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Identification of New Genes Related to Virulence of *Xanthomonas axonopodis* Pv. *Citri* during Citrus Host Interactions

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Abstract

A mutant library of the bacterium *Xanthomonas citri* subsp. *citri* strain 306 pathotype A (*Xac*), the causative agent of most aggressive Asiatic type A citrus canker, was screened regarding altered canker symptoms after inoculations into *Citrus sinensis* and *Citrus limonia* host leaves. Twenty-six mutants have shown phenotypic virulence changes and have respectively knocked out gene identified by sequencing. *In vivo* growth curves were obtained for nine mutants to quantify how the mutations could affect pathogen's adaptability to growth inside and attack host plant infected tissue. Among identified genes in mutated strains, we could find those that until now had not been reported as being involved in *Xac* adaptation and/or virulence, such as predicted to encode for xylose repressor-like protein (*XAC*Δ*xylR*), Fe-S oxidoreductase (*XAC*Δ*aslB*), helicase IV (XACΔhelD), ubiquinol cytochrome c oxidoreductase iron-sulfur subunit (XACΔpetA), chromosome partitioning protein (XACΔparB) and cell division protein FtsB (XACΔftsB), in addition to genes predicted to encode for hypothetical proteins. The new genes found in this study as being relevant to adaptation and virulence, improve the understanding of *Xac* fitness during citrus plant attack and canker symptoms development.

Keywords

Xanthomonas axonopodis Pv. *Citri*, Mutants, Impaired Virulence

*These authors contributed equally to this work.

1. Introduction

Citrus canker is caused by various species of the bacterial genus *Xanthomonas*, and the most aggressive canker is induced by *Xanthomonas citri* subsp. *citri* pathotype A (*Xac* = *Xanthomonas axonopodis* pv. *citri*) [1], which is responsible for the so-called Asiatic or type A Canker. The disease symptoms evolve as initial water soaking followed by hyperplasia and necrosis at the site of infection, lead to the dispersion of the pathogen to other infection sites across plant host tissues [2]. Given its importance as a pan-tropical plague, *Xac* genome was completely sequenced [3]. Since then, conducting large-scale studies has prioritized the functional elucidation of genes associated with adaptation to host citrus plants, or those involved in virulence and/or pathogenesis.

Although less effective than *Xac*, it is worth mentioning that the variants *X. citri* subsp. *citri* A* and A^W, as well as *X. fuscans* subsp. *aurantifolli* strains B and C also cause citrus canker [4] [5] [6]. Their importance is attributed to their ability to infect only specific species of citrus, since they are restricted to certain regions in the world or vary considerably in their virulence induction profiles which are generally weaker than that observed for canker A. For these reasons, the genomes of these bacteria were also completely or partially sequenced [4] [7] [8], thus enabling a comparative genomic analysis would guide the search for genes that possibly explain the role of biological variants, leading to a better characterization of the pathosystem as well as its relationships with respective hosts.

With the aim of gaining a better understanding of the repertoire of genes and proteins associated with *Xac* adaptation and virulence-induction in citrus, a series of studies have described the effect of mutations in specific genes impairing pathogen virulence and physiological responses of plant host under pathogen infection [9] [10] [11] [12] [13]. However, there are few published studies that jointly describe the discovery of a new gene repertoire related to pathogenicity and virulence in the *Xac* genome, which represents a breakthrough in the understanding of the relationship of this phytopathogen with its compatible hosts. A previous study based on the *in planta* analysis of *Xac* pathotype A mutants has led to the identification of 44 mutated genes in strains that show total or partial inability to induce the *Xac* virulence phenotype in the host plant [14]. Another study involving the same organism described 292 mutants showing a massive reduction in virulence phenotypes [15]. More recently, seven new genes associated with virulence in *X. citri* subsp. *citri* 29-1 have been also isolated from a library of mutants [16].

As the random mutant *in planta* screening approach has been proved to be useful for the discovery of novel genes involved in *Xac* pathogenicity and virulence processes, in the present study, a new set of 1300 random *Xac* mutant strains are inoculated into citrus host plants, and twenty-six strains have showed none or reduced canker symptoms induced in infected hosts, being the mutated loci to these mutants identified by sequencing. A quantitative assessment comparing wild type *Xac* with mutants through *in planta* growth curves pointed to remarkable reductions on the proliferation of mutant strains inside host infected tissue. Beside functionally assigned mutated genes, special emphasis was given to eleven hypothetical genes, never before related to adaptation or virulence processes in this patho system.

2. Materials & Methods

2.1. Bacterial Strains and Growth Conditions

The wild type *Xac* strain 306 (wt), obtained from the phytopathogenic bacteria collection of the Laboratory of Bacteriology of the Agronomical Institute (IAPAR, Paraná state, Brazil), was stored in agar slant tube containing phosphate buffered medium (calcium nitrate 0.5 g/L, ferrous sulfate 0.5 g/L, sodium phosphate dibasic 2 g/L, peptone 5 g/L, sucrose 20 g/L, agar 20 g/L and adjusted to pH 6.6) at room temperature. Mutants obtained by random insertion using the EZ::Tn5 < KAN-2 > Tnp Transposome Kit (Epicentre Technologies), were maintained in Glycerol-TS growing medium (tryptone 10 g/L, sucrose 10 g/L, sodium glutamate 1 g/L, glycerol 150 ml/L and kanamycin antibiotic 100 μg/mL) at −80˚C. The growth experiments for *Xac* wt and mutants were performed in TSA medium (tryptone 10 g/L, sucrose 10 g/L and monosodium glutamate 1 g/L) at 28˚C, with addition of agar (15 g/L) where solid medium was required. Cells were grown over night in test tubes containing 3 mL of culture medium, at 28˚C with shaking at 180 rpm before inoculation. In the case of mutants, kanamycin was added to the culture medium to final concentration of 100 μg/mL for solid medium or 50 μg/mL for liquid medium.

2.2. *In Vitro* Mutagenesis

A set of *Xac* strain 306 mutants was obtained by random insertion of the Tn5 transposon [14] [17] with an EZ::Tn5 KAN-2 Tnp Transposome Kit™, according to the instructions of the manufacturer (Epicentre Technologies). The transposon was inserted by electroporation (2500 V, 25 μF, 200 ohms, 0.2 cm cuvette width). Transformed colonies were selected on solid TS culture medium containing kanamycin and mutants were picked and transferred individually to 96-well microtitre plates containing liquid TS culture medium with kanamycin and 20% (v/v) glycerol. After growing for 2 days at 28˚C with shaking at 200 rpm, the plates were stored at −80˚C.

2.3. *In Vivo* Virulence Test

The mutants were individually multiplied in solid TS medium with kanamycin, while the wild-type strain (wt) was multiplied in medium without kanamycin. After growth at 28˚C for 48 h, the mutants and wt strain were individually resuspended in 1 mM $MgCl_2$ end each had their cellular concentration adjusted to an optical density of 0.3 at 600 nm, corresponding to approximately 10^8 CFU/mL [18]. The bacterial suspension was infiltrated by pressing with a needleless syringe in one point of the left abaxial side of young *Citrus sinensis* end *Citrus limonia* leaves. The wt strain (control) was inoculated on the right side of the same leaf using the same concentration and conditions. After inoculation, plants were grown in a room at 28˚C with artificial light 3000 lux with 12 h photoperiod by 16 days. Evaluation of symptoms was carried out over 16 days (4, 8, 12 and 16 days after inoculation) and documented by digital photography. Those mutants that were unable to induce the pathogenic process or led to a difference in the symptoms, compared to that observed for the wt strain were selected.

