Xanthomonas citri subsp. citri and Xanthomonas arboricola pv. pruni: Comparative analysis of two pathogens producing similar symptoms in different host plants

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Abstract

Comparative studies in Xanthomonas have provided a vast amount of data that enabled to deepen in the knowledge of those factors associated with virulence and Xanthomonas plant interaction. The species of this genus present a wide range of host plants and a large number of studies have been focused to elucidate which mechanism are involved in this characteristic. In this study, comparative genomic and phenotypic analysis were performed between X. citri subsp. citri (Xcc), one of the most studied pathogens within Xanthomonas, and X. arboricola pv. pruni (Xap), a pathogen which has aroused great interest in recent time. The work was aimed to find those elements that contribute to their host divergence despite the convergence in the symptoms that each species cause on Citrus spp. and Prunus spp., respectively. This study reveals a set of genes that could be putatively associated with the adaptation of these pathogens to their hosts, being the most remarkable those involved in environmental sensing systems such as the case of the TonB-dependent transporters, the sensors of the two-component system and the methyl accepting chemotaxis proteins. Other important variants were found in processes related to the decomposition of the cell wall as could be appreciated by their dissimilar set of cell-wall degrading enzymes. Type three effectors, as one of the most important factors in delineating the host specificity in Xanthomonas, also showed a different array when comparing both species, being some of them unique to each pathogen. On the other hand, only small variations could be connected to other features such as the motility appendages and surface adhesion proteins, but these differences were accompanied by a dissimilar capacity to attach on host and non-host leaf surface. The molecular factors found in this work provide the basis to perform a more in-depth functional analyses that unveil those actual factors associated with pathogenesis and host specificity in Xcc and Xap.
Introduction

*Xanthomonas* is a large genus of bacteria that encompasses species associated with plants causing serious economic losses in significant crops. Among the plant diseases due to xanthomonads, two are considered specially relevant in fruit trees, *Xanthomonas citri* subsp *citri* (*Xcc*), the causal agent of citrus bacterial canker (CBC) and *Xanthomonas arboricola* pv. *pruni* (*Xap*) that produces bacterial spot of stone fruits and almond (BSF) [1–3]. CBC is considered one of the major threat for citriculture and it is endemic in many citrus production zones, even though, *Xcc* is not present in other areas such as the Mediterranean basin or California in USA, where these bacteria are regulated as quarantinable [4]. BSF is worldwide distributed with a presence in most *Prunus*-growing regions, however *Xap* is still a quarantine organism in some areas like the European Union [5,6]. CBC is defined as a tropical or subtropical disease; meanwhile, BSF occurs mostly in temperate areas. *Xcc* and *Xap* are spread by wind-blown rains and may enter to the plant by injuries or through the stomata, multiplying inside the tissue and being disseminated when proper humidity and temperature condition are present in the environment. Both, *Xcc* and *Xap*, cause lesions in leaf, fruit, stems and branches that visually may like quite similar between the two [2,3].

Studies to elucidate the mechanisms involved in the infection process have been conducted mainly in CBC but recently also in BSF, which has gained much more attention during the last few years with the prediction of putative virulence factors [1,7]. The availability of genomic information in *Xcc* and *Xap* has permitted the study of their secretion systems and effectors; particular attention has been paid on the type III secretion system (TSS3) and all its components that play a major role in virulence. Moreover, other factors involved in the different stages of the infection have been separately studied or predicted in both species [7–9].

One of the most intriguing topic in *Xanthomonas* genus is the high degree specialization of the species or even the host specificity within the species that may comprise multiple pathovars [10–13]. The reason why some members of *Xanthomonas* are able to infect specifically a host and no others, and why close genetically strains or species long differ in the host, is constantly under debate but not completely solved [12,14,15]. Comparative analyses on the genomes of *Xcc* and *Xanthomonas campestris* gave some clues on host specificities at the beginning of the omic era [16]. Moreover, either in *Citrus* or in *Prunus* species, the pathogenic strains of *Xcc* and *Xap* may coexist with other xanthomonads, less or non-aggressive, that produce no severe symptoms or no symptoms at all. The comparison with those strains in the same host has helped to identify some factors involved in pathogenicity in these two models [8,17]. Herein, we have been going further and compared two particular xanthomonads models that have in common their availability to infect woody plants producing similar symptoms but differing in their host species and environmental conditions. The goal was to get an idea of the common mechanisms that underline the infections to produce similar effects in their different hosts and try to elucidate those that may contribute for the host selection. Comparative genomic analysis has been made based on data available in databases but also increasing the number of *Xap* genomes by obtaining two from strains that showed different motility features. The work was aimed to identify those genes specifically absent or present in each bacterial species and try to associate the differential genome content to phenotypic characters that could play a role predominantly in the early stages of the virulence process.

Results

Genome sequencing of *X. arboricola* pv. *pruni* strains CITA 9 and CITA 99

To expand the available genomes batch of *X. arboricola* pv. *pruni*, whole genome sequences of *X. arboricola* pv. *pruni* strains CITA 9 and CITA 99 were obtained (Table 1). These strains
were selected based on their motility on semisolid surfaces and ability to cause disease on almond, peach and plum [8].

Main genomic features of CITA 9 and CITA 99 are shown in Table 2. A total of 41,846,862 reads were obtained from the sequencing of CITA 9, while 26,414,106 reads were obtained after sequencing of CITA 99. These reads were assembled in a draft genomes of 5,061,994 bp and 5,075,205 bp for CITA 9 (fold coverage 96x, 57 contigs with a N50 of 148,657 bp) and CITA 99 (fold coverage 97x, 60 contigs with N50 147,514 bp), respectively. Automatic annotation of the genome sequences, with the NCBI Prokaryotic Genome Annotation Pipeline, found a total of 4,445 total coding sequences, 4,152 protein coding sequences, 5 complete rRNAs [5S (2), 16S (1), 23S (2)] and 51 tRNAs for the draft genome sequence of CITA 9, meanwhile CITA 99 showed a total of 4,431 coding sequences with 4,147 protein coding sequences, 5 complete rRNAs [5S (3), 16S (1), 23S (1)] and 51 tRNAs. In addition, complete genome sequence of the potential virulence associated plasmid pXap41 [19] was found in the draft genome sequence of both strains (Table 2).

### Table 2. Genome sequence information and statistics of *X. arboricola pv. pruni* strains CITA 9 and CITA 99.

| Property/Attribute          | CITA 9                  | CITA 99                  |
|----------------------------|-------------------------|--------------------------|
| Strain synonyms            | IVIA 3161-2-1; CFBP 7100| IVIA 3161-2-1; CFBP 7100 |
| Sequencing platform        | Illumina HiSeq platform | Illumina HiSeq platform  |
| Fold coverage              | 96x                     | 97x                      |
| Assemblers                 | CLC and MIRA            | CLC and MIRA             |
| Genome annotation          | NCBI-PGAP               | NCBI-PGAP                |
| Locus tag                  | EJK96                   | EJL05                    |
| GenBank ID                 | RWYS000000000           | RWYT00000000             |
| Genome size (bp)           | 5,061,994               | 5,075,205                |
| DNA G+C (%)                | 65.4                    | 65.4                     |
| Total genes                | 4,539                   | 4,525                    |
| Protein coding sequences   | 4,445                   | 4,431                    |
| RNA genes                  | 94                      | 94                       |
| Genes with function prediction | 3,651                 | 3,633                    |
| Genes assigned to COGs     | 2,184                   | 2,181                    |
| Genes with Pfam domains    | 2,247                   | 2,241                    |
| Genes with signal peptides | 612                     | 611                      |
| Genes with transmembrane helices | 970                 | 975                      |
| CRISPR repeat unit         | 0                       | 0                        |
| Plasmids                   | 1 (pXap41)              | 1 (pXap41)               |
Average nucleotide identity (ANI) among the genome sequence from the two strains, obtained here, and the strains of Xap available in databases, was over 99%, confirming all of them as part of the same bacterial species (S1 Table).

