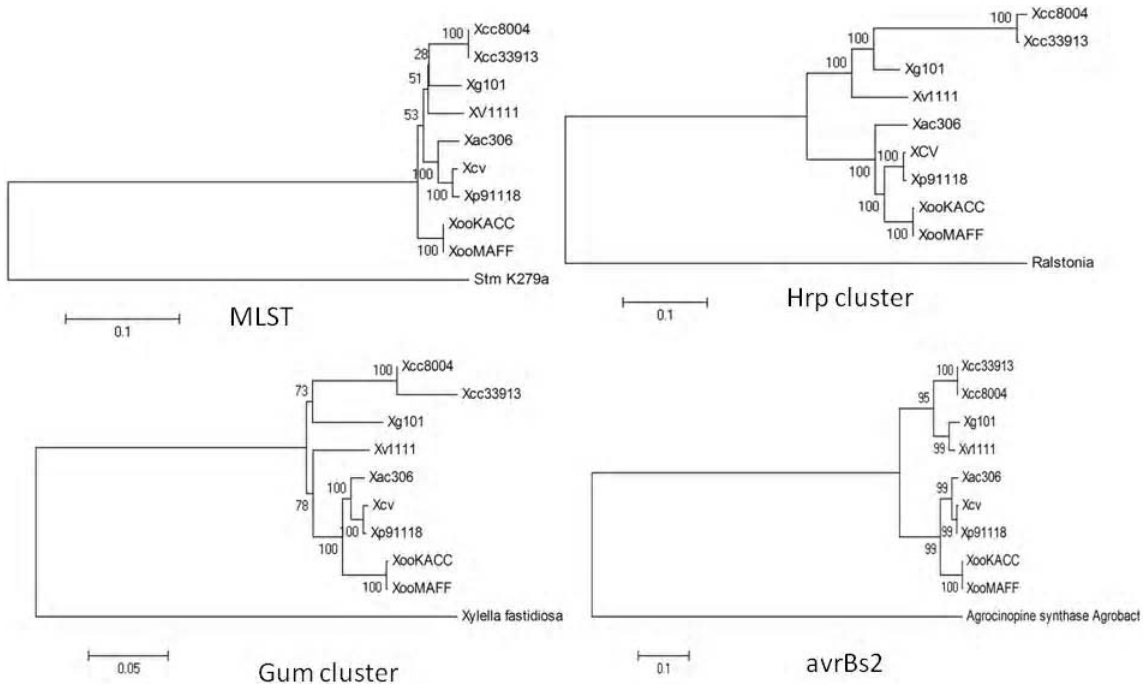

Figure 7 Correlation between phylogenies based on Multi-Locus Sequence Typing (MLST) core genome and pathogenicity clusters: Concatenated amino acid sequences of the six genes *fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, *lepA* from four bacterial spot pathogen strains along with other sequenced xanthomonads are considered in the analysis. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.

pepper pathogens *Xcv*, *Xv* and *Xg* possess similar LPS biosynthesis cluster, part of the LPS cluster from *Xp* is similar to the one from *Xac* (Figure 8). *Xv* contains few effectors, including *xopAG* (*avrGf1*) and *xopAI* the latter of which was previously found to be unique to citrus pathogens *Xac*, *Xaub* and *Xauc* [25]. *Xg* has a number of effectors homologous to *P. syringae* type III effectors suggesting probable horizontal transfer of these effectors. *Xg* contains a unique T4SS along with the one that is exclusive to *Xp*, *Xv* and *Xg*. *Xp* has two T6SSs, as found in *Xcv*. *Xv* has only one T6SS which is similar to that of *Xac*. *Xg* has no T6SS as seen for *Xcc* (Figure 8). While *Xg* and *Xv* show close relationship to *Xcc* based on whole genome comparisons, few pathogenicity clusters mentioned above seem to be conserved among tomato/pepper xanthomonads.

Type III effectors have been investigated for their contribution to pathogenicity and host-range specificity. In addition to homologs of the known effectors, we identified novel effectors in the draft genomes. By comparing effector repertoires of tomato pathogens, two possible candidate pathogenicity determinants, *xopF1* and *xopD*, were identified, of which *xopD* is responsible for delaying symptom development, and in turn, is important for pathogen survival. Unique genes present in *Xg* include the novel effectors *xopAO*, *xopAQ*, *xopAS* and an *avrBs1* member as well as a few other virulence factors, which have been characterized in other plant pathogens and which could explain the aggressive nature of *Xg* on pepper. Each species contains at least three unique type III effectors, which could explain host preferences among the strains and their aggressiveness on tomato/pepper. Comparison of the LPS clusters between the four species revealed significant variation. *Xp* has acquired a novel LPS cluster during evolution, which might be responsible for its predominance and its limited host range. As seen from the *in planta* growth assay of *Xp ΔavrXv3* mutant carrying the LPS O-antigen from *Xcv*, the LPS cluster from pepper pathogens can be a contributor to the increased *in planta* growth of *Xp ΔavrXv3* mutant on pepper, but is not the absolute virulence determinant. Use of the XA21 receptor similar to the