2.4. Identification of Mutated Genes

The identification of mutated genes follows all procedures and steps (DNA extraction, transformation of *E. coli* with the recombinant plasmid, sequencing of mutated genes and Southern blotting validation) previously described by [14].

2.5. Bacterial *in Planta* Growth Curves

In planta growth curve experiments were carried out with *Xac* wt and nine mutants with altered virulence (XAC0068, XAC0732, XAC2265, XAC3278, XAC0075, XAC1491, *XAC*Δ*aslB*, XAC2457 and XAC3906). Three different clones (biological replicates) of each mutant or wt strains were multiplied in solid TS culture medium as described above, with the addition of kanamycin for mutant clones. After growth at 28˚C for 48 h, each culture was individually resuspended in 1 mM $MgCl_2$ and the cellular concentration was adjusted to an optical density of 0.001 at 600 nm (10^5 CFU/mL). Then, each triplicate of bacterial suspension for a given strain (mutants or wt) was infiltrated at three distinct locations (technical replicates) on the abaxial side of young *Citrus limonia* leaves using a 1cc needleless tuberculin syringe and the inoculated area per leaf was enough to take a disk of 0.75 cm in diameter. Quantitative analyses were performed 0, 2, 4, 6, 8, 10, 12 and 14 days after inoculation. At each time, 3 disks of 0.75 cm in diameter were removed from different inoculated leaves for each clone (three biological replicates) and transferred to an eppendorf tube containing 1 mL of 1 mM $MgCl_2$ cold solution. Immediately, each leaf disk was ground individually on ice with a pestle. Serial dilutions of 10^{-1} to 10^{-7} were prepared and 10 µL of each dilution was dropped on three plates (technical triplicate) of solid TS medium containing kanamycin (except for the wt strain) using the microculture technique. Plates were kept at 28˚C for 36 hours, and isolated colonies were counted. The results were expressed as the average of the three biological replicates (with three technical triplicate) ± standard deviation. The presented results correspond to the arithmetic average of nine repetitions for each analyzed strain.

2.6. Analysis of Orthologous Genes and Evolutive Inference of Disrupted Genes

The nucleotide sequence of twenty-six *Xac* disrupted gene were compared to non-redundant database (*nr*) using the blastx alghoritm [19] to search for the presence of orthologous of these genes in twenty-five genomes belonging to four genera of Xanthomonadaceae family: *Xanthomonas*, *Xylella*, *Stenotrophomonas* and *Pseudoxanthomonas*, with all the genomes been present in the Kegg database [20]. An E-value lower than $1e^{-20}$, was the criteria to consider a gene presenting in the chosen genomes (**Table 1**).

A presence-absence matrix was constructed and analyzed using the Tree View software (Page, 1996), selecting the heat map option. To the heat map result was incorporated an analysis based on the production of two dendograms based on presence and absence of genes by species and based on the distribution of orthologous genes in a given specie. This allowed defining 4 sets of orthologous genes in the genomes. The evolutionary analysis of these orthologous genes was done by parsimony analysis, using a vertical cladogram where selected strains represent each one of the twenty-five ge-

Table 1. General information of genomes used in this study.

Abbreviation	*Genome strain*	*Assembly Ref Seq*	*Host or niche*	*Disease or isolation*	*Ref PMID*
Xac	*X. axonopodis* pv. *citri* 306	GCA_000007165.1	Citrus	Citrus canker	12024217
Xcc	*X.campestris* pv. *campestris* ATCC 33913	GCF_000007145.1	Brassica and Arabidopsis	Black rot	12024217
Xoo	*X.oryzae* pv. *oryzae* KACC 10331	GCA_000007385.1	Rice	Bacterial blight	15673718
Xcb	*X.campestris* pv. *campestres* 8004	GCA_000012105.1	Crucifers	Black rot	15899963
Xcv	*X.campestris* pv. *vesicatoria* 85-10	GCA_000009165.1	Pepper and tomato	Bacterial spot	16237009
Xom	*X.oryzae* pv. *oryzae* MAFF 311018	GCA_000010025.1	Rice	Bacterial blight	*JARQ* 39(4), 275 - 287 (2005)
Xca	*X.campestris* pv. *campestris*B100	GCA_000070605.1	Crucifers	Black rot	18304669
Xop	*X.oryzae* pv. *oryzae* PXO99A	GCA_000019585.2	Rice	Bacterial blight	18452608
Xal	*X.albilineans* GPE PC73	GCA_000087965.1	Sugarcane	Leaf scald	20017926
Xax	*X.alfalfae* sub sp. *citrumelonis* F1	GCA_000225915.1	Citrus	Bacterial spot	21908674
Xcp	*X.campestris* pv. *raphani* 756C	GCA_000221965.1	cruciferous and solanaceous	Leaf spot	21784931
Xor	*X.oryzae*pv. *oryzicola* BLS256	GCA_000168315.3	Rice	Leaf streak	21784931
Xao	*X.axonopodis* Xac29-1	GCA_000348585.1	Citrus sp.	Citrus canker	
Xci	*X.citri* subsp. *citri* Aw12879	GCA_000349225.1	Restricted-Host-Range OF Citrus Canker	Citrus canker	23682143
Xfa	*X.fastidiosa* 9a5c	GCA_000006725.1	Citrus	Variegated chlorosis	10910347
Xft	*X.fastidiosa* Temecula1	GCA_000007245.1	Grapevine	Pierce's disease	12533478
Xfm	*X.fastidiosa* M12	GCA_000019325.1	Almond	Leaf scorch	20601474
Xfn	*Xfn-X.fastidiosa* M23	GCA_000019765.1	Almond and grapevine	Leaf scorch and Pierce's disease	
Xff	*X.fastidiosa* subsp. *Fastidiosa* GB514	GCA_000148405.1	Grapevine	Pierce's disease	Southwestern Entomologist 35(3):479-483. 2010
Sml	*S.maltophilia* K279a	GCA_000072485.1	Human	Bloodstream infection	18419807
Smt	*S.maltophilia* R551	GCA_000020665.1	Human	Infection	
Buj	*S.maltophilia* JV3	GCA_000020665.1	Plants	Biocontrol agents	19060168
Smz	*S.maltophilia* D457	GCA_000284595.1	Human	Opportunistic pathogen	22689246
Psu	P.*suwonensis* 11-1	GCA_000185965.1	Environment	Isolated from cotton waste compost	
Psd	*P.spadix* BD-a59	GCA_000233915.4	Environment	BTEX degrader	22207748

nomes, already grouped according to the profile of taxa separation based on 16S-rGNA or gyrB gene sequences [21] [22], and the analysis were based on the presence or absence of the 26 mutated genes in the genomes.