**Comparative genomics of X. arboricola pv. pruni and X. citri subsp. citri**

Genome comparative analysis of seven Xap and 43 Xcc strains (S1 Table), permitted to find a total of 222,751 protein coding sequences that were clustered, according to pairwise blast comparisons, in 7,506 gene clusters. Based on a gene/genome distribution previously proposed [20], these genes were distributed as follows: 2,447 genes were shared by 100% of the strains, 614 genes were present in 95%, 2,184 were present in the genome sequence of at least 15% of the strains and 2,127 were present in less than 15% of the strains; from these “cloud genes”, 1,036 were found only in one single genome (Fig 1A).

From the entire orthologous gene clusters found, 3,712 were shared by Xap and Xcc strains; 1,104 were unique to Xap and 2,690 were only found in the genome sequences of Xcc (Fig 1B). Representative sequences of each one of the gene clusters, were classified into general protein functions according to the COG database; as a result, from the 3,712 shared by both species, only 2,402 corresponded to complete gene sequences of the COG database (without any truncation in the N or C protein terminus) and from these, 2,033 had a COG function assigned being those associated with the transcription process (13.6%) the most abundant (Fig 2).

A high number of the specific sequences for Xap and Xcc were annotated as hypothetical proteins according to the NCBI database. Taking into account only those complete protein sequences with a COG functional annotation (81 and 92 protein sequences in Xap and Xcc, respectively), they were mostly identified as proteins with a transcriptional function (12% and 18% in Xap and Xcc respectively) (Fig 2). According to the annotation of the unique protein sequences of each species, those 23 with a potential virulence function in Xap consisted in eleven annotated as TBDTs (sequences ID at NCBI database: WP_052152606.1, GAE49251.1, GAE59124.1, GAE52322.1, KCX01026.1, KCW99720.1, KPN11333.1, KCW99074.1, KCW99075.1, WP_081327680.1, KCW98622.1), one MCP (KCW98291.1), one cellulase (GAE53805.1), two lipases (DK27_22630, KCW98617), one xylanase (KCX01616.1), three components of the T3SS (KCX00174.1, GAE61338.1, GAE53745.1) and four potential effectors...
On the other hand, in Xcc, it was found 28 putative virulence protein sequences comprising four TBDTs (AAM38897.1, AGI07591.1, AAM35882.1, AAM35579.1), six STCRs (AAM37649.1, AAM37020.1, AAM36995.1, AAM36994.1; AGI08218.1, AAM3798.1), one MCP (AGI08281), one adhesin (AAM38948.1), one cellulase (AGI09979.1), one component of a T4SS (AAM39283.1, AAM39284.1, AAM39285.1, AAM39286.1, AAM29287.1, AAM39290.1, AAM39291.1, AAM39292.1, AAM39293.1), two components of the T3SS (AAM35284.1, AAM35307.1) and four effector proteins (AGI06427.1, AGI10546.1, CEH61339.1, AAM35178.1). Finally, when analyzing the similarity of the 50 bacterial strains based on the distribution of each one of the gene clusters in their genome sequences, both species were clearly separated as two different taxa as represented by the similarity tree and the distribution of the strains in the space according to the two most important components on the principal component analysis (Fig 1C and 1D). These results have been also confirmed by the average ANI values observed. Within species ANI values over 99% were shown, meanwhile ANI average value among genome sequences of Xap and Xcc was below 90% (S1 Table).

In addition to the potentially virulence-associated genes found in each one of the analyzed species, a set of 410 genes previously described in Xanthomonas, related to the whole disease process were searched in all the 50 strains selected by using the BLAST algorithm. Tabular BLAST results were filtered and only those gene sequence alignments with a percentage of identical matches (identity) and a percentage of aligned sequence length (query coverage) over 80% were considered as present in each one of the 50 genomes. The distribution of those genes found in Xap and Xcc strains by the abovementioned approach is described below.

**Comparative analysis based on putative virulence-associated genes in Xanthomonas spp**

**a) Surface structure and appendages.** Infection in CBC or BSF seems to be started by bacterial attachment and colonization of host tissues through surface structures and appendages. Moreover, prominent roles of motility and chemotaxis were identified at the beginning of the genomic studies in Xanthomonas [16]. Both species present a total of thirty orthologs that conformed the complex of genes involved in flagella and its regulation and no differences were shown between Xap and Xcc. In addition, Xap and Xcc include genes for type IV fimbriae
(pili) and fimbrial adhesins, however *Xcc* showed thirty one of them, meanwhile from *Xap*, only twenty genes could be identified, all of them in common with *Xcc*. The eleven genes specifically found in *Xcc* were identified as *pil* or *fim* genes. Moreover, four genes related to non-fimbrial adhesins are shared between both groups, showing *Xcc* an additional specific protein (homolog to XCV2672). Therefore, although both type of strains contain mostly similar structures for adhesion to plant surface, minimum differences on adhesions may give rise for a specific interaction with the host.

**b) Environmental sensing and response.** Environmental sensing by bacteria is also a main factor affecting the virulence and bacterial behavior and may play a role on the host selection. Herein, a comparison was made on environmental sensors used by the bacteria to respond to the different surrounding conditions.

Five genes for TBDTs are shared between *Xap* and *Xcc* but three more were identified as specific in *Xap* (XCC1719, XCC3595 and XCC4162) and six in *Xcc* (XAC0291, XAC0852, XAC2185, XAC2193, XAC3050 and XAC4062). The orthologue to XAC3050, which corresponds to a TonB receptor, was identified in 100% of the *Xcc* strains analyzed but it was not found in any of *Xap* strains.

A large repertoire of STCRs was found either in *Xap* or *Xcc*. This repertoire involves fifty-two genes shared by the strains of both species and seventeen specific ones for *Xcc* (XAC0326, XAC2192, XCV3165, XCV2623, XAC0136, XAC1282, XAC3643, XAC1075, XAC2167, XCV2111, XAC1819, XAC2804, XCV3166, XCV4223, XCV2105, XOO3875, XCV3267).

Different batches of MCPs were identified in both groups of strains. Twenty MCPs are mutual and were found at least in one strain of each species; two class I MCPs receptors are present only in *Xap* (homologs to XCC0276 and XCC0324) and other three are specific of *Xcc* (homologs to XCV1938, XCV1939, XCV1942). Besides, seven of the twenty three MCPs identified in *Xcc*, are present in all the strains of this group, meanwhile only one (homolog to XCV1778), out of the twenty two identified in *Xap*, is common to every strain. Moreover, this gene has been also identified in 100% of the *Xcc* strains, indicating a putative and essential common role in both species.

To reveal if the environmental sensors analyzed play a role in the host specificity of *Xap* and *Xcc*, their profiles were analyzed by a similarity and cluster analysis based in presence/absence of the gene sensors and according to Jaccard’s coefficient (Fig 3). This analysis revealed two main major clusters that correlated with the two *Xanthomonas* species evaluated, however particular profiles were shown in some cases for strains of *Xap* isolated from Japan (MAFF 302420 and MAFF 301427). The mean level of similarity between main clusters corresponding to *Xap* and *Xcc* were 24.6±7.1% for TBDTs, 59.8±7.1% for STCRs and 56.6±9.8 for MCPs. Meanwhile, similarities within *Xcc* and *Xap* groups were for TBDTS of 85.0±12.05 and 76.2±16.0, for STCR of 98.0±2.6 and 76.3±15.0 and for MCPs, 92.5±6.0 and 68.0±17.8, respectively. The most apparent difference between *Xap* and *Xcc* was in TBDTS profiles, which may indicate a major role of these structures in their plant host differentiation.

*Xanthomonas* uses a cell-to-cell communication or ‘quorum sensing’ (QS) system for promoting collective behaviors which coordinate the expression of a multitude of genes and this depends of the diffusible signal factor (DSF). *Xap* and *Xcc* contain the ten genes involved in synthesis, perception of the DFS and transduction of the signal, although *Xap* presented an additional gene (*rpfl*) that is related to its regulation (analogue to XCC1847).