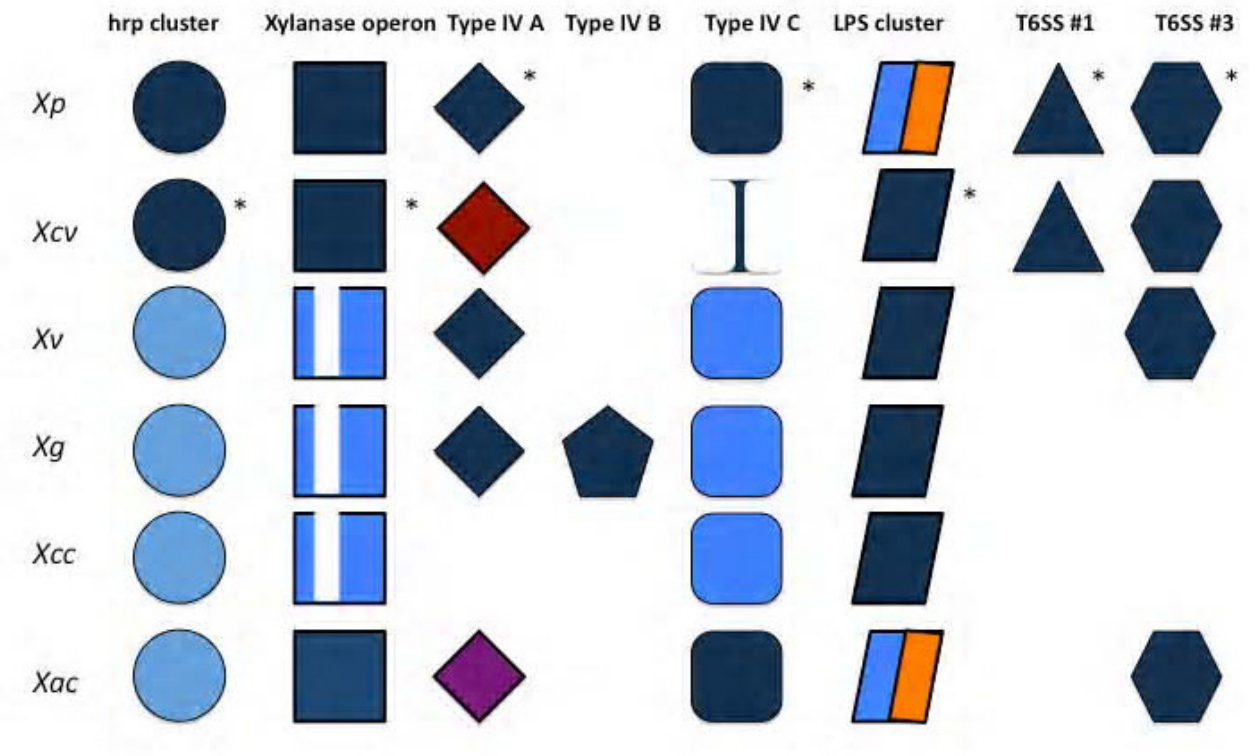

Figure 8 A diagrammatic representation of relationship among bacterial spot xanthomonads, *Xac* and *Xcc* with respect to presence or absence of pathogenicity clusters. Similar color shade indicates high identity and similar cluster organization. Lower sequence identities compared to the reference are indicated by faded gray shades. Reference strain is indicated by asterisk next to the symbol. The absence of certain part of cluster is indicated by white. In the case of LPS cluster, *Xv* and *Xac* contain novel cluster regions in the C terminal region which is indicated by a different color. *Xac* and *Xcv* contain a plasmid borne type IV cluster. Although it differs from type IVA present in other bacterial spot xanthomonads, *Xac* and *Xcv* cluster is mentioned here under type IVA with different colors. A blank space indicates complete absence of gene cluster in that particular species. A more detailed representation of individual clusters can be found in figures 2 through 5.

Xoo-rice system in *Xcv* - tomato/pepper could be one of the ways to confer resistance to xanthomonads due to presence of a similar AX21 peptide and a functional rax system in *Xcv*. Common and unique genes encoding enzymes involved in cell wall deconstruction are candidates for further study to define host preference and virulence.

In conclusion, comparison of draft genomes obtained by next generation sequencing has allowed an in-depth study of diverse groups of bacterial spot pathogens at the genomic level. This analysis will serve as a basis to infer evolution of new virulent strains and overcoming existing host resistance. The knowledge of potential virulence or pathogenicity factors is expected to aid in devising effective control strategies and breeding for durable resistance in tomato and pepper cultivars.

Methods

Genome sequencing

Xv, *Xp* and *Xg* were sequenced by 454-pyrosequencing [27] at core DNA sequencing facility, ICBR, University of Florida. *Xanthomonas* isolates were grown overnight in nutrient broth. Genomic DNA was isolated using CTAB-NaCl extraction method [97] and resuspended in TE buffer (10 mM Tris pH 8, 1 mM EDTA pH 8). Libraries of fragmented genomics DNA were sequenced on 454-Genome Sequencer, FLX instrument at Interdisciplinary Center for Biotechnology Research (ICBR) at UF. *De novo* assemblies were constructed using 454 Newbler Assembler [27]. The three draft genomes were obtained with around 10× coverage.

For Illumina sequencing, the *Xanthomonas* strains were purified from single-colony and grown overnight in liquid cultures. Genomic DNA was isolated by phenol extraction and precipitated twice with isopropanol, and finally dissolved in TE buffer. DNA was then purified by cesium chloride density gradient centrifugation and precipitated with 95% ethanol, then dissolved in TE buffer. Libraries of fragmented genomic DNA with adapters for paired-end sequencing were prepared according to the protocol provided by Illumina, Inc. with minor modifications. The libraries were sequenced on the 2G Genome Analyzer at Center of Genome Research & Biocomputing at Oregon State University and post-processed using a standard Illumina pipeline [28]. We obtained approximately 8-10 million 60-bp reads for each genome, providing roughly 95× predicted coverage.

Assembly and annotation

De novo assembly was generated on Newbler assembler (version 2.3; 454 Life Science, Branford, CT) using 454-sequencing reads for each genome. CLC workbench [29] was used in the next step for combining 454-based contigs with illumina reads, wherein, 454 based contigs were used as long reads to fill in gaps generated during combined *de novo* assembly. These combined assemblies of each genome were uploaded on IMG-JGI (Joint Genome Institute, Walnut Creek, California) server for gene calling. The gene prediction was carried out using GeneMark. Pfam, InterPro, COGs assignments were carried out for identified genes. Pathogenicity clusters described in the paper were manually annotated.

Whole genome comparisons

We aligned draft genomes against reference *Xanthomonas* genomes using nucmer [31] of MUMmer program (version 3.20) and dnadiff was used to calculate percentage of aligned sequences. We have also compared genomes using the MUM index [30] to measure distances between two genomes. The maximal unique exact matches index (MUMi) distance calculation was performed using the Mummer program (version 3.20). Mummer was run on concatenated contigs or replicons (achieved by inserting a string of 20 symbols 'N' between contig or replicon sequences) of each genome. The distance calculations performed using the MUMi script are based on the number of maximal unique matches of a given minimal length shared by two genomes being compared. MUMi values vary from 0 for identical genomes to 1 for very distant genomes [30].