3. Results and Discussion

Studies carried out with random mutant libraries proved to be important tools for the discovery of new gene functions or the involvement of genes with specific functions, already defined in other essential biological processes, for the survival of pathogens within the plant, or virulence induction. The *in planta* screening of 1300 *Xac* mutant strains, allowed the selection of twenty-six on the basis of defective canker symptoms induced in infected hosts and evaluated in terms of the structure and composition of their respective mutated genes, in addition to their location and organization in the *Xac* genome. These results brought improvement to our understanding about the origin of these genes, especially with respect to genes categorized as hypothetical, which corresponded to 42.3% (n = 11) of these mutants (**Figure 1(a)**). The analysis of the genomic region where these genes are located revealed that three genes defined as hypothetical are located into putative lateral gene transfer islands (**Figure 1(b)**). The remaining 15 knockout genes were categorized as showing putative functions. Importance was given to genes related to DNA replication or repair, which accounted for a total of 4 mutants

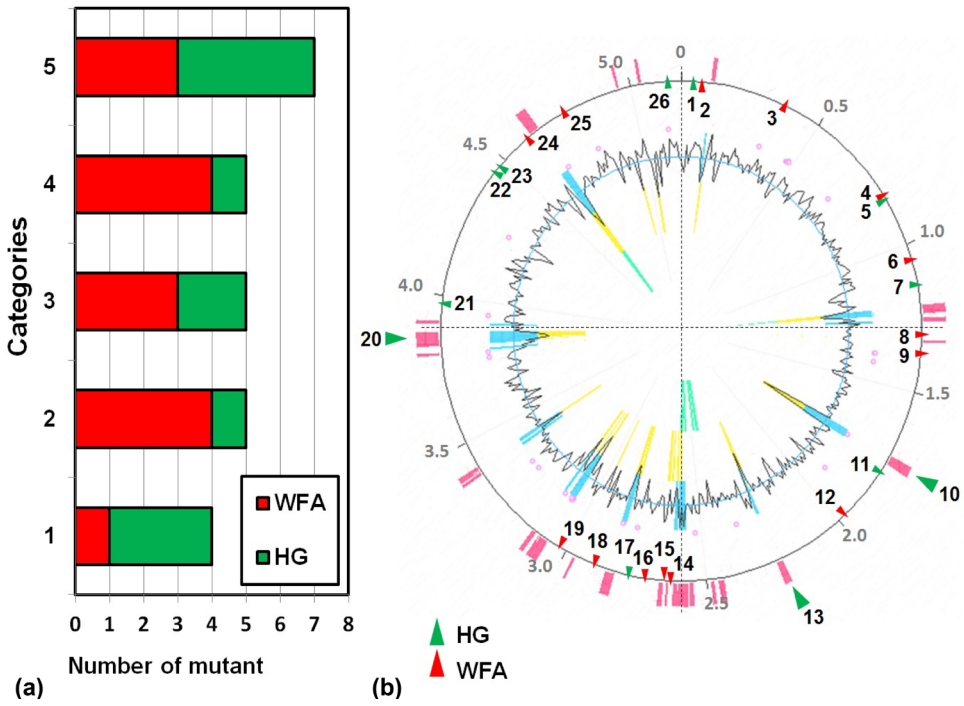

Figure 1. Mutants general features after inoculation in *Citrus sinensis* and *Citrus limonia* hosts. (a) Classification of mutants into phenotypical categories: 1—Nonpathogenic mutants; 2—Mutants showing partial loss of pathogenicity; 3—Mutants with partial loss of necrosis and total loss of hyperplasia; 4—Mutants presenting decreased hyperplasia and/or necrosis; 5—Mutants showing increased necrosis. (b) Localization of the disrupted genes in *XAC* genome. The likely genomic islands (red strips) were determined by the program IslandViewer (Dhillon, *et al.* 2013). Each one of the twenty-six mutants is pointed by triangles on the circle representing XAC chromosome. ((a) and (b)) HG—Hypothetical gene (green); WFA—With function assigned (red).

(15.4%). Based on the analysis of the composition of protein domains associated with genes defined as hypothetical, it was also possible to understand and assign potential functions to their encoded proteins. For example, although the protein product of the gene *XAC*4321 has low complexity, it exhibits another domain that encodes a signal peptide, thus suggesting its potential to be secreted by the bacterium (**Table 2**).

To facilitate the functional and structural description, the selected mutants were classified into five categories based on observed changes in their phenotypic profile (total or partial absence of the virulence phenotype) and infected host (*C. sinensis* and *C. limonia*).

3.1. Mutants Showing Total Loss of Virulence in *C. sinensis* and *C. limonia*

Out of twenty-six mutants analyzed, four (15.4%) knockouts led to a complete loss of virulence in both evaluated hosts (**Table 2**, **Figure 2**), three of them correspond to hypothetical genes (ΔXAC0068, ΔXAC0732 and ΔXAC3278), and one is predicted to encode a helicase IV (*XAC*Δ*helD*).

In plant growth curves analysis conducted to the strain ΔXAC0068, corresponding to a mutated hypothetical gene, it has shown less than 1000 times smaller growth in the citrus leaf parenchyma when compared to *Xac* wild type strain. This clearly demonstrates that the mutation affected the adaptive process of this strain within the inner intercellular spaces of the host plant (**Figure 2(a)**). Sequence analysis of the *XAC*0068 gene using blastx [19] demonstrated 99% to 76% identity with four other genes present in different bacteria: *XAC*29_00345, XCAW_00457, Acav_1091, F11_07645 and Rru_A1480. The first two genes correspond to orthologs of *X. axonopodis Xac*29-1 [7] and *X. citri* subsp. *citri* Aw12879 [8], both canker-causing agents in citrus plants. The third organism with an orthologous sequence was *Acidovorax avenae* subsp. *avenae*, a member of the beta-proteobacteria responsible for causing disease in many plants of economic importance, such as rice, oats, sugar cane, and corn [23]. The fourth organism in which an ortholog was identified was *Rhodospirillum rubrum*, an autotrophic alpha-proteobacterium which inhabits a wide variety of environments such as lakes, streams, mud and sewage [24] [25]. The common feature among these four orthologs is the presence of the NTP_transf_5 motif, belonging to the nucleotidyl transferase group involved in DNA repair [26]. It is interesting to notice that these similarities found in three different classes of bacteria (gamma, beta and alpha) belonging to such diverse ecological niches could suggest the occurrence of horizontal gene transfer between these species. This hypothesis is supported by the fact that no orthologs of XAC0068 gene was found in any other species of the genus *Xanthomonas* other than those mentioned above, ruling out the possibility of vertical inheritance. The presence of this gene in the genomes of species related to canker disease might indicate a functional role during interaction with citrus hosts.

Growth curve analysis for ΔXAC0732 mutant compared to wild type profile has shown a 10^2 reduction in bacterial titration, thus suggesting virulence impairment (**Figure 2(b)**). The gene knocked out in this mutant proved to be unique to the genus *Xanthomonas*, since blastx analysis [19] showed similarity with only five hypothetical

Table 2. General characteristics of studied mutants.