**c) Type secretion systems II and III.** Both species of *Xanthomonas* present genes associated with functional T2SS and T3SS and therefore the mechanisms to secrete a battery of degrading enzymes and virulence effectors.

*Xcc* and *Xap* share 13 genes related to the T2SS machinery but showing in addition two genes only found in *Xap* (XCC0664 and XCC3419), corresponding to genes xcsJ and xpsI, associated to pseudopilins and maybe related to the secretion of different substrates [21].
On the T3SS, Xap and Xcc share 17 genes of hrp (hypersensitive response and pathogenicity) gene cluster. However, Xcc showed additional eleven genes hrc (hrp-conserved) or hpa (hrp-associated), some of them related to putative chaperones that may assist the effector secretion (S2 Table).

d) Degradative enzymes. Plant cell wall is one of the main barriers between the host plant and the plant pathogen. As most of the phytopathogenic bacteria, Xap and Xcc are extracellular parasites that occupy intercellular spaces and need to decompose the surrounding cell-walls mainly composed by pectin and cellulose. Xcc and Xap have a repertoire of genes that codify for enzymes with cellulolytic, hemi-cellulolytic and pectolytic activities. In case of cellulolytic enzymes, in Xap, twelve genes for cell wall degradation were found, eleven of them are shared between Xcc and Xap, but one specific gene was identified with this activity in each group, XCC3881 in Xap and XAC3516 in Xcc. Seven orthologues for pectolytic enzymes were found in both species, four of them are shared by both species and two enzymes were identified specifically in Xcc (XCC0644 and XCC0645) and one in Xap (XCC3459). Moreover, a total of eleven common orthologs were found for hemi-cellulolytic enzymes in both bacterial species.

e) Type III effectors and other Type III secreted proteins. Due to the importance of the T3SS on the Xanthomonas [14], the profile of T3Es and other Type III secreted proteins (T3SPs) of Xap and Xcc was also predicted. Xcc showed a larger repertoire conformed by 27 T3Es and 2 T3SPs [HpaA (XAC0400) and HrpW (XAC2990)] (Fig 4). From these, 10 [XopA/Hpa1 (XAC0416), XopAP (XCV3138/XAC2990), XopAU (XAC1171/XCV1196), XopAW (XAC2949), XopE3 (XAC3224), XopI (XAC0754), XopK (XAC3085), XopN (XAC2786), XopQ (XAC4333) and XopX (XCV0572/XAC0543)] were found in all the 43 strains analyzed. On the other hand, 12 T3Es were found in the genome sequences of Xap strains (Fig 4); from these, nine [AvrBs2 (XAC0076/XCC0052), AvrXccA2 (XCCB100_177), XopAF/AvrXv3 (XCAW_b00003), XopAQ (XAC3514), XopAW (XAC2949), XopE3 (XAC3224), XopL (XAC3090), XopN (XAC2786), XopZ1 (XAC2009)] were present in all the genome sequences of this species. From the T3Es present in Xap only AvrXccA2 was unique in this species. Sequences associated with other 10 T3Es (XopAF, XopAH, XopAI, XopAK, XopQ, XopE2, XopF1, XopG, XopQ and XopV) and to the T3SPs HrpW and HpaA were found in Xap strains but they seem to be incomplete, truncated or showed a percentage of coverage or identity lower than 80% when comparing with those genes listed in S2 Table. Therefore, in order to perform the comparative analyses between both species, they were considered as completely absent in Xap. Finally, those effectors determined as TAL effectors [AvrBs3/PthA (XACA0022), PthA2 (XACA0039), PthA3 (XAC0015), PthA4 (XAC0065)] were only found in some strains of Xcc (Fig 4).

Biofilm on abiotic surfaces

Xcc and Xap formed biofilms when they were cultured in either LB, a nutrient rich broth, or XVM2, which simulates apoplast condition and induces virulence factors (Fig 5). When compared to Xcc strain 306, Xap strain CITA 33 seemed to produce larger amount of biofilm. In addition, Xap strain formed significantly (P<0.05) more biofilm when grown in LB medium than in XVM2 medium, meanwhile Xcc strain showed the opposite behavior revealing more aggregation in the apoplastic simulated condition which involves nutrient limitation and lower bacterial growth.
Biofilm on biotic surfaces

Bacterial aggregation on biotic leaf surfaces was also observed in both *Xanthomonas* strains evaluated. Moreover, our results revealed that it occurred in all the plant species analyzed, host or non-host, but with different aggregation patterns. For each plant pathogen bacterial strain, distribution pattern was different according to the incubation period tested.

*Xcc* strain 306, a natural pathogen of lemon, was able to establish fibers connecting the bacterial cell with the plant epidermis at 4 dpi (days post inoculation). After 7 dpi this strain showed developed structures not only connected to the leaf surface but also forming well defined aggregates. When *Xcc* 306 was inoculated onto *P. dulcis*, no bacterial aggregates were detected at 4 or 7 dpi; however it was able to produce lateral adhesion filaments attached to the plant surface after 7 dpi. *Citrus lemon* leaves were susceptible to be colonized by Xap strain CITA 33 and some bacterial aggregates were observed. Furthermore, at 7dpi the presence of bacterial cells of CITA 33 was confirmed on the leaf surface of citrus species and a biofilm mature structure was observed (Fig 6).

![Biofilm formation by Xanthomonas citri subsp. citri strain 306 and Xanthomonas arboricola pv. pruni strain CITA 33 on polypropylene surface quantified by absorbance of crystal violet stain.](https://doi.org/10.1371/journal.pone.0219797.g005)
Fig 6. Scanning electron microscopy of Xanthomonas citri subsp. citri strain 306 and Xanthomonas arboricola pv. pruni strain CITA 33 onto the leaf surface of Citrus lemon (CL) and Prunus dulcis (PD) after 4 and 7 dpi. Scale bar 5 μm.

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Bacterial survival onto leaf surfaces seemed to be more related with the host than with the bacterial species inoculated onto the surface (Fig 7). No differences in survival were shown between both species on the different hosts, however the number of culturable cells for both strains was higher on almond compared to lemon leaves.

**Discussion**

This work was aimed to improve the knowledge of the possible mechanisms involved in the infection of two *Xanthomonas* species and to determine those that may play a key role in host range. *Xcc* and *Xap* produce similar symptoms in their hosts, which are in both cases woody plants. Moreover, the disease cycles for citrus bacterial canker and bacterial spot of stone fruits, seem to be analogous and may incorporate an epiphytic phase at early stage of the infection. Although biofilm formation is not an essential step in CBC, *Xcc* may form organized bacterial aggregates that facilitate the disease progress and contribute to the bacterial survival and virulence [22–25]. Furthermore, *Xap* multiplies epiphytically on leaf forming biofilm structures that may assist in the infection development [2]. In addition, both, *Xap* and *Xcc*, contain secretion systems to discharge virulence factors that trigger the infection processes in later stages [1,2].
Since the beginning of the “omics” studies in *Xanthomonas* [16], comparative analyses within and among *Xanthomonas* species, have facilitated our understanding of the pathogenic processes as well as unveiled those molecular factors involved in virulence and host range determinacy [10,26].

In this work, first, a comparative genomic analysis of complete genomes available for *Xap* and *Xcc* was performed in order to elucidate general differences between the two bacterial groups. Unsurprising, average nucleotide identities among *Xap* and *Xcc* strains was below 90%, meanwhile the same within the species was around 99%, confirming the taxonomic different adscription of such species inside the *Xanthomonas* genus according to standards [27,28]. However, 77% of the orthologous gene clusters found in *Xap* were shared with *Xcc*, and in *Xcc*, 58% of them were shared with *Xap*. Therefore 23% and 42% of the gene clusters were unique for each species.