Phylogenetic analysis

MLST sequences (*fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, *lepA*) for all the genomes were obtained in concatenated form from PAMDB website http://pamdb.org. Genes and their corresponding amino acid sequences spanning *gum*, *hrp* cluster were downloaded from NCBI genbank sequences of sequenced genomes. Amino acid sequences of proteins of these clusters for *Xcv* and *Xcc* were used as query to search for homology against draft genomes of *Xp, Xv* and *Xg*. The amino acid sequences were then concatenated for each pathogenicity cluster and then aligned using CLUSTALW ignoring gaps. Neighbour-joining trees were constructed with boostrap value for 1000 replicates using MEGA4 [98]. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 2723 positions in the final dataset.

Phylogeny reconstruction

Species tree. We used a supermatrix approach as in previous work [25]. Protein sequences of six *Xanthomonas* genomes (ingroups) and the *S. maltophilia* R551-3 genome (outgroup) were clustered in 5,096 families using OrthoMCL [99]. We then selected families with one and only one representative from each of the ingroup genomes and at most one outgroup protein, resulting in

2,282 families. Their sequences were aligned using MUSCLE [100] and the resulting alignments were concatenated. Non-informative columns were removed using Gblocks [101], resulting in 792,079 positions. RAxML [102] with the PROTGAMMAWAGF model was used to build the final tree.

Prediction of effector repertoires, cloning of candidate effectors and confirmation using avrBs2 reporter gene assay

A database was created collecting all the known plant and animal pathogen effectors. Using all these known effectors as query, tblastn analysis was performed against all contigs of the draft genomes of *Xv*, *Xg* and *Xp* with e-value of 10^{-5} [103]. Pfam domains were searched for possible domains found in known effectors in predicted set of ORFs of draft genome sequences. Candidate effectors were classified according to the nomenclature and classification scheme for effectors in xanthomonads recently [24]. Candidate effectors showing < 45% identity at amino acid level to the known effectors were confirmed for their translocation using *avrBs2* reporter gene assay.

N-terminal 100 amino acid region along with upstream 500 bps sequence of candidate genes were PCR amplified using primers with BglII restriction sites at the 5' ends. Following digestion with BglII, PCR amplicons were ligated with BglII-digested pBS(BglII::$avrBs2_{62\text{-}574}$::*HA*) (courtesy of Dr. Mary Beth Mudgett, Stanford university), and later transformed into *E. coli* DH5α. In-frame fusions were confirmed by DNA sequencing using F20 and R24 primers. BamHI-KpnI fragments containing the candidate gene fused to *avrBs2* was then cloned into pUFR034. Resulting plasmids were then introduced into *Xcv* pepper race 6 (*TED3* containing mutation in *avrBs2*) by tri-parental mating. The resulting *Xcv* strains were inoculated on *Bs2* pepper cv. ECW 20R and kept at 28°C in growth room. After 24 hours, strong HR was indicating successful translocation of candidate effector fusions.

Cloning of pepper specificity genes in *Xp*

The three genes mentioned above were cloned individually and in combination in pLAFR3 vector and conjugated in *Xp* 91-118 Δ*avrXv3* mutant PM1. The PM1 transconjugants with the three individual genes and combined ones along with virulent pepper race 6 strain were infiltrated at 10^5 CFU/ml concentration in pepper cv. ECW and leaves were sampled at every 48 hours after inoculation. The samples were plated on nutrient agar, incubated at 27°C and CFU/ml counts were enumerated. Experiment was carried out in triplicate and repeated three times.

Database submission

The draft genome sequences of *Xanthomonas vesicatoria* ATCC 35937 (*Xv*) have been deposited at DDBJ/EMBL/GenBank under accession number AEQV00000000. The draft genome sequences of *Xanthomonas perforans* 91-118 (*Xp*) have been deposited at DDBJ/EMBL/GenBank under accession number AEQW00000000. The draft genome sequences of *Xanthomonas gardneri* ATCC 19865 (*Xg*) have been deposited at DDBJ/EMBL/GenBank under accession number AEQX00000000. The version described in this paper is the first version, AEQV01000000, AEQW01000000, AEQX01000000. All three draft genomes will be released upon manuscript acceptance.

Additional material

Additional file 1: Table S1: General features of the sequencing data and of the *de novo* assemblies of draft genomes of *Xv*, *Xp* and *Xg* using individual sequencing methods.

Additional file 2: Figure S2: 1a) Phylogenetic tree based on MUMi indices; 1b) Distance matrix based on MUMi indices. MUMi program was used to calculate pairwise distances between draft genomes and reference *Xanthomonas* genomes.

Additional file 3: Table S3: Whole genome comparisons using MUMmer dnadiff program. % coverage of the aligned contigs and % identities of the respective contigs against reference genomes has been shown for each draft genome.

Additional file 4: Figure S4: AvrBs2-based HR assay confirms translocation of novel effectors. Hypersensitive response reaction indicating presence of translocation signal was recorded 24 hrs after inoculation on pepper cv. ECW20R with candidate effectors *xopZ2* (a), *avrBs1* (b), *xopG* (d), *xopAM* (e), *xopAO* (f) conjugated in race 6 strain along with control race 6 strain (c). All the strains showed water-soaking on pepper cv. ECW after 48 hrs after inoculation

Additional file 5: Table S5: Evidence of the horizontal gene transfer using Alien_hunter analysis.

Additional file 6: Table S6: Genes/contigs representing T6SS in draft genomes as compared to *Xcv*.

Additional file 7: Table S7: Domain architecture and distribution of proteins with HD-GYP, GGDEF and/or EAL domains encoded by genomes of different *Xanthomonas* strains.

Additional file 8: Table S8: Genes unique to *Xp*, grouped in clusters.

Additional file 9: Table S9: Genes common to all pepper pathogens but absent from *Xp*.

Additional file 10: Table S10: Genes unique to *Xg*. Genes of special interest are highlighted in red and yellow.