Mutant (ID)	Locus_tag	G.Name	Function[A]	COG[B]	Category[A]	NCBI ID	Start Pos	Stop Pos	*Size*(*aa*)	*Domains*	*C.sinensis*[C]		*C.limoni*[C]	
											Hyp	Nec	Hyp	Nec
1 (ΔXAC0068)	XAC0068	----	Hypothetical protein	S	VIII.B	1154139	80281	81369	362	Low complexity	0	0	0	0
2 (XACΔ*xylR*)	XAC0075	*xylR*	Xylose repressor-like protein	K	I.D.2	1154146	90593	91750	385	ROK	0	-	0	0
3 (XACΔ*metE*)	XAC0336	*metE*	5-methyl tetrahydropteroyl triglutamate-homocysteine methyltransferase	E	II.A.2	1154407	401371	402399	342	Meth_synt_1	=	=	=	+
4 (XACΔ*mdp*)	XAC0726	*mpd*	Methyl parathion hydrolase	R	I.A.2	1154797	866395	865373	340	Lactamase_B	-	=	-	+
5 (ΔXAC0732)	XAC0732	----	Hypothetical protein	S	VIII.B	1154803	872669	873469	266	Low complexity	0	0	0	0
6 (XACΔ*rnk*)	XAC0903	*rnk*	Regulator of nucleoside diphosphate kinase	F	I.D.2	1154974	1065358	1064918	146	GreA_GreB	-	-	-	-
7 (ΔXAC1008)	XAC1008	----	Conserved hypothetical protein	S	VIII.A	1155079	1163422	1162994	142	Hydrolase_2	=	0	-	0
8 (XACΔ*recR*)	XAC1111	*recR*	Recombination proteinr ecr	S	III.A.3	1155182	1262303	1262896	197	RecR + TOPRIM	=	-	=	-
9 (XACΔ*yagT*)	XAC1189	*yagT*	Ferredoxin	C	I.C.3	1155260	1358150	1357572	192	Fer2 + Fer2_2	0	-	0	-
10 (ΔXAC1491)	XAC1491	----	Hypothetical protein	S	VIII.B	1155562	1723743	1724402	219	Low complexity	-	0	0	0
11 (ΔXAC1531)	XAC1531	----	Conserved hypothetical protein	S	VIII.A	1155602	1769598	1769005	197	UPF0029 + DUF1949	0	-	0	-
12 (XACΔ*ftsB*)	XAC1720	*ftsB*	Conserved hypothetical protein	S	VIII.A	1155791	1982074	1982439	121	DivIC	0	-	0	-
13 (XACΔ*aslB*)	XAC1927	*aslB*	Fe-Soxidoreductase	C	I.C.3	1155998	2251490	2252668	392	Elp3	0	-	0	0
14 (XACΔ*cynX*)	XAC2234	*cynX*	MFS transporter	R	V.A.7	1156305	2618139	2616955	394	MFS_1 + two transmembrane	-	-	-	-
15 (XACΔ*orf8*)	XAC2243	*orf8*	Plasmid-related protein	K	VI.B	1156314	2624846	2627146	766	HhH1	=	=	=	+
16 (XACΔ*helD*)	XAC2265	*helD*	Helicase IV	L	III.A.1	1156336	2648746	2651565	935	UvrD-helicase	0	0	0	0
17 (ΔXAC2344)	XAC2344	----	Conserved hypothetical protein	S	VIII.A	1156415	2740051	2739809	80	YCII	=	+	=	+
18 (XACΔ*petA*)	XAC2457	*petA*	Ubiquinol cytochrome C oxidoreductase, iron-sulfur subunit	C	I.C.1	1156528	2861546	2860902	214	UCR_Fe-S_N + Rieske	0	-	0	0
19 (XACΔ*gumI*)	XAC2578	*gumI*	GumI protein	M	VII.E	1156649	3038840	3037791	349	Low complexity	0	-	0	-
20 (ΔXAC3278)	XAC3278	----	Hypothetical protein	S	VIII.B	1157349	3859131	3858688	147	BLUF	0	0	0	0
21 (ΔXAC3368)	XAC3368	----	Conserved hypothetical protein	S	VIII.A	1157439	3967496	3968215	239	Peptidase_M23	0	-	0	-
22 (ΔXAC3777)	XAC3777	----	Conserved hypothetical protein	S	VIII.A	1157848	4451497	4451318	59	Low Complexity	=	+	=	+
23 (ΔXAC3815)	XAC3815	----	Conserved hypothetical protein	S	VIII.A	1157886	4487859	4489103	414	DUF3667 + four transmembrane	=	=	=	+
24 (XACΔ*parB*)	XAC3906	*parB*	Chromosome partitioning protein	D	V.B	1157977	4591768	4592694	308	ParB	-	-	0	0
25 (XACΔ*pepB*)	XAC3987	pepB	Leucine aminopeptidase	O	III.C.3	1158058	4678747	4680129	460	Peptidase_M17	=	+	=	+
26 (ΔXAC4321)	XAC4321	----	Hypothetical protein	S	VIII.B	1158392	5106045	5107586	513	Signal peptide + Low complexity	=	+	=	+

[B]C: Energy production and conversion; D: Cell cycle control, cell division, chromosome partitioning; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; K: Transcription; L: Replication, recombination and repair; M: Cell wall/membrane/envelope biogenesis; O: Post-translational modification, protein turnover, and chaperones; R: General function prediction; S: Function unknown. [C]Hyp - Hyperplasia; Nec - Necrosis; "0" - Total absence of symptoms; "+" – Increased symptoms; "-" – decreased symptoms; "=" equal to wild type *Xac* induced symptoms.