Herein, genes involved in surface structure and appendages were identified in *Xap* and *Xcc*. Both bacteria contain mostly similar structures, for instance, they enclose the same flagella machinery, although motility described in the two models is not identical. Even though swimming motility has been confirmed in *Xcc* and *Xap* [8,25], surface motility appeared to be different between them. *Xap* seems to present typical swarming flagella-dependent motility and some of the strains show dendritic-like patterns [8], meanwhile, *Xcc* can spread on semisolid agar surfaces by way of sliding motility, a mechanism independent of flagella [23]. In addition, both species showed genes related to the Type IV pilus that is associated to twitching motility [29,30] and it was described either in *Xap* or *Xcc* [8,31,32]. Nevertheless, differences were identified in *pil* and *fim* genes involved on pili or fimbria structure. Fimbria have been described in different *Xanthomonas* to be involved in early stage of the infection mediating specific adherence to the host [33]. Our results on adhesion to host and non-host leaf surface are congruent with this genomic difference, presenting dissimilar aggregation patterns when these species were or not inoculated on their plant host surface. However, these species survive similarly on leaf regardless the host in the line of results recently published [34]. This might be due to the short time incubation period evaluated that made impossible to determine the actual survival ability of the bacteria, or because experimental conditions were artificially favorable for them, resulting in an environment where their survival structures were not needed for population persistence. As emphasized in previous work, further investigation is needed to determine the effect of the environmental conditions for the bacterial survival on non-host surface [34].

Variable responses to the environmental conditions have been observed in both species in previous studies. In *Xcc*, chemotaxis, mediated by MCPs, was indicated to be fundamental to the process of colonization of plant tissue during the disease induction [38], meanwhile strains of *Xcc* and *Xap* from different hosts, show a variable response to certain synthetic compounds and also distinct MCPs profiles [8, 39]. The response to the environmental composition and
its posterior motility towards or against the chemical present is directed by MCPs, whose profile could be associated with the host specificity, as well as with the coevolution developed during the plant-microbe interactions [37]. As occurred with TBDTs and STCRs, most MCPs are shared by Xap and Xcc, although some differences could be elucidated. For instance, orthologs of MCP XCC0324 were specifically identified in Xap, and had only been described in pathogenic and non-pathogenic X. campestris (Xc) [37,40]. This MCP has been proved to restrict the host range of Xc to bean, garden stock and tomato [40] and a similar role could have on Xap. In addition, Xap also possess the MCP XCC0276 that had just been found in X. campestris [37].

STCRs are able to sense both extracellular and intracellular signals and have been also described in Xcc to participate in several processes, many of them related with biofilm formation, chemotaxis and motility [35,41–43]. Xap and Xcc showed similar SCTR patterns but some differences were revealed between them that might be related to the specific interaction with the host and its selection.

To determine which of the environmental sensors described above might play the major role in defining the host range, similarity matrices based on the sensor presence/absence were calculated. The lowest similarity between Xcc and Xap resulted from TBDTs profiles, indicating a possible source of specialization on them as a main character in sensing specific signal to the host in the infection process. This would be supported by the presence of conserved genes within the same species that would consist the core for TBDT within it.

Both species, Xcc and Xap, have the systems and factors required to survive and colonize plants, like flagella, exopolysaccharide, motility and biofilm formation etc. However, the variation in the environmental sensors will lead a variable response in different plant hosts; even if both species are able to survive on plant surfaces, the resulting combination of several sensors, in different strains, may induce different responses, as occur during biofilm formation on both LB and XVM2 or on host and non-host leaf surfaces. Higher biofilm formation on LB media by Xap indicates better aggregation when bacterial population increases. Xap is a real epiphytic bacteria that grow well on leaf surface, meanwhile for Xcc plant surface is a hostile environment where the bacteria show a restricted growth. The different biofilm abilities in high and low nutrient content media is concurrent with their different lifestyle, being Xap a good epiphytic bacteria compared to Xcc which is more efficient colonizing the apoplastic space than the leaf surface [22].

As previously described, xanthomonads harbour a large repertoire of cell-wall degrading enzymes which are secreted by T2SS, showing disparities within the genus or among strains that infect specific tissues into the same plant (as the case of pathovars oryzae and oryzicola of X. oryzae) [15,44]. According to the comparative analyses performed here, not remarkable variants were found in the case of the composition of T2SS, but interesting disparities were found between both species in the repertoire of the potential cell-wall degrading enzymes. Xap and Xcc may enclose different mechanisms to obtain nutrients, the first possess a higher number of enzymes because looks like it relies on those enzymes to break the cell and obtain nutrients [8,45,46], however in Xcc PthA effector contributes to break the plant cell and those cell wall degrading enzymes are used to further degrade the components released into the apoplast [47,48]. Mutants in Xcc affected in the T2SS, with cellulose activity truncated, only show a slight delay of symptoms after leaf inoculation [47]. All these evidences on cell wall degrading enzymes suggest their different role in Xcc and Xap. However, further functional analysis of the specific genes identified here for each species are required in order to determine if these variants reflects the preference of each species for their specific plant host as has been established in other phytopathogenic organisms [49].
In addition to the genes that could lead to a specific adaptation to their respective hosts in Xap and Xcc, previous comparative studies in Xanthomonas have identified the repertoire of T3Es as the major factor that contribute to virulence and host specificity [13,50]. In Xanthomonas the core set of T3Es seems to be conformed by seven effectors (AvrBs2, XopK, XopL, XopQ, XopR, XopX and XopZ). In our analyses this set of genes was found in most of the strains of Xcc but not in all of them. On the other hand, when searching these effectors in Xap (by sequence similarity with the genes of the list available at S2 Table), only five effectors of the core set were found with coverage/identity over the selected cutoff (80%). Meanwhile, homologous sequences to xopK (XAC3085) and xopQ (XAC4333) were found in every strain analyzed but their identity was lower than 80%. When comparing the sequences of these two effectors to the sequence of other Xanthomonas species, such as X. campestris ATCC 33913, they showed a coverage/identity over 90%. Therefore, it could be possible that these two effectors are actually in Xap but they showed dissimilarities to those found in Xcc due to their divergent evolutionary history [51]. Functional analysis are mandatory in order to determine if these two effectors, as well as the other effectors of Xap with similarity values under the cutoff selected, are operational T3Es. Recently, it has been described that the core set of T3Es of the three main pathogenic pathovars of X. arboricola is constituted by ten genes and in this study it was possible to find all of them with the exception of xopAV [51]. Additionally, in their recent analyses of the potential set of T3Es present in X. arboricola based on a machine-learning approach, up to 57 predicted T3Es were predicted for the pathogenic strains of X. arboricola therefore [51], it is possible for that reason, that the number of effectors described here could be underestimated. Further new analysis based in this novel approach, as well as functional or transcriptomic assays, could complement the work performed here to elucidate the real role of all these potential effectors in the host-pathogen interactions.

It is important to note that our study have permitted to find a group of T3Es that differentiate Xap and Xcc that could be of great interest in order to study their adaptation to different host plants. This is the case of those considered as TAL effectors (avBrs3/ptHA, ptHA2, ptHA3, ptHA4), as well as those non-TAL effectors xopE1, xopA, xopAD, xopAE, xopAG, xopAP, xopAU, xopC1, xopI, xopP found only in Xcc and avrXccA2 that is present only in Xap. Currently, it is necessary to go beyond in the knowledge about TAL effectors in Xap. All genome sequences for Xap have been obtained by next generation sequencing, which is generally unable to identify this type of effectors. Consequently, to complete these genomes by resequencing the studied strains with other approaches that permitted to obtain longer reads, such as PacBio technology, is mandatory in order to determine the presence and variability of TAL effectors in Xap as has been demonstrated in other xanthomonads [52].

The comparative genomic work presented here gives some clues of the mechanisms that may play a role in the interaction of two important Xanthomonas with their hosts. However, in order to boost the knowledge related to the dynamics of its genome and to refine the understanding of its host specificity, it is mandatory to go further, improving the quality of the genomes, performing precise transcriptomic analyses, and more important, supporting the “omic” results by definitive functional studies to demonstrate the current mechanism of each of the genes involved in infection processes and host range.