List of abbreviations

Xcv: *Xanthomonas euvesicatoria* strain 85-10; formerly, *Xanthomonas campestris* pv. *vesicatoria* strain 85-10; *Xv*: *Xanthomonas vesicatoria* strain 1111 (ATCC 35937); *Xp*: *Xanthomonas perforans* strain 91-118; *Xg*: *Xanthomonas gardneri* strain 101 (ATCC 19865); *Xoo*: *Xanthomonas oryzae* pv. *Oryzae; Xcc*: *Xanthomonas campestris* pv. *Campestris; Xcm*: *Xanthomonas campestris* pv. *musacearum* NCPPB4381; *Xvv*: *Xanthomonas vasicola* pv. *vasculorum* NCPPB702; *Xac*: *Xanthomonas citri* subsp. citri strain 306; formerly, *Xanthomonas axonopodis* pv. *citri* strain 306; *Xaub*: *Xanthomonas fuscans* subsp. *aurantifolii* B strain; *Xauc*: *Xanthomonas fuscans* subsp. *aurantifolii C* strain; *Xoc: Xanthomonas oryzae pv. Oryzicola; Xca: Xanthomonas campestris*

pv. *Armoracie; Xalb: Xanthomonas albilineans; Sm: Stenotrophomonas maltophilia*.

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Authors' contributions
JBJ conceived the project. JBJ and BJS oversaw genomic sequencing. JBJ provided the strains. KK did genome assembly. NP, RPR, RK, VC, PBP, FB annotated pathogenicity clusters in the genomes. DJN and BJS helped create an effector database. NP carried out effector analysis and confirmed them experimentally. NP, BAV, RK, FFW and JBJ interpreted effector analysis. NP, RPR, RK, VC, JFP, PBP, JMD, MS, TM did analyses of different pathogenicity clusters of the three genomes and helped writing corresponding sections in the manuscript. NP oversaw the experimental validations of the pathogenicity clusters. NFA created ortholog families, and did phylogenetic analysis based on all orthologus families of the three draft genomes and the reference genomes. JCS did MUMi analysis and constructed phylogenetic tree based on that analysis. NP generated the GenBank files. DJN, BJS, FFW, RK, JBJ, BAV, JFP, and JCS helped with data analyses. NP and JBJ wrote the final manuscript. All authors approved the final manuscript.

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doi:10.1186/1471-2164-12-146
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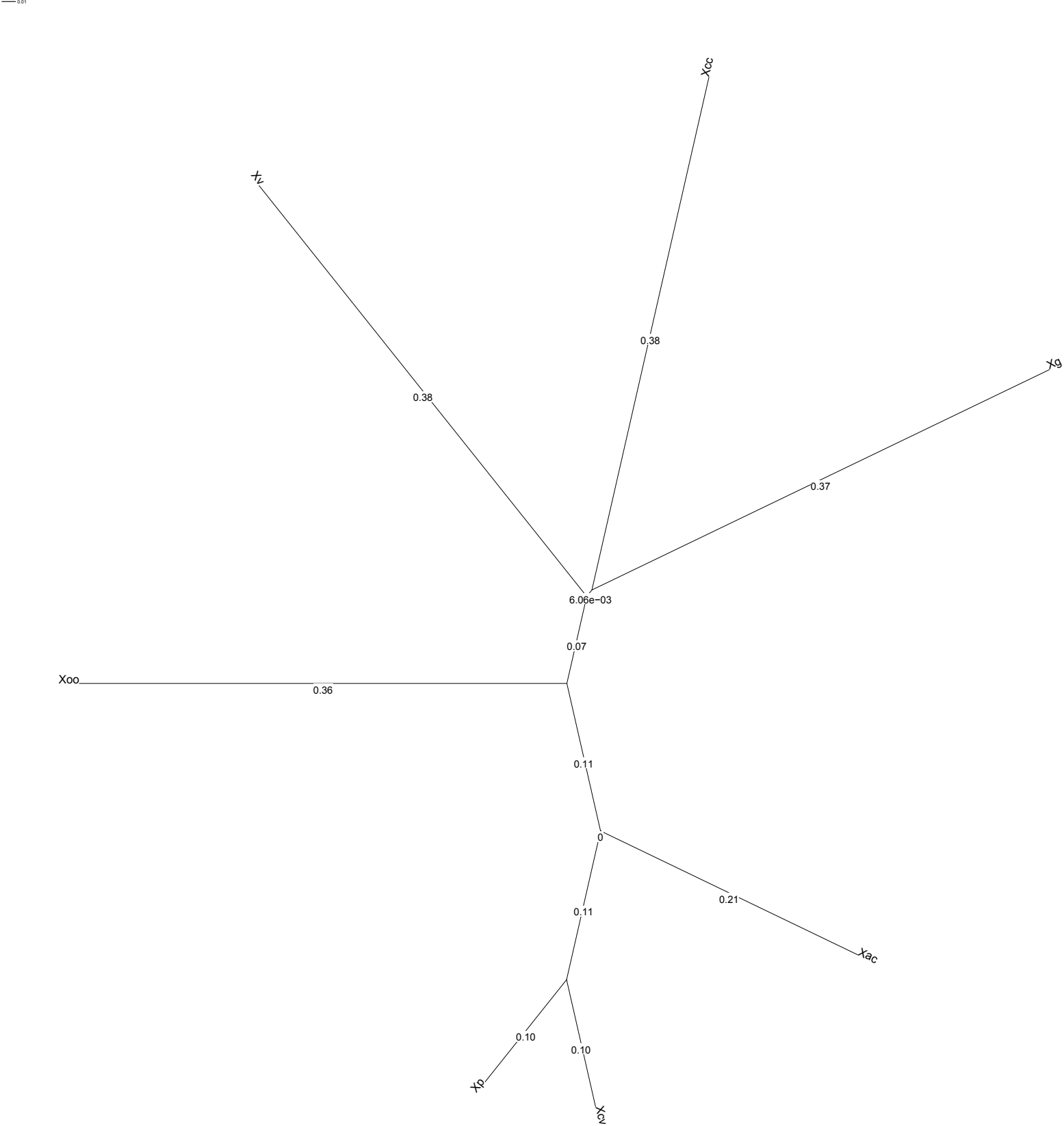
Additional file 1 – Table S1: General features of the sequencing data and of the *de novo* assemblies of draft genomes of *Xv*, *Xp* and *Xg* using individual sequencing methods.