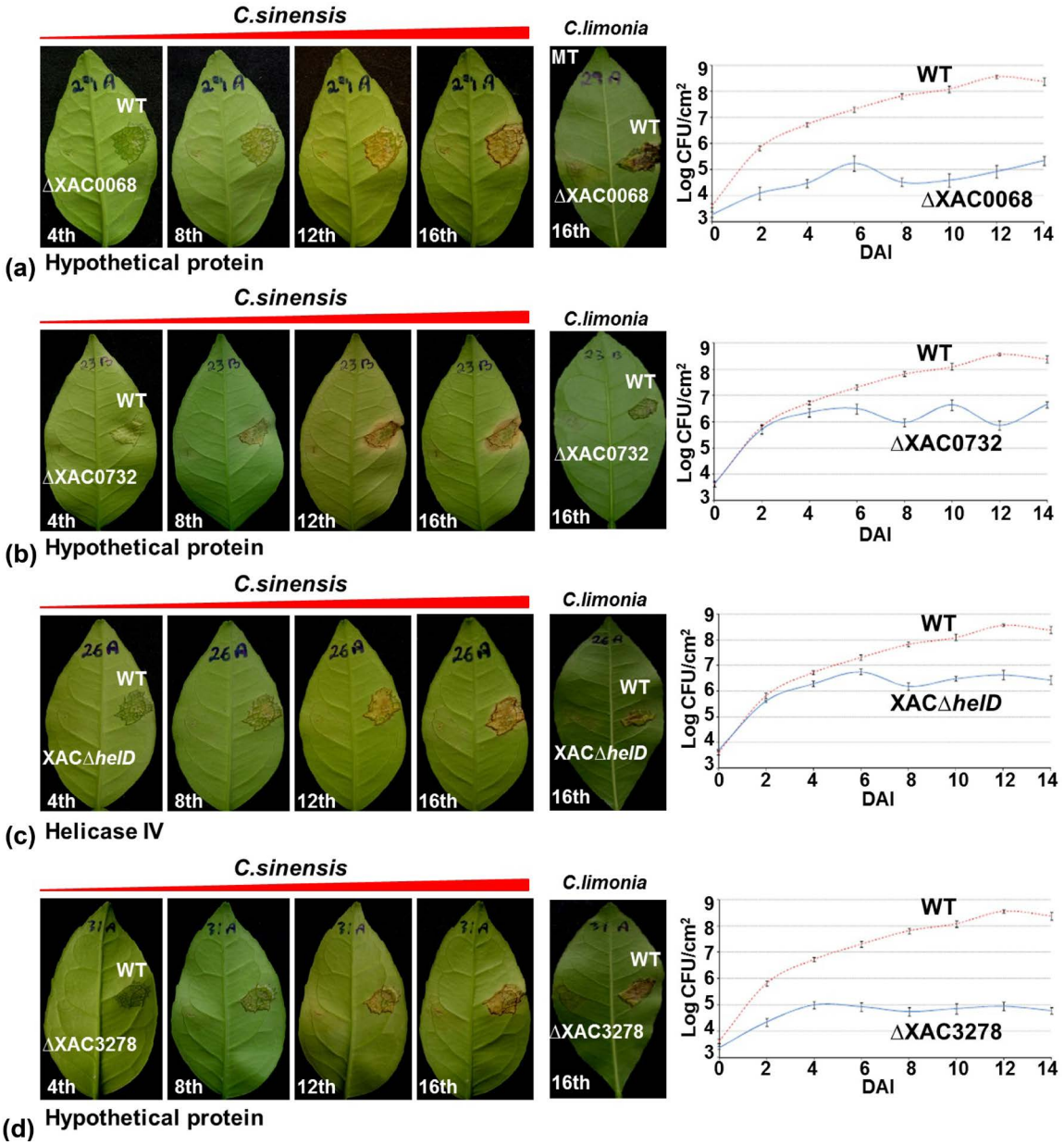

Figure 2. Phenotype and growth curve *in vivo* of the mutants that led to total loss of the virulence in *Citrus sinensis* and *Citrus limonia*. In each of the four analyzed mutants ((a) to (d)), it's possible to follow the virulence progression over the 16 days in *C. sinensis* (at days 4, 8, 12 and 16 after infection), while for *C. limonia* only the virulence phenotype at the 16 days after infection is presented. The *in vivo* growth curve presented is for *C. sinensis*.

genes of this genus: XAC29_03725, XCAW_03850, XFF4834R_chr07400, XACM_0730 and XCV0786. The two sequences with higher degrees of identity (99%) were again found in *X. axonopodis Xac*29-1 and *X. citri* subsp. *citri* Aw12879, respectively [8], which are strains capable of inducing canker symptoms in citrus hosts. The third ortholog was found in *X. fuscans* subsp. *fuscans*, a specie capable of causing damage in leguminous plants, among which bean as the most important one [27]. The fourth orthologous sequence was found in *X. alfalfae* subsp. *citrumelonis* (*X. axonopodis* pv. *citrumelo*), which specifically attacks rootstocks of the 'Swingle' citrumelo (*Citrus paradisi* x *Poncirus trifoliata*) [28], and the fifth ortholog was found in *X. campestris* pv. *ve-*

sicatoria, which causes disease in peppers and tomatoes [29].

The *XAC*Δ*helD* mutant, whose XAC2265 knocked-out gene encodes Helicase IV (HelD), showed a 100-fold smaller *in planta* growth than wild type strain profile (**Figure 2(c)**). Although it is known that HelD belongs to a group of enzymes that play a crucial role in homologous recombination and DNA repair [30], it has also been recently described it as part of the transcriptional machinery in *Bacillus subtilis*, where it is involved in adaptation to the environment. This is because *helD* mutants in *B. subtilis* showed a reduced ability to adapt and grow when cells, in stationary phase, are diluted in rich medium [31]. Structurally, HelD has six conserved motifs (UvrD-helicase, UvrD_C, UvrD_C_2, AAA_19, AAA_30, AAA_12), all related to replication, recombination and DNA repair [26]. In bacteria of the genus *Brucella*, *in vivo* experiments on goat hosts demonstrated that mutant bacteria for an UvrD-helicase showed reduced virulence ability to attack the animal host. The authors attribute this reduced virulence phenotype to the oxidative conditions encountered by the bacteria within the host defense phagosome cells, where the absence of UvrD undermines bacterial DNA repair system when subjected to oxidative stress during host defense to pathogen infection [32]. During *Xac* attack to citrus host an analogous situation is established, where an oxidative stress due to plant resistance response against pathogen attack is also induced, suggesting a similar virulence role to *Xac*'s HelD as detoxifying protein. It is worth noting that orthologs of HelD with a high degree of sequence homology were observed in only two other species of *Xanthomonas* capable of infecting citrus, *X. axonopodis Xac*29-1 [7] and *X. citri* subsp. *citri* Aw12879 [8], which could indicate a putative speciation of this gene in these organisms.

The *in planta* curve of ΔXAC3278 mutant evidenced a 4000 times smaller growth when compared with the wild type strain, clearly demonstrating that the mutation compromised the virulence and fitness of this strain within the plant (**Figure 2(d)**). The knock out gene for this mutant is only present in the genus *Xanthomonas*, since sequence homology analysis revealed only four orthologous genes of the genus *Xanthomonas*: *X. axonopodis Xac*29-1 (XAC29_16710), *X. citri* subsp. *citri* Aw12879 (XCAW_01591), *X. fuscans* subsp. *fuscans* (XFF4834R_chr22200) and *X. alfalfae* subsp. *citrumelonis* (XACM_2229). All four orthologous genes share the BLUF (sensors of Blue Light Using FAD) domain, which is present in several proteins (mostly bacterial) and is involved in the repression of photosynthesis genes in response to blue light (possibly oxide-reduction reactions) using flavin adenine dinucleotide (FAD) as cofactor [33]. BLUF domain is similar to cryptochromes, which are blue light photoreceptors involved in stem growth, cotyledon expansion, and flowering induction in plants as well as in biological clock synchronization (circadian rhythms) in animals, and are encoded by Cry1 and Cry2 genes in plants [33] [34]. Genes containing the BLUF domain have been shown to be involved in functions related to survival and virulence of *Xac* during host plant colonization [35]. Interestingly, this gene is inserted into a genomic region demonstrated to result from horizontal transfer events. Apart from being acquired in these genomes, the function of this gene in these strains seems fundamental for the interaction and successful attack to plant hosts, as previously described for the XAC0068 gene.