Material and methods
Bacterial strains and growth conditions
Xanthomonas strains used in this study are listed in Table 1 and were routinely cultured at 27°C for 48 h on Luria Bertani (LB) medium (1.5% agar) or in LB broth (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract and 5 g l⁻¹ sodium chloride). X. arboricola pv. pruni strains CITA 9 and CITA...
99 (= IVIA 3161-2-1 = CFBP 7100) were isolated from symptomatic leaves of Prunus persica cv. Merrill O’Henry and from Prunus amygdalus cv. Rumbeta, respectively, and their whole genome sequenced. These two bacterial strains are available and conserved in the collections of Centro de Investigación y Tecnología de Aragón (CITA, Aragón, Spain) and Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Genomic data from the remaining xanthomonads have been previously published [7,8,10,18,53] and are available in public databases PATRIC 3.5.27 (www.patricbrc.org) and NCBI (www.ncbi.nlm.nih.gov).

Whole genome sequencing and genome comparative analyses

DNA from strains CITA 9 and CITA 99 was obtained from 30 ml of pure and fresh bacterial culture by using QIAamp DNA minikit (Qiagen, Barcelona, Spain). DNA quality and quantity were determined by electrophoresis in 1% agarose gel and by using the Qubit fluorometer (Invitrogen) according to the manufacturer instructions. Two samples of 73.6 ng μl⁻¹ (CITA 9) and 71.8 ng μl⁻¹ (CITA 99) of DNA were submitted for sequencing at STAB VIDA Next Generation Sequencing Laboratory (Caparica, Portugal), with the Illumina HiSeq platform, using 100 bp paired-end sequencing reads.

Quality of the obtained reads was assessed with the CLC Genomics Workbench (CLC v. 9.0.1) and only those reads with up to two ambiguous nucleotides, an error probability of less than 0.01, and with a minimum length of 30, were maintained for genome assembly. De novo assembly was performed with CLC and MIRA 4.0 and the assembly data produced by both software was merged with Sequencher 5.4 taking into account only those contigs with a length of at least 2,000 bp. Contigs with a minimum overlap of 100 bp and a minimum match percentage of 99% were merged and those contigs that were exclusively present in one of the assemblies were discarded. Finally, the high quality reads were mapped against the merged contigs to determine if the alignments were accurate and to manually correct possible misassemblies. Automatic annotation of the draft genomes was conducted with the NCBI Prokaryotic Genome Annotation Pipeline [54] and with the Prokka annotation pipeline [55] using a specific database constructed with all the reference complete genome sequences of Xanthomonas available at NCBI’s database. Output results from the Prokka pipeline (faa, fsa and gff files) were utilized as input data in order to determine similarities in the coding sequences (CDS) among all the genomes analyzed, as well as to calculate their average nucleotide identity based on the blast algorithm (ANI). The genome comparative analyses was performed with the Roary v. 3.11.2 [56], the Micropan v. 1.2 [57] and the Pyani v. 0.2.7 (available at https://github.com/widdowquinn/pyani) pipelines. The assignment of species-specific CDS to each cluster orthologous group (COG) was performed with the conserved domain database at NCBI using an expected value threshold of 0.001 [58].

Additionally, variation among the studied bacterial strains was assessed in respect of the presence of 410 genes with a putative or with known pathogenicity function previously reported in Xanthomonas (S2 Table) [12,16,21,31,37,59–73]. These genes encompassed environmental sensors (TonB- dependent transporters -TBDTs-, sensors of the two-component regulatory system -STCRs- and methyl accepting chemotaxis proteins -MCPs-), adherence and motility genes (structure and regulation of flagella, type IV pilus and non-fimbrial adhesins), production of xanthan gum, quorum sensing, cell wall degrading enzymes, secretion systems (types II, III and IV) as well as their associated effectors. To determine the presence/absence of these genes, they were searched with BLASTp by using an identity and coverage cutoff of 80%. Nucleotide sequences of the CITA 9 and CITA 99 genomes are available from the GenBank, DDBJ and EMBL databases (accession numbers: RWYS0000000 and RWYT0000000).
Cluster analysis for environmental sensor profiles

Profiles of environmental sensors were obtained for each strain. Presence/absence of TBDTs, STCRs and MCPs were converted to a binary form and strains pairwise similarity was calculated according to the Jaccard’s coefficient and subjected to Unweigh Pair Group Method (UPGMA) cluster analysis. All the previous was computed using NTSYS 2.11T (Exeter Software, Setauket, NY).

Biofilms on inert surface

Bacterial adhesion and biofilm formation were measured by a polypropylene 96-well plate assay as previously described [25,74]. Suspensions of strains Xcc 306 and CITA 33 in logarithmic growth phase, after incubation in LB, were washed twice and inoculated into LB or XVM2 culture media (20 mM NaCl, 10 mM (NH4)2SO4, 5 mM MgSO4 7H2O, 1 mM CaCl2, 0.16 mM KH2PO4, 0.32 mM K2HPO4, 0.01 mM FeSO4, 10 mM fructose, 10 mM sucrose, 0.03% casein hydrolysed) onto polypropylene 96-well plates. LB was used as a medium with high nutrient content and XVM2 as a medium that mimics apoplastic condition and induces the expression of virulence-associated genes [75,76].

Adhesion to the well surface was estimated after static bacterial growth in 200 μl of LB or XVM2 media for 72 h at 27˚C. After this time, the medium was removed, and the microplates were incubated for an additional 72 h. Biofilm formation was measured after rinsing the plates with sterile distilled water (SDW) and staining with 0.3% crystal violet (CV) for 15 minutes. Excess stain was removed by rinsing the plates with SDW. Residual CV was solubilized by the addition of 200μL of 20:80 acetone:ethanol to each well and the absorbance quantified using a microplate reader set at A570 nm wavelength. Absorbance values for each strain were calculated as the means of three readings of five wells from three different assays. Means comparisons were performed by analysis of variance (ANOVA) and separated by Student-Newman-Keuls (SNK) multiple range test using Statgraphics Plus for Windows 4.1 (Statistical Graphics, Rockville, MD).

Biofilms on biotic surface

The same Xanthomonas strains used above were used to evaluate biofilm formation on biotic surfaces. For inoculation, 30 ml of overnight LB cultures at 27˚C were centrifuged 15 minutes at 1,400 g and washed twice with 10 mM MgCl2. A final concentration of 108 cfu ml⁻¹ achieved by adjusting absorbance to 0.1 was used for inoculations that were performed on 24 h detached fully expanded leaves of Citrus x limon (L.) Burm. f. (pro. sp.) and Prunus dulcis (Mill.) D. A. Webb. Plant leaves were superficially sterilized by immersion in 70% ethanol for 1 minute, 30 seconds in 0.05% sodium hypochlorite and thereafter washed three times with SDW. Leaf disks of 120 mm diameter were placed on 1.5% bacteriological agar plates and inoculated by adding 20 μl of 108 cfu ml⁻¹ bacterial suspension. 10 mM MgCl2 was used as negative control. The plates were incubated at 27˚C using a light/dark photoperiod of 16/8 hours during 4 and 7 days. After incubation, non-attached bacterial population was washed with 0.1 M KPO4 buffer and bacterial aggregates fixed overnight at 4˚C in 3% glutaraldehyde in KPO4 buffer. The post fixed treatment was performed in 2% of OsO4 in 1 M KPO4 buffer. After washing twice in 0.1 M KPO4 buffer and twice in SDW, samples were dehydrated in an ethanol series and critical point dried with CO2. The samples were coated with gold-palladium and observed in a Jeol JSM 6400 scanning electron microscope at the Electron Microscopy National Center of Complutense University of Madrid (UCM). Each combination plant-strain combination was performed in two discs and two plates and the assay was conducted twice.
Bacterial persistence on biotic surface

Bacterial survival was evaluated on leaf discs by colony counting. Bacteria attached to the leaf surface were harvested by shaking individually each plant sample in 1 ml SDW for 30 minutes. Serial dilutions from $10^{-1}$ to $10^{-6}$ were prepared and three drops of 20 μl of each dilution were plated onto two LB agar plates and incubated at 27˚C for 48 h. Colony forming units (cfu) in each drop were counted and the average cfu ml$^{-1}$ was calculated and used to quantify the viable and culturable population.