	Xanthomonas vesicatoria (*Xv*)	*Xanthomonas perforans* (*Xp*)	*Xanthomonas gardneri* (Xg)
De novo assembly based on 454 data			
Number of reads	397,923	476,897	408,422
Number of bases	39,726,904	45,692,099	38,828,836
Mean length of reads	99.84	95.81	95.07
Max length of reads	351	240	239
Min of length of reads	35	35	35
Number of assembled contigs	4,181	2,360	4,540
Mean length of contigs	1,235	2,036	1,060
N50	701	317	767
Length of the longest contig	17,234	38,931	32,542
Total length of contigs	5,167,430	4,805,047	4,816,042
Calculated genome coverage	7.78X	8.95X	7.61X
De novo assembly based on Solexa data			
Number of reads	10,567,052	7,470,680	8,463,426
Number of bases	608,552,220	448,240,800	507,805,560
Max length of reads	60	60	60
Min of length of reads	60	60	60
Number of assembled reads	10,142,537	7,150,918	8,184,012
Number of assembled contigs	568	492	722
Mean length of contigs	9,275	9,866	7,261

N50	84	68	112
Length of the longest contig	69,349	78,549	82,425
Total length of contigs	5,268,663	4,854,240	5,242,611
Calculated genome coverage	115X	88X	94X

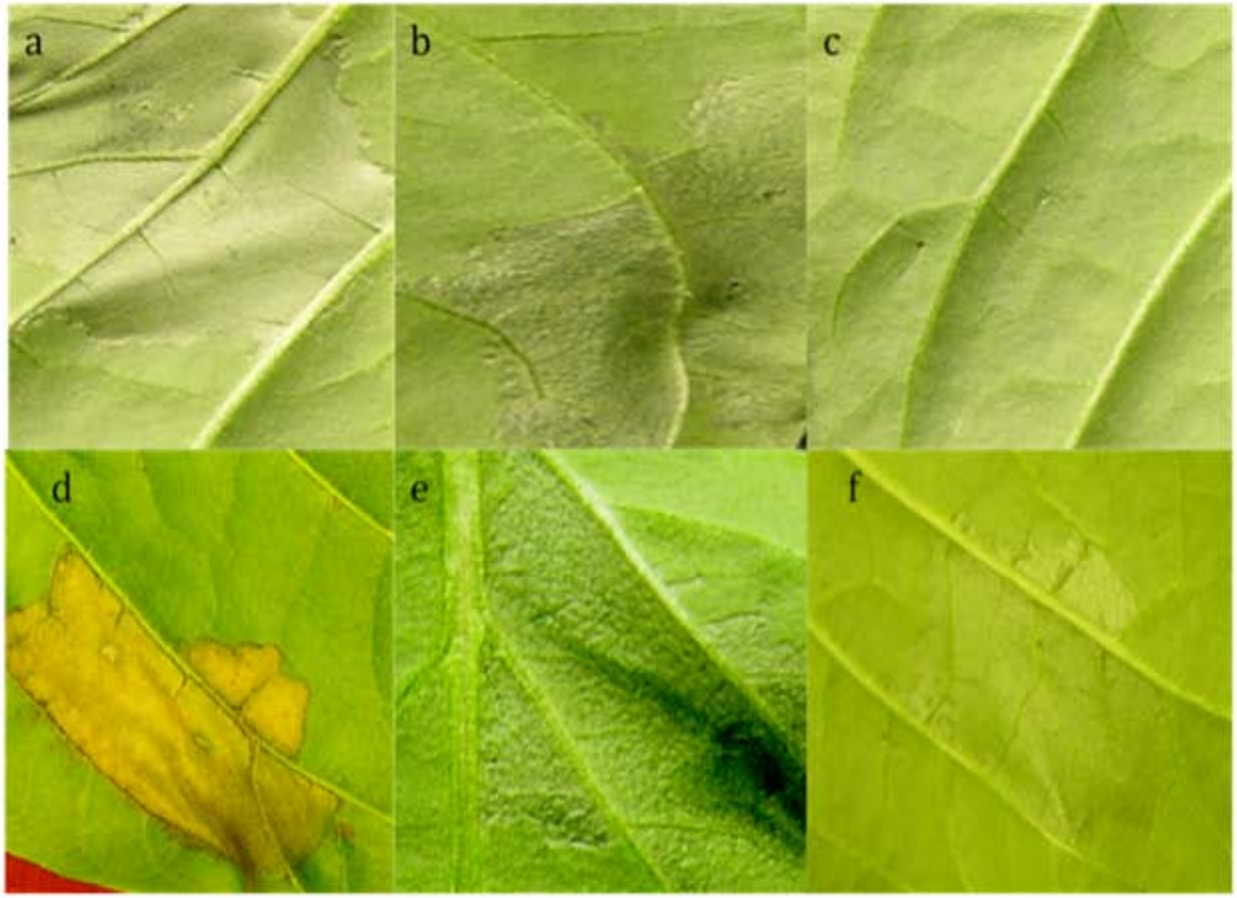
Additional file 2 – Figure S2: 1a) Phylogenetic tree based on MUMi indices; 1b) Distance matrix based on MUMi indices. MUMi program was used to calculate pairwise distances between draft genomes and reference *Xanthomonas* genomes.


0.01
Xcc
Xv
0.38
0.38
Xg
0.37
6.06e−03
0.07
Xoo
0.36
0.11
0
0.11
0.21
Xac
0.10
0.10
Xc
XcV

	Xac	Xcc	Xoo	Xcv	Xv	Xp	Xg
Xac	0	0.77	0.67	0.41	0.76	0.41	0.75
Xcc	0.77	0	0.81	0.77	0.77	0.77	0.75
Xoo	0.67	0.81	0	0.67	0.80	0.67	0.79
Xcv	0.41	0.77	0.67	0	0.76	0.19	0.76
Xv	0.76	0.77	0.80	0.76	0	0.76	0.76
Xp	0.41	0.77	0.67	0.19	0.76	0	0.76
Xg	0.75	0.75	0.79	0.76	0.76	0.76	0

Additional file 3 – Table S3: Whole genome comparisons using MUMmer dnadiff program. % coverage of the aligned contigs and % identities of the respective contigs against reference genomes has been shown for each draft genome.