3.2. Mutants Contributing to Partial Loss of Virulence in *C. sinensis* and a Total Loss in *C. limonia*

Five mutants (19.2%), from the twenty-six analyzed in this study, were classified into this category. None of them induced canker symptoms when inoculated in *C. limonia* host. However, when inoculated in *C. sinensis*, they caused minor necrosis (*XAC*Δ*xylR* and *XAC*Δ*petA*), mild hyperplasia (ΔXAC1491), or minor necrosis accompanied by mild hyperplasia (*XAC*Δ*parB*) (**Table 2**, **Figure 3**).

The mutant *XAC*Δh*xylR*, whose XAC0075 knocked-out gene encodes a xylose repressor-like protein (XylR), showed an approximately 100-fold smaller cell number increase in *C. sinensis* host, when compared to the wild type strain, demonstrating that the mutant suffered significant changes in its cellular metabolism (**Figure 3(a)**). An analysis using the blastx algorithm revealed that this gene is unique, with a very high sequence homology to all sequenced *Xanthomonas* and several *Pseudomonas syringae* pathovars, a causal agent of diseases in tomatoes, beans, rice, and soybeans [19] [36]. In its structure, the deduced protein has a ROK domain (**Table 2**), which function is related to carbohydrates metabolism and transport, including glucokinase activity and possibly the production of glycoprotein compounds, such as virulence related xanthan gum [37] [38] [39]. A study investigating the promoter activities of plant-modulated bacterial gene expression revealed a PIP-BOX-homologous sequence on the XAC0075 gene [40]. The PIP-box or Plant-Promoter-Inducible Box is a consensus sequence of nucleotides (TTCGC...N15...TTCGC) present in the promoter region of some genes responsible for the expression of pathogenicity and virulence factors [41]. In *X. campestris* pv. *campestris*, a phytopathogen responsible for disease in crucifers, it was demonstrated the role of XyIR in regulating xylan synthesis and control of pathogen survival in the epiphytic phase, also participating as a regulator of genes related do pathogenic process [42].

The mutant ΔXAC1491, whose knocked-out gene encodes a hypothetical protein, showed an approximately 100-fold reduced growth when compared to the wild strain, suggesting a relationship of this gene function with the *Xac* adaptation process inside the host plant (**Figure 3(b)**). Sequence similarity analysis showed that orthologs for this gene have been found only in the genomes of *X. citri* subsp. *citri* strains 306 and Aw12879 and *X. axonopodis Xac*29-1 [19]. The fact that this gene is inserted in a so-called horizontal gene transfer island aids to justify its exclusivity.

The mutant XACΔaslB which knocked out gene (XAC1927) encodes a Fe-S oxidoredutase (aslB) has showed an *in planta* growth 100 times lower when compared to wild type XAC306 strain (**Figure 3(c)**). This gene is located at a putative transfer island and was previously seem [14] as being expressed only during *in vivo* contact with citrus plant host.

The mutant *XAC*Δ*petA*, whose knocked-out gene XAC2457 encodes the ubiquinol-cytochrome oxidoredutase protein (PetA), showed an approximately 1000-fold smaller growth when compared to the wild type strain inoculated in *C. sinensis* (**Figure 3(d)**). The PetA protein, along with PetB and PetC, are encoded by in tandem genes in the *Xac* genome (XAC2457 to XAC2455), belong to the cytochrome c-reductase complex and are directly involved in electron transfer during oxidative phosphorylation.

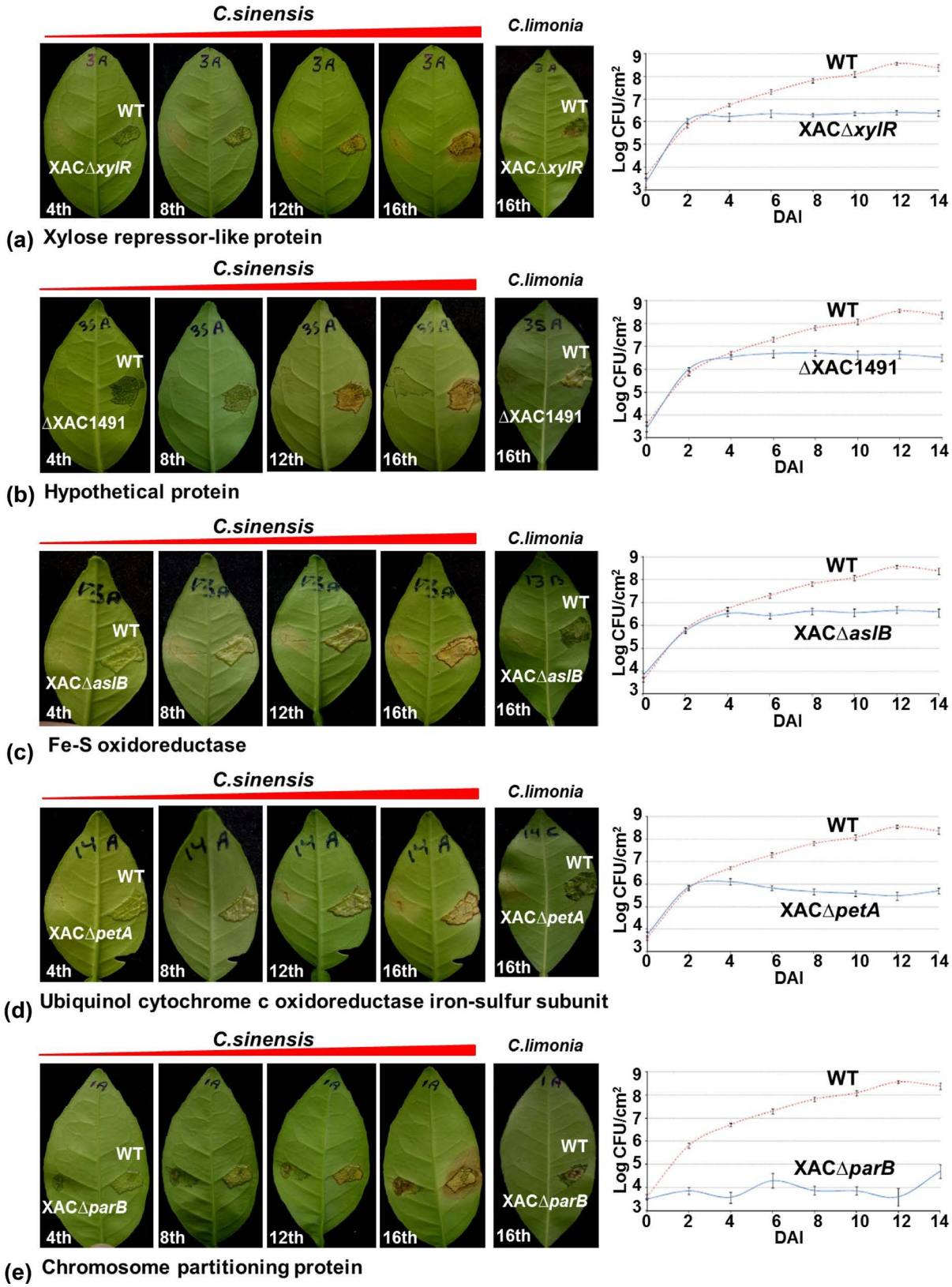

Figure 3. Phenotype and growth curve *in vivo* of mutants that led to partial loss of virulence in *Citrus sinensis* and total loss in *Citrus limonia*. In each of the five analyzed mutants ((a) to (e)), it's possible to follow the virulence progression over the 16 days in *C. sinensis* (at days 4, 8, 12 and 16 after infection), while for *C. limonia* only the virulence phenotype at the 16 days after infection is presented. The *in vivo* growth curve presented is for *C. sinensis*.