Supporting information

S1 Table. Pairwise average nucleotide identity (ANI) comparison among the studied strains of Xanthomonas arboricola pv. pruni (Xap) and Xanthomonas citri subsp. citri (Xcc).

S2 Table. Presence of virulence-associated genes in Xanthomonas arboricola pv. pruni (Xap, 7 strains) and Xanthomonas citri subsp. citri (Xcc, 43 strains).

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References

1. Ference CM, Gochez AM, Behlau F, Wang N, Graham JH, Jones JB. Recent advances in the understanding of *Xanthomonas citri* ssp. *citri* pathogenesis and citrus canker disease management. Mol Plant Pathol. 2018; 19: 1302–1318. https://doi.org/10.1111/mpp.12638 PMID: 29105297

2. Garita-Cambreron J, Palacio-Bielsa A, Cubero J. *Xanthomonas arboricola* pv. *pruni*, causal agent of bacterial spot of stone fruits and almond: its genomic and phenotypic characteristics in the *X. arboricola* species context. Mol Plant Pathol. 2018; 19, 2053–2065. https://doi.org/10.1111/mpp.12679 PMID: 29575564

3. Graham JH, Gottwald TR, Cubero J, Achor DS. *Xanthomonas axonopodis* pv. *citri*. Factors affecting successful eradication of citrus canker. Mol Plant Pathol. 2004; 5: 1–15. https://doi.org/10.1046/j.1364-3703.2004.00197.x PMID: 20565577

4. *Xanthomonas axonopodis* pv. *citri*. EPPO Bull. 2005; 35: 289–294. https://doi.org/10.1111/j.1365-2338.2005.00835.x

5. EFSA. Scientific Opinion on pest categorisation of *Xanthomonas arboricola* pv. *pruni* (Smith, 1903). EFSA J. 2014; 12: 1–25. https://doi.org/10.2903/j.efsa.2014.3857

6. *Xanthomonas arboricola* pv. *pruni*. EPPO Bull. 2006; 36: 129–133. https://doi.org/10.1111/j.1365-2338.2006.00925.x

7. Garita-Cambreron J, Palacio-Bielsa A, López MM, Cubero J. Pan-genomic analysis permits differentiation of virulent and non-virulent strains of *Xanthomonas arboricola* that cohabit *Prunus* spp. and elucidate bacterial virulence factors. Front Microbiol. 2017; 8: 573. https://doi.org/10.3389/fmicb.2017.00573 PMID: 28450982

8. Garita-Cambreron J, Palacio-Bielsa A, López MM, Cubero J. Comparative genomic and phenotypic characterization of pathogenic and non-pathogenic strains of *Xanthomonas arboricola* reveals insights into the infection process of bacterial spot disease of stone fruits. PLoS One; 2016; 11: e0161977. https://doi.org/10.1371/journal.pone.0161977 PMID: 27571391

9. Jalan N, Kumar D, Andrade MO, Yu F, Jones JB, Graham JH, et al. Comparative genomic and transcriptome analyses of pathotypes of *Xanthomonas citri* subsp. *citri* provide insights into mechanisms of bacterial virulence and host range. BMC Genomics; 2013; 14: 551. https://doi.org/10.1186/1471-2164-14-551 PMID: 23941402

10. Gordon JL, Lefeuvre P, Escalon A, Barbe V, Cruveiller S, Gagnevin L, et al. Comparative genomics of 43 strains of *Xanthomonas citri* pv. *citri* reveals the evolutionary events giving rise to pathotypes with different host ranges. BMC Genomics. 2015; 16: 1098. https://doi.org/10.1186/s12864-015-2310-x PMID: 26699528

11. Parkinson N, Aritua V, Heeney J, Cowie C, Bew J, Stead D. Phylogenetic analysis of *Xanthomonas* species by comparison of partial gyrase B gene sequences. Int J Syst Evol Microbiol. 2007; 57: 2281–2287 https://doi.org/10.1099/ijs.0.65220-0

12. Ryan RP, Vorhö lter F-J, Potnis N, Jones JB, Van Sluys M-A, Bogdanove AJ, et al. Pathogenomics of *Xanthomonas*: understanding bacterium-plant interactions. Nat Rev Microbiol. Nature Publishing Group; 2011; 9: 344–355. https://doi.org/10.1038/nrmicro2558 PMID: 21478901

13. Schwartz AR, Potnis N, Timilisina S, Wilson M, Patané J, Martins J, et al. Phylogenomics of *Xanthomonas* field strains infecting pepper and tomato reveals diversity in effector repertoires and identifies determinants of host specificity. Front Microbiol. Frontiers; 2015; 6: 535. https://doi.org/10.3389/fmicb.2015.00535 PMID: 26089818

14. Boulanger A, Noël LD. *Xanthomonas* whole genome sequencing: phylogenetics, host specificity and beyond. Front Microbiol. Frontiers; 2016; 7: 1100. https://doi.org/10.3389/fmicb.2016.01100 PMID: 27470197

15. Jacques M-A, Arlat M, Boulanger A, Boureau T, Carrère S, Cesbron S, et al. Using ecology, physiology, and genomics to understand host specificity in *Xanthomonas*. Annu Rev Phytopathol. 2016; 54: 163–187. https://doi.org/10.1146/annurev-phyto-080615-100147 PMID: 27296145

16. Da Silva ACR, Ferro JA, Reinach FC, Farah CS, Furlan LR, Quaggio RB, et al. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature. 2002; 417: 459–463. https://doi.org/10.1038/417459a PMID: 12024217

17. Jalan N, Aritua V, Kumar D, Yu F, Jones JB, Graham JH, et al. Comparative genomic analysis of *Xanthomonas axonopodis* pv. *citrumelo* F1, which causes citrus bacterial spot disease, and related strains provides insights into virulence and host specificity. J Bacteriol. 2011; 193: 6342–6357. https://doi.org/10.1128/JB.05777-11 PMID: 21908674

18. Garita-Cambreron J, Sena-Vélez M, Palacio-Bielsa A, Cubero J. Draft Genome Sequence of *Xanthomonas arboricola* pv. *pruni* strain Xap33, causal agent of Bacterial Spot Disease on almond. Genome Announc. 2014; 2: e00440–14. https://doi.org/10.1128/genomenA.00440-14 PMID: 24903863
19. Pothier JF, Vorhölter FJ, Blom J, Goesmann A, Pühler A, Smits THM, et al. The ubiquitous plasmid pXap41 in the invasive phytopathogen Xanthomonas arboricola pv. pruni: Complete sequence and comparative genomic analysis. FEMS Microbiol Lett. 2011; 323: 52–60. https://doi.org/10.1111/j.1574-6968.2011.02352.x PMID: 21732961

20. van Vliet AHM. Use of pan-genome analysis for the identification of lineage-specific genes of Helicobacter pylori. FEMS Microbiol Lett.; 2017; 364: fnw296. https://doi.org/10.1093/femsle/fnw296 PMID: 28011701

21. Szczesny R, Jordan M, Schramm C, Schulz S, Cogez V, Bonas U, et al. Functional characterization of the Xcs and Xps type II secretion systems from the plant pathogenic bacterium Xanthomonas campes-tris pv vesicatoria. New Phytol. 2010; 187: 983–1002. https://doi.org/10.1111/j.1469-8137.2010.03312.x PMID: 20524995