Genome comparison		% of contigs of draft genome aligned	% of average identity for the aligned sequences
Xp	Xcv	85.57	98.1
	Xac	85.91	93.8
	Xcc	74.23	87.36
	Xoo MAFF	77.32	90.5
Xg	Xcv	78.44	88.57
	Xac	79.71	88.05
	Xcc	83.33	88.83
	Xoo MAFF	72.83	87.9
Xv	Xcv	83.11	87.86
	Xcc	76.35	87.37
	Xac	80.07	87.90
	Xoo MAFF	69.26	87.68


a
b
c
d
e
f

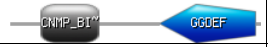
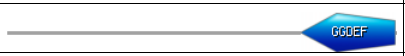
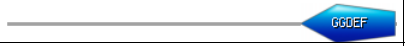
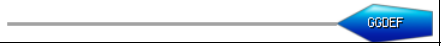
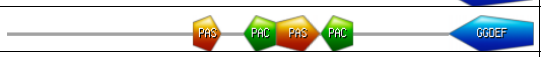
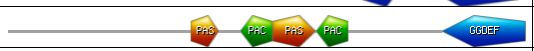
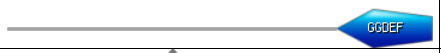
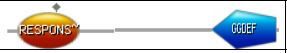
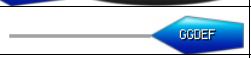
Additional file 5 – Table S5: Evidence of the horizontal gene transfer using alien hunter analysis.

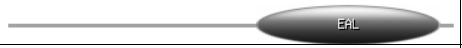
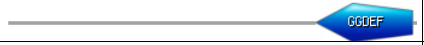
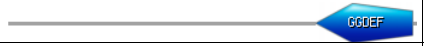
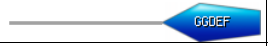
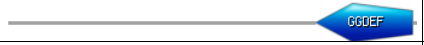
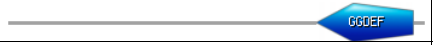
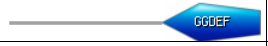
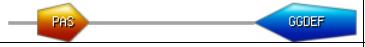
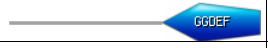
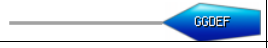
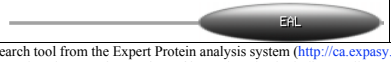
Gene/ Gene cluster	Locus tag	Score for Alien Hunter (Threshold = 12.496)	% GC	tRNA/transposase/mobile genetic elements in the vicinity	Evidence of HGT
avrBs1	XGA_0724	32.031	45	Transposase	Good
xopAO	XGA_1250	13.735	48	Predicted to be located on plasmid	Good
xopAS	XGA_0764/XGA_0765	19.844	59	Transposase	Good
xopG	XGA_4501 XVE_4777 XCV1298	21.272	50	ISxac2 transposase in *Xcv*	Good
xopAQ	XGA_2091	Does not belong to anomalous region	51	Could not be predicted	Weak
xopZ2	XGA_2762 XVE_3221	Does not belong to anomalous region	69	IS30 transposase 3000 bps apart	Weak
LPS cluster	XPE_3787 to XPE_3795	Belongs to anomalous region	50	No	Good
Bacteriocin cluster	XPE_0786 to XPE_0790	Belongs to anomalous region	50	tranposase	Good

Additional file 6 – Table S6: Genes/contigs representing T6SS in draft genomes as compared to *Xcv*.

T6SS subtype #1		**T6SS subtype #3**			
Xp	XCV homologs	Xp	XCV homologs	Xv	XCV homologs
Contig 33	XCV2120-XCV2127(N)	Contig 120	XCV4244-XCV4236(N)	Contig 233	XCV4244-XCV4216
Contig 287	XCV2127(i)	Contig 287	XCV4236(i)		
Contig 288	XCV2127(i)	Contig 288	XCV4236(i)		
Contig 291	XCV2127(i)	Contig 291	XCV4236(i)		
Contig 238	XCV2127(i)	Contig 238	XCV4236(i)		
Contig 254	XCV2127(i)	Contig 254	XCV4236(i)		
Contig 90	XCV2127(C)-XCV2137(N)	Contig 44	XCV4236(C)-XCV4216		
Contig 240	XCV2137(C)-XCV2144	no homolog	XCV4215	no homolog	XCV4215
		Contig 116	XCV4214-XCV4209(N)	Contig 183	XCV4214-XCV4212(N)
		Contig 133	XCV4209(C)-XCV4206(N)	Contig 148	XCV4213(N)-XCV4206(N)
		Contig 233	XCV4206(i)		
		Contig 195	XCV4206(C)	Contig 175	XCV4206(C)

Additional file 7 – Table S7. Domain architecture and distribution of proteins with HD-GYP, GGDEF and/or EAL domains encoded by genomes of different *Xanthomonas* strains