Since oxidative phosphorylation is an energetic process common to aerobic organisms, it is evident that *petA* is shared by the vast majority of them [19]. Therefore, the loss of its functionality would result in a reduction of bacterial titration, which is clearly observed in the growth curve and, consequently, related to pathogen's virulence.

The mutant *XAC*Δ*parB*, whose knocked-out gene (XAC3906) encodes the protein for chromosome division (ParB), showed a dramatic *in planta* growth reduction, approximately 10,000 times, when compared to the wild type strain (**Figure 3(e)**). ParB and ParA protein functions are related to plasmid partitioning during cell division. The absence of these genes in *Caulobacter crescentus* inhibited the molecular processes responsible for the early events of cellular division [43]. Besides that, virulence factors can be hosted on pathogenic bacteria plasmids [44], and indeed that's the case for *Xac* where functional partitioning of plasmids containing virulence genes can be determinant to successful pathogen attack to host. Based on gene expression techniques, the ParB protein was shown to be induced in *Xac* after 12 hours growth in a XAM1 pathogenicity-promoting medium [14], revealing its importance in this process.

3.3. Mutants Leading to a Partial Reduction in Necrosis and Total in Hyperplasia in *C. sinensis* and *C. limonia*

Five mutants (19.2%) (*XAC*Δ*ftsB*, *XAC*Δ*yagT*, *XAC*Δ*gumI*, ΔXAC3368 and ΔXAC1531) were unable to induce hyperplasia and lead to a reduced necrosis in both hosts evaluated (*C. sinensis and C. limonia*) (**Table 2**, **Figure 4**).

The *XAC*Δ*ftsB* mutant (**Figure 4(a)**) was knocked out for the gene XAC1720, whose functional protein corresponds to a protein involved in cellular division (FtsB). This protein, together with FtsL (XAC0773) and FtsQ (XAC0782) are small membrane proteins that create a macromolecular complex capable to promote the coordinated invagination of the plasma membrane and peptidoglycan wall and, therefore, lead to the construction of the cellular division septum [45]. The blastx algorithm-based analysis [19] showed that the XAC1720 gene has orthologs with high sequence identity (99% to 88%) only within the genera *Xylella*, *Xanthomonas* and *Stenotrophomonas*, which correspond to phytopathogens or opportunistic human pathogens gamma-proteobacteria [20].

For ΔXAC3368 mutant (**Figure 4(b)**) the knocked-out gene is designated as hypothetical. This gene is showed as being specific to the genus *Xanthomonas*. Although hypothetical, the XAC3368 gene has two domains, the ATPase_gene1 that encodes for a small peptide (about 100 amino acids) with long hydrophobic tails found in several bacterial genomes and that possibly have an enzymatic function, and the Peptidase_M23, a Gly-Gly endopeptidase, which also has an enzyme function [46].

The *XAC*Δ*yagT* mutant (**Figure 4(c)**) was knocked out for XAC1189 gene, whose functional protein is predicted to be a water-soluble ferredoxin protein associated to iron-sulfur (2Fe-2S) accumulations and an important role during electron transfer in several enzymatic reactions, including the carrying of electrons during photosynthesis in bacteria, seaweed and plants [47] [48]. Orthologs of the XAC1189 gene, with a high homology, were found in a large number of proteobacteria (gamma, beta, delta and alpha). However, almost a complete sequence identity was observed in the genus *Xanthomonas*.

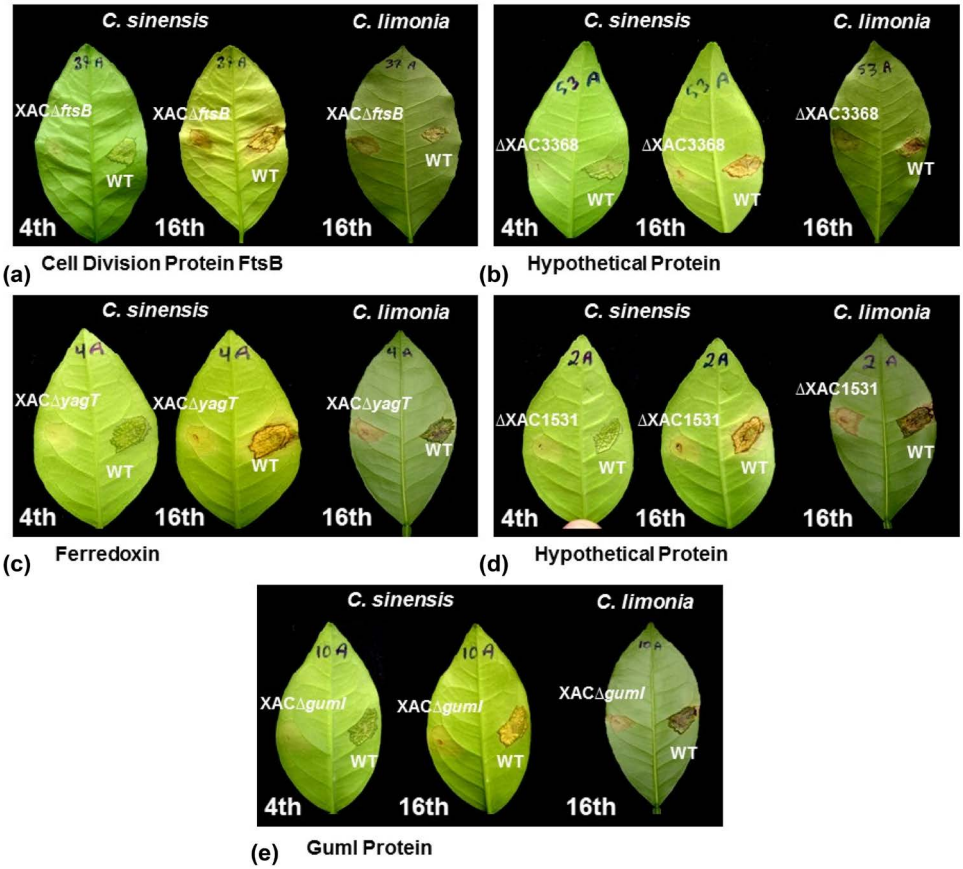

Figure 4. Phenotype of mutants that led to partial loss of necrosis and total loss of hyperplasia in *Citrus sinensis* and *Citrus limonia*. In *C. sinensis* virulence symptoms at days 4 and 16 after infection are presented, while in *C. limonia* only the virulence phenotype at the 16 days after infection is presented.

The ΔXAC1531 (**Figure 4(d)**) mutant is knocked out in a hypothetical gene specific to genus *Xanthomonas* and contain the unknown function UPF0029 and DUF1949 domains (**Table 2**). Despite of inducing a necrotic lesion at the site of infection this mutant is not able to induce hyperplasia on infected plant leaf tissue.