22. Cubero J, Geil I, Johnson EG, Redondo A, Graham JH. Unstable green fluorescent protein for study of Xanthomonas citri subsp. citri survival on citrus. Plant Pathol. 2011; 60: 977–985. https://doi.org/10.1111/j.1365-3059.2011.02450.x

23. Dunger G, Guzzo CR, Andrade MO, Jones JB, Farah CS. Xanthomonas citri subsp. citri type IV pilus is required for twitching motility, Biofilm Development, and Adherence. Mol Plant-Microbe Interact. 2014; 27:1132–1147. https://doi.org/10.1094/MPMI-06-14-0184-R PMID: 25180689

24. Rigano LA, Siciliano F, Enrique R, Sendin L, Filippone P, Torres PS, et al. Biofilm formation, epiphytic fitness, and canker development in Xanthomonas axonopodis pv. citri. Mol Plant-Microbe Interact. 2007; 64: 1222–1230. https://doi.org/10.1094/MPMI-20-10-1222 PMID: 17918624

25. Sena-Velez M, Redondo C, Geil I, Ferragud E, Johnson E, Graham JH, et al. Biofilm formation and motility of Xanthomonas strains with different citrus host range. Plant Pathol. 2015; 64: 767–775. https://doi.org/10.1111/ppa.12311

26. Jalan N, Kumar D, Yu F, Jones JB, Graham JH, Wang N. Complete Genome Sequence of Xanthomonas citri subsp. citri Strain A”12879, a restricted-host-range citrus canker-causing bacterium. Genome Announc. 2013; 1: e00235-13-e00235-13. https://doi.org/10.1128/genomeA.00235-13 PMID: 23692143

27. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, et al. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int J Syst Evol Microbiol. 2018; https://doi.org/10.1099/ijsem.0.02516 PMID: 29292687

28. Richter M, Rossello-Mora R. Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci. 2009; 106:19126–19131 https://doi.org/10.1073/pnas.0906412106 PMID: 19855009

29. Burdman S, Bahar O, Parker JK, Fuente LD La. Involvement of Type IV Pili in pathogenicity of plant pathogenic bacteria. Genes (Basel). 2011; 2: 706–735. https://doi.org/10.3390/gene2040706 PMID: 24710288

30. Mattick JS. Type IV Pili and Twitching Motility. Annu Rev Microbiol. 2002; 2002; 56:289–314. https://doi.org/10.1146/annurev.micro.56.021302.160938 PMID: 12142488

31. Dunger G, Llontop E, Guzzo CR, Farah CS. The Xanthomonas type IV pilus. Curr Opin Microbiol. Elsevier Ltd; 2016; 30: 88–97. https://doi.org/10.1016/j.mib.2016.01.007 PMID: 26874963

32. Sena-Vélez M, Redondo C, Graham JH, Cubero J. Presence of extracellular DNA during biofilm formation by Xanthomonas citri subsp. citri strains with different host range. PLoS One. 2016; 11: e0156695. https://doi.org/10.1371/journal.pone.0156695 PMID: 27248687

33. van Doorn J, Boonekamp PM, Oudega B. Partial characterization of fimbriae of Xanthomonas campes-tris pv. hyacinthi. Mol plant-microbe Interact. 1994; 7: 334–344. https://doi.org/10.1099/mpmi-7-033 PMID: 7912121

34. Zarei S, Taghavi SM, Hamzehzarghani H, Osdaghi E, Lamichhane JR. Epiphytic growth of Xanthomonas arboricola and Xanthomonas citri on non-host plants. Plant Pathol. 2018; 67: 660–670. https://doi.org/10.1111/ppa.12769

35. Li J, Wang N. Genome-wide mutagenesis of Xanthomonas axonopodis pv. citri reveals novel genetic determinants and regulation mechanisms of biofilm formation. PLoS One. 2011; 6: e21804. https://doi.org/10.1371/journal.pone.0021804 PMID: 21750733

36. Zimaro T, Thomas L, Marondezde C, Garavaglia BS, Gehring C, Ottado J, et al. Insights into Xanthomonas axonopodis pv. citri biofilm through proteomics. BMC Microbiol. 2013; 13:186 https://doi.org/10.1186/1471-2180-13-186 PMID: 23924281

37. Mnedbi-Hajni N, Darrasse A, Pigné S, Durand K, Fouteau S, Barbe V, et al. Sensing and adhesion are adaptive functions in the plant pathogenic xanthomonads. BMC Evol Biol. 2011; 11: 67. https://doi.org/10.1186/1471-2148-11-67 PMID: 21396107
38. Moreira LM, Facincani AP, Ferreira CB, Ferreira RM, Ferro FC, et al. Chemotactic signal transduction and phosphate metabolism as adaptive strategies during citrus canker induction by *Xanthomonas citri*. Funct Integr Genomics. 2015; https://doi.org/10.1007/s10142-014-0414-z PMID: 25403594

39. Sena-Vélez M. Mecanismos implicados en las etapas iniciales de infección en la cancrosis de los cítricos provocada por *Xanthomonas citri* subsp. *citri*. 2015. PhD Thesis. Opto Biotecnología. Universidad Politécnica de Madrid. http://oa.upm.es/38610

40. Indiana A. Rôles du chimiotactisme et de la mobilité flagellaire dans la fitness des Xanthomonas. 2014. PhD Thesis. Biologie végétale. Université d’Angers, 2014. Français

41. He YW, Boon C, Zhou L, Zhang LH. Co-regulation of *Xanthomonas* transcription factor involved in the regulation of chemotaxis, biofilm formation and virulence in *Xanthomonas citri* subsp. *citri*. J Bacteriol. 2011; 193: 1590–1599. https://doi.org/10.1128/JB.01415-10 PMID: 21257774

42. Yan Q, Wang N. The ColR/ColS two-component system plays multiple roles in the pathogenicity of the citrus canker pathogen *Xanthomonas citri* subsp. *citri*. Front Microbiol. 2015; 7: 4164–4176. https://doi.org/10.1146/4162-2920.12684 PMID: 25346091

43. Yaryura PM, Conforte VP, Malamud F, Roeschlin R, de Pino V, Castagnaro AP, et al. XbmR, a new transcription factor required by *Xanthomonas oryzae* pv. *oryzicola* but not by *X. oryzae* pv. *oryzae* in rice. Microbiol. (United Kingdom). 2012; 158: 2372–2383. https://doi.org/10.1099/mi.0.059964–0

44. Aarrouf J, Garcin A, Lizzi Y, El Maattaoui M. Immunolocalization and histocytological effects of *Xanthomonas arboricola* pv. *pruni* on naturally infected leaf and fruit tissues of peach (*Prunus persica*L. Batsch). J Phytopathol. 2008; https://doi.org/10.1111/j.1439-0434.2007.01364.x45

45. Socquet-Juglard D, Kamber T, Pothier JF, Christen D, Gessler C, Duffy B, et al. Comparative RNA-Seq Analysis of Early-Infected Peach Leaves by the Invasive Phytopathogen *Xanthomonas arboricola* pv. *pruni*. PLoS One. 2013; 8. https://doi.org/10.1371/journal.pone.0054196 PMID: 23342103

46. Bapista JC, Machado MA, Homem RA, Torres PS, Vojnov AA, Do Amaral AM. Mutation in the xpsD gene of *Xanthomonas campescens* pv. *oryzae* affects cellulose degradation and virulence. Genet Mol Biol. 2010; https://doi.org/10.1590/S1415-475720090006000110 PMID: 21637619

47. Brunings AM, Gabriel DW. *Xanthomonas citri*: Breaking the surface. Mol Plant Pathol. 2003; https://doi.org/10.1046/j.1364-3703.2003.00163.x49

48. King BC, Waxman KD, Nenni N V., Walker LP, Bergstrom GC, Gibson DM. Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. Biotechnol Biofuels. 2011; 16; 4:4. https://doi.org/10.1186/1754-6834-4-4 PMID: 21324176