Domain architecture of proteins	Xcc8004	Xcc33913	XooKACC10331	XooPXO99A	XooMAFF311018	Xcv	Xv	Xp	Xg
	XC0249	XCC0239	absent	absent	absent	XCV0266	0573	1195	1780
	XC0362	XCC0350	XOO4220	ORF03945	XOO3989	XCV0363	3039	4492	3433
	XC0420	XCC0407	XOO0111	ORF03778	XOO0067	XCV0450	2497	2956	1448
	XC0496	XCC0484	XOO0520	ORF02944	XOO0483	XCV0529	1437	3029	2716
	XC0613	XCC3546	absent	absent	absent	XCV0645	2531	4167	4044[1]
	XC0637	XCC3523	XOO4021	ORF04155	XOO3792	XCV0667	2531	3570	5056
	XC0641	XCC3519	XOO4016[b]	ORF04147	XOO3786	XCV0672	0665	3574	4680
	XC0675	XCC3486	XOO3988	ORF04275	XOO3759	XCV0699	1226	4018	4301
	XC0831	XCC3333	XOO0933[a]	ORF02615[a]	XOO0855[a]	XCV3588	1507	1922	2867
	XC1036	XCC3120	absent	absent	absent	absent	absent	absent	absent
	XC1383	XCC2731	XOO1440	ORF02019	XOO1324	XCV3048	5029[2]	1275	0529
	XC1411	XCC2703	XOO1467	ORF04753	XOO1368	XCV3023	2771	0357	5134
	XC1476	XCC2641	XOO1551	ORF04826	XOO1439	XCV2973	3314	1829	1596
	XC1582	XCC2563	XOO3246	ORF01278	XOO3074	XCV2860	1735	0013	2269
	XC1755	XCC2361	XOO02798	ORF00476	XOO2640	XCV2672	0992	0406	1397
	XC1766	XCC2350	XOO2787	ORF00466	XOO2627	XCV2660	0980	0394	2619
	XC1803	XCC2313	absent	absent	absent	XCV2623	0942	3324	5028
	XC1824	XCC2291	XOO2725	ORF00403	XOO2570	XCV2595	3188	3346	3744
	XC1841	XCC2274	XOO2708	ORF00384	XOO2553	XCV2579	3205	1807	2022
	XC2161	XCC2023	absent	absent	absent	XCV2102	1554	2913	3058
	XC2226	XCC1959	XOO2561	ORF01021	XOO2420	XCV2041	4978[3]	3128	1884
	XC2228	XCC1958	XOO2563	ORF01019	XOO2422	XCV2039	4980	3130	1886
	XC2274	XCC1913	XOO2614	ORF00967	XOO2473	XCV1985	4032	1307	1461
	XC2275	XCC1912	XOO2615	ORF00965	XOO2474	XCV1983	4030	1306	1460
	XC2276	XCC1911	XOO2616	ORF00964	XOO2475	XCV1982	absent	1305	1459
	XC2324	XCC1865	XOO2860	ORF00058	XOO2715	XCV1929	2941	0537	0024

	XC2335	XCC1854	XOO2871	ORF00070	XOO2725	XCV1917	2924	0522	2956
	XC2459	XCC1777	XOO2354	ORF00678	XOO2236	XCV1825	5096	0307	3401
	XC2715	XCC1520	absent	absent	absent	XCV1610	3722	1158	2000
	XC2793	XCC1445	XOO2330[a]	ORF00647	XOO2206[a]	XCV1546	0243	3986	1105
	XC2795	XCC1443	absent	ORF00649	XOO2208	XCV1544	0241	3988	1961
	XC2866	XCC1372	absent	absent	absent	XCV1477	171	4823	4622
	XC2946	XCC1294	XOO1879	ORF01741	XOO1775	XCV1398	2082	4745	1494
	XC3163	XCC1086	absent	absent	absent	XCV1210	1545	221	0886
	XC3800	XCC3729	absent	absent	absent	absent	absent	absent	absent
	XC3829	XCC3759	absent	absent	absent	absent	absent	absent	absent
	XC3962	XCC3877	XOO4445[a]	ORF03877[a]	XOO4185[a]	XCV4051	0685	2596	0663
	XC4313	XCC4224	absent	ORF04275	absent	XCV4470	4882	4444	4813
	absent	absent	XOO2331	ORF00647	absent	absent	absent	absent	absent
	absent	absent	absent	absent	absent	XCV2971	3316	1827	1598
	absent	absent	absent	absent	absent	XCVd0150[c]	absent	absent	absent

[1]Domains were predicted using the prosite research tool from the Expert Protein analysis system (http://ca.expasy.org/prosite). This analysis revealed all proteins with the conserved HD domain; the subset of these proteins with the HD-GYP domain was identified by checking for the presence of the GYP motif and by BLASTP alignments. The proteins also contain a variety of input domains including cyclic nucleotide binding domain (CNMP_BI), PAS, PAC, and uncharacterized domains or undefined regions are marked by a /. Complete domain descriptions and functions of other domains are given at http://ca.expasy.org/prosite. [a] Proteins with N-terminal truncations; [b] Gene has an internal stop codon. [c]Plasmid-borne.

Additional file 8 – Table S8: Genes unique to *Xp*, grouped in clusters.

Locus tag in *Xp*/ Gene OID	Distribution of flanking genes	Function
Cluster 1- LPS cluster genes		
XPE_3787 to XPE_3795	Present in *Xcv*	Lipopolysaccharide biosynthesis cluster
Cluster 2- Chemotaxis protein histidine kinase inactivated by transposase carrying 3 genes (in yellow) along with it in *Xp*.		
XPE_4460	In all 4	chemotaxis protein histidine kinase
XPE_4461	In all 4	transposase
XPE_4462		Fe-S oxidoreductase
XPE_4463		
XPE_4464		
XPE_4465	In all 4	chemotaxis protein histidine kinase
Cluster 3- Carrying unique genes in *Xp* not present in any plant pathogens		
XPE_1809		Transposase
XPE_1810		TIR-like domain, cyclic nucleic acid binding domain
XPE_1811		Hypothetical protein
XPE_1812		Hypothetical protein
XPE_1813		Hypothetical protein
Cluster 4- *avrXv4* and phage genes in the neighbourhood		
Cluster 5- *XopC* from *Xcv* is replaced by other unique genes in *Xp*		
XPE_3067	present in 306	hypothetical protein
XPE_3068	present in 306	hypothetical protein
XPE_3069	XAC2120	
XPE_3070	mdmC from Xac306	Predicted O-methyltransferase
XPE_3071	not called in gene calling in 306	Hypothetical protein
Cluster 6- Carrying bacteriocin genes		
XPE_0786 to XPE_0790		
Cluster 7- flanked by phage integrase		
XPE_2401		Predicted transcription regulator containing HTH domain
XPE_2402		Uncharacterized protein conserved in bacteria
XPE_2403	present in *Xv*	
XPE_2404		
XPE_3894		plasmid mobilization system relaxase
XPE_3895	XCV1122	
XPE_3896	52% hypothetical	predicted ATPase

	protein [Legionella pneumophila str. Corby]	
XPE_3897		hypo protein from Legionella
XPE_3898	present in *Xv, Xg*	
XPE_3899	in *Xv*, XCV1116	
XPE_3900	*Xcc*B100_3109	exonuclease VII
XPE_3901	present in *Xg*	
Cluster 8- Upstream flanking genes are conserved in order in all xanthomonads; while downstream are transposase genes in *Xcv.*		
XPE_3601	present in all xanthomonads	
XPE_3602		
XPE_3603		
XPE_3604		
XPE_3605		
XPE_3606		
XPE_3607		
XPE_3608		
XPE_3609		
cluster 9- Flanking genes conserved in *Xcv*		
XPE_3366	XAUB_37550 95%	hypothetical protein
XPE_3367	XCV0352	
XPE_3368	XCV0353	
XPE_3369	no hit to any plant pathogen	hypothetical protein
XPE_3370	no hit to any plant pathogen	hypothetical protein
XPE_3371	no hit to any plant pathogen	hypothetical protein
XPE_3372	no hit to any plant pathogen	hypothetical protein
XPE_3373	no hit to any plant pathogen	Activator of Hsp90 ATPase homolog 1-like protein.
XPE_3374	no hit to any other plant pathogen	hypothetical protein
Cluster 10- Upstream and downstream flanking genes present in *Xcv*		
XPE_1376	no hit to any other plant pathogen	
XPE_1377	No hit to any other plant pathogen	
XPE_1378	no hit to any other plant pathogen	
XPE_1379	no hit to any other plant pathogen	
XPE_1380		EF-hand calcium binding protein

Cluster 11- Upstream, downstream flanking genes also present in *Xcv*		
XPE_0135	XAC3183	Hypothetical protein
XPE_0136	XAC3182	Hypothetical protein
Cluster 12- Flanking genes present in Xcv. In place of following unique genes, Xcv contains ISXac3 transposase.		
XPE_0734		signal peptide, transmemb helices
XPE_0735		hypothetical protein
XPE_0736	Xccb100_0356, vasculorum and musacearum	hypothetical protein
Cluster 13		
XPE_2183	In Xoo	Type I site-specific restriction-modification system, R (restriction) subunit and related helicases
XPE_2185	Xoo	Type I restriction-modification system methyltransferase subunit
XPE_2187	Xoo	Uncharacterized conserved protein
XPE_2190	Xoo	Uncharacterized conserved protein
XPE_2192	Xoo	Type I restriction-modification system methyltransferase subunit
XPE_2194	Xoo	Type I site-specific restriction-modification system, R (restriction) subunit and related helicases
XPE_2195	Xoo, Xoc	hypothetical protein

Additional file 9 – Table S9: Genes common to all pepper pathogens but absent from *Xp*.

Locus tag in Xcv85-10	Gene symbol	Product name	Evidence to be involved in pathogenicity/ virulence
Genes with the known functions			
XCV2278		Pectate lyase precursor	
XCV3713	wxcL	Glycosyltransferase	
XCV3715	wxcN	Putative membrane protein involved in synthesis of cell surface polysaccharide	
XCV3716	wxcO	Putative carbohydrate translocase	Alavi, SM *et. al*., 2008 in *X. fuscans* – bean pathosystem and this study.
XCV3718	gmd	GDP-mannose 4,6-dehydratase (EC: 4.2.1.47)	
XCV3720	wxcB	Putative protein kinase	
XCV3722	wzm	O-antigen ABC transporter permease	
XCV4257	rpmB	LSU ribosomal protein L28P	
XCV1298		Type III effector (homolog of hopH1 from *Pseudomonas syringae*)	
XCV1839		Hypothetical protein	This study
XCVc0007	kfrA	KfrA protein	
XCV0510	hsdS1	Type I site-specific deoxyribonuclease (specificity subunit)	
XCV0513	hsdM1	Type I site-specific deoxyribonuclease (modification subunit)	
XCV2820		Putative type IV pilus assembly protein PilV	
XCV3312		Transcriptional regulator, AraC family	
XCV2191		Putative DoxD-like family membrane protein	

Genes coding for mobile genetic elements		
XCVb0012		Putative ISxac3 transposase (fragment)
XCVb0018	tnpR	Tn5045 resolvase
XCVc0040		Site-specific recombinase/resolvase family protein
XCVd0025		ISxac3 transposase (fragment)
XCVd0071		Phage integrase family protein
XCVd0097	tnpA	Tn5044 transposase
XCVd0109	tnpR	Tn5045 resolvase
XCVd0115		Tn5044 traposase
XCV0355		ISxac3 transposase
XCV0619		Transposase
XCV0706		ISxac3 transposase
XCV1118		ISxac3 transposase
XCV1553		Phage-related integrase
XCV1698		ISxac3 transposase
XCV1843		ISxac3 transposase
XCV1848		Putative integrase/recombinase
XCV2158		ISxac3 transposase
XCV2217		Phage-related integrase
XCV2261		Phage-related integrase
XCV2263		ISxac3 transposase (fragment)
XCV2273		Tn5044 transposase
XCV2295		Putative ISxac3 transposase (fragment)
XCV2439		Tn5044 trasposase
XCV2453		Filamentous phage Cf1c protein
XCV2461		Filamentous phage phiLf related protein
XCV2474		Filamentous phage Cf1c protein
XCV2477		ISXac3 transposase
XCV2484		Phage-related integrase
XCV2615		Integrase
XCV2690		ISxac3 transposase
XCV2712		Putative transposase (fragment)
XCV2867		ISxac3 trasposase
XCV3384		ISxac3 trasposase

XCV3397	ISxac3 trasposase
XCV3410	ISxac3 trasposase

Genes with function unknown
XCVd0055
XCV0648
XCV1188
XCV1189
XCV1187
XCV1303
XCV1596
XCV1937
XCV2455
XCV2857
XCV2958
XCV3162
XCV3326
XCV3986
XCV4135
XCV4262
XCV4421