49. Haji A, Brin C, Hunault G, Lardeux F, Lemaire C, Manceau C, et al. A «repertoire for repertoire» hypothesis: Repertoires of type three effectors are candidate determinants of host specificity in *Xanthomonas*. PLoS One. 2009; 14; 4: e6632. https://doi.org/10.1371/journal.pone.0006632 PMID: 19680562

50. Merda D, Briand M, Bosis E, Rousseau C, Portier P, Barret M, et al. Ancestral acquisitions, gene flow and multiple evolutionary trajectories of the type three secretion system and effectors in *Xanthomonas* plant pathogens. Mol Ecol.; 2017: 26: 5939–5952. https://doi.org/10.1111/mec.14343 PMID: 28689687

51. Grau J, Reschke M, Erkes A, Streubel J, Morgan RD, Wilson GG, et al. AnnoTALE: bioinformatics tools for identification, annotation and nomenclature of TALEs from *Xanthomonas* genomic sequences. Sci Rep.; 2016; 6: 21077. https://doi.org/10.1038/srep21077 PMID: 26876161

52. Garita-Cambronerio J, Palacio-Bielisa A, López MM, Cubero J. Draft Genome Sequence of Two Strains of *Xanthomonas arboricola* Isolated from *Prunus persica* Which Are Dissimilar to Strains That Cause Bacterial Spot Disease on Prunus spp. Genome Announc. 2016; 4. https://doi.org/10.1128/genomeA.00974-16 PMID: 27609931

53. Angioli S V, Gussman A, Klimek W, Cochrane G, Field D, Garrity GM, et al. Toward an online repository of standard operating procedures (SOPs) for (Meta)genomic Annotation. Omi A J Integr Biol. 2008; 12:137–41. https://doi.org/10.1089/omi.2008.0017 PMID: 18416670

54. Seemann T, Prokka: Rapid prokaryotic genome annotation. Bioinformatics. 2014; 30: 2068–2069. https://doi.org/10.1093/bioinformatics/btu153 PMID: 24642063

55. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: Rapid large-scale prokaryote pan genome analysis. Bioinformatics. 2015; 31: 3691–3693. https://doi.org/10.1093/bioinformatics/btv421 PMID: 26198102

56. Snipen L, Liland KH. micropan: an R-package for microbial pan-genomics. BMC Bioinformatics. 2015; 16: 79. https://doi.org/10.1186/s12859-015-0517-0 PMID: 25888166
58. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. Nucleic Acids Res. 2015; 43: D222–D225. https://doi.org/10.1093/nar/gku1221 PMID: 25414356

59. Cesbron S, Briand M, Essakhi S, Gironde S, Bouroue T, Manceau C, et al. Comparative genomics of pathogenic and nonpathogenic strains of Xanthomonas arboricola unveil molecular and evolutionary events linked to Pathoadaptation. Front Plant Sci. 2015; 6: 1126. https://doi.org/10.3389/fpls.2015.01126 PMID: 26734033

60. Chevanne FF, Hughes KT. Coordinating assembly of a bacterial macromolecular machine. Nat Rev Mol Cell Biol. 2008; 9: 455–465. https://doi.org/10.1038/nrm2384

61. Filloux A. The underlying mechanisms of type II protein secretion. Biochim Biophys Acta—Mol Cell Membr. 2004; 1694: 163–179. https://doi.org/10.1016/j.bbamcr.2004.05.003 PMID: 15546665

62. Guglielmini J, Néron B, Abby SS, García-Bárcena MP, la Cruz F de, Rocha EP. Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. Nucleic Acids Res. Oxford University Press; 2014; 42: 5715–5727. https://doi.org/10.1093/nar/gku194 PMID: 24623814

63. Guo Y, Figueiredo F, Jones J, Wang N. HrpG and HrpX Play Global Roles in Coordinating Different Virulence Traits of Xanthomonas axonopodis pv. citri. Mol Plant-Microbe Interact.; 2011; 24: 649–661. https://doi.org/10.1094/MPMI-09-0209 PMID: 21261465

64. Hajri A, Potthier JF, Fischer-Lê Saux M, Bonneau S, Poussier S, Bouroue T, et al. Type III effector gene distribution and sequence analysis provide new insights into the pathogenicity of plant-pathogenic Xanthomonas arboricola. Appl Environ Microbiol. American Society for Microbiology; 2012; 78: 371–84. https://doi.org/10.1128/AEM.00128-11 PMID: 22104102

65. He YW, Zhang LH. Quorum sensing and virulence regulation in Xanthomonas campestris. FEMS Microbiol Rev. 2008; 32: 842–857. https://doi.org/10.1111/j.1574-6976.2008.00120.x PMID: 18557946

66. Li R-F, Lu G-T, Li L, Su H-Z, Feng G, Chen Y, et al. Identification of a putative cognate sensor kinase for the two-component response regulator HrpG, a key regulator controlling the expression of the hrp genes in Xanthomonas campestris pv. campestris. Environ Microbiol.; 2014; 16: 2053–2071. https://doi.org/10.1111/1462-2920.12207 PMID: 23906314

67. Nascimento R, Gouran H, Chakraborty S, Gillespie HW, Almeida-Souza HO, Tu A, et al. The type II secreted Lipase/Esterase LesA is a key virulence factor required for Xylella fastidiosa pathogenesis in grapevines. Sci Rep.; 2016; 6: 15898. https://doi.org/10.1038/srep15898 PMID: 27655034

68. Potnis N, Krasileva K, Chow V, Almeida NF, Patil PB, Ryan RP, et al. Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper. BMC Genomics. BioMed Central; 2011; 12: 146. https://doi.org/10.1186/1471-2164-12-146 PMID: 21396108

69. Subramoni S, Suárez-Moreno ZR, Venturi V. Lipases as Pathogenicity Factors of Plant Pathogens. Handbook of hydrocarbon and lipid microbiology. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010. pp. 3269–3277. https://doi.org/10.1007/978-3-540-77587-4_248

70. Vandromme J, Cottny B, Baeyen S, De Vos P, Maes M. Draft genome sequence of Xanthomonas axonopodis pv. citri. J Bacteriol. 2005; 187: 1201–1205. https://doi.org/10.1128/JB.187.3.1201-1205.2005 PMID: 15669897

71. Vorhöltter F-J, Schneier S, Goesmann A, Krause L, Bekel T, Kaiser O, et al. The genome of Xanthomonas campestris pv. campestris B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. J Biotechnol. 2008; 134: 33–45. https://doi.org/10.1016/j.jbiotec.2007.12.013 PMID: 18304669

72. Wang L, Rong W, He C. Two Xanthomonas Extracellular Polgalacturonases, PhgAxc and PhgBxc, are regulated by Type III secretion regulators HrpX and HrpG and are required for virulence. Mol Plant-Microbe Interact. 2008; 21: 555–563. https://doi.org/10.1094/MPMI-21-5-0555 PMID: 18993615

73. White FF, Potnis N, Jones JB, Koebnik R. The type III effectors of Xanthomonas. Mol Plant Pathol. John Wiley & Sons, Ltd (10.1111); 2009; 10: 749–766. https://doi.org/10.1111/j.1364-3703.2009.00590.x PMID: 19849782

74. O’Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study biofilms. Methods Enzymol. 1999; 310: 91–109. https://doi.org/10.1016/S0076-6879(99)10008-9 PMID: 10547784

75. Astúa-Monge G, Freitas-Astúa J, Bacocina G, Roncoletta J, Carvalho SA, Machado MA. Expression profiling of virulence and pathogenicity genes of Xanthomonas axonopodis pv. citri. J Bacteriol. 2005; 187: 1201–1205. https://doi.org/10.1128/JB.187.3.1201-1205.2005 PMID: 15669897

76. Wengelnik K, Marie C, Russel M, Bonas U. Expression and localization of HrpA1, a protein of Xanthomonas campestris pv. vesicatoria essential for pathogenicity and induction of the hypersensitive reaction. J Bacteriol. 1996; 178, 1061–1069. https://doi.org/10.1128/jb.178.4.1061-1069.1996 PMID: 8576039

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