The *XACΔgumI* mutant (**Figure 4(e)**) with gene XAC2578 knocked out is responsible for encoding the GumI protein. This gene belongs to a group of 14 genes involved in the synthesis of xanthan gum, an extracellular polysaccharide (EPS) related to induced watersoaking and hyperplasia phenotype during host tissue colonization by *Xanthomonas* [49]. Xanthan gum is a virulence factor, since EPS-deficient mutants are usually avirulent [50] [51] [52].

3.4. Mutants Contributing to a Reduction in Hyperplasia and/or Necrosis in *C. sinensis* and *C. limonia*

Five mutants (*XACΔrnk*, *XACΔmpd*, ΔXAC1008, *XACΔrecR* and *XACΔcynX*) caused hosts to present different phenotypic changements in relation to wild type *Xac* induced canker lesion (**Table 2**, **Figure 5**).

The *XACΔrnk* mutant (**Figure 5(a)**) has the gene XAC0903 knocked-out, which is responsible for encoding a nucleoside diphosphate kinase (Ndk) regulator. Orthologs

for *rnk* of *Xac* have only been found in bacteria of the genus *Xanthomonas*. Ndk is known to be involved in bacterial pathogen adaptation to host during infection [53].

The *XAC*Δ*mpd* mutant (**Figure 5(b)**) had the XAC0726 gene, responsible for encoding the methyl parathion hydrolase, knocked out. Similarity analysis revealed that this gene is highly specific, since orthologs have been found only in *X. axonopodis Xac*29-1, *X. citri* subsb *citri* Aw12879 and *X. fuscans* subsp. *fuscans*. The methyl parathion hydrolase (MPH) protein is an enzyme that catalyzes the degradation of a wide range of organophosphate compounds, among them the methyl parathion, which is one of the most commonly used organophosphate fertilizers worldwide, especially in fruit and vegetable farming [54].

The ΔXAC1008 mutant (**Figure 5(c)**), which is responsible for coding a hypothetical protein, knocked out. Similarity analysis demonstrated that this gene has orthologs only in bacteria of the genus *Xanthomonas* and *Stenotrophomonas* [20]. It has a hydrolase_2 domain that, in *B. subtilis*, has been shown to be involved in cell wall hydrolysis [52]. Studies on genetic expression analyses showed that this protein is induced in *Xac* after 3 days of being inoculated into the plant [14], highlighting its importance in the pathogenic process.

The *XAC*Δ*recR* mutant (**Figure 5(d)**) was knocked-out for the gene XAC1111, which is responsible for encoding the Recombination protein RecR, which is involved

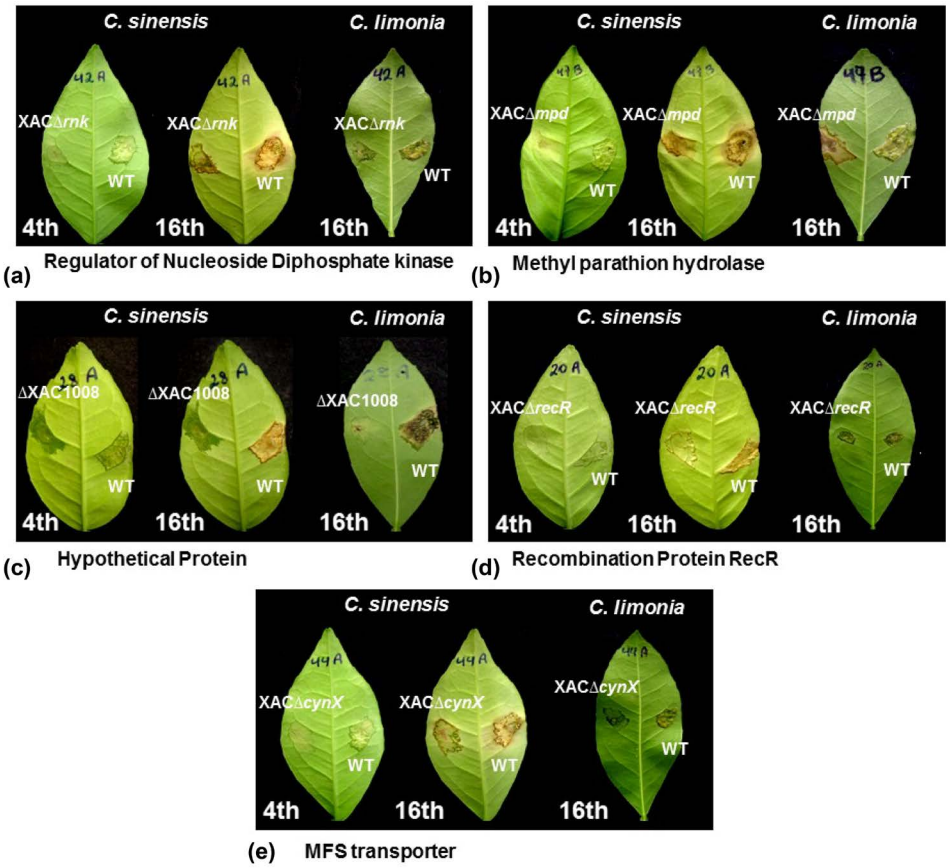

Figure 5. Mutants that led to decrease of hyperplasia and/or necrosis in *Citrus sinensis* and *Citrus limonia*. In *C. sinensis* virulence symptoms at days 4 and 16 after infection are presented, while in *C. limonia* only the virulence phenotype at the 16 day after infection is presented.

in DNA repair and replication. Similarity analysis demonstrated that this gene has orthologs only in the genera *Xylella*, *Xanthomonas*, *Stenotrophomonas* and *Pseudoxanthomonas* belonging to the family Xanthomonadaceae.

The *XAC*Δ*cynX* mutant (**Figure 5(e)**) was knocked-out for the gene XAC2234, which encodes the MFS transporter protein, a membrane protein found in all organisms as transporter (entry and exit) of various substrates, such as sugars, drugs, metabolites, oligosaccharides and amino acids [55]. Nevertheless, similarity analysis showed that the XAC2234 gene is the most specific among all the mutants assessed in this study. We found only one ortholog in *X. axonopodis Xac*29-1. Gene expression techniques demonstrated that this protein is repressed in *X. citri* after 24 hours growth in a XAM1 pathogenicity-inducing medium [14], suggesting a role for this protein in the early phytopathogenic process.

3.5. Mutants Contributing to Increased Necrosis in *C. sinensis* and *C. limonia*

Seven mutants (26.9%) (*XAC*Δ*orf8*, *XAC*Δ*pepB*, *XAC*Δ*metE*, ΔXAC2344, ΔXAC3777, ΔXAC3815 and ΔXAC4321) showed an increased capability to induce plant necrosis (**Table 2** and **Figure 6**). Knowing that the necrosis process induced in infected plant tissue is one of the defense mechanisms that the plant uses to isolate and kill pathogens [56] [57], we can infer that perhaps these genes play a role in the initial process of pathogenicity by conferring a "camouflage" to the pathogen. Therefore, the absence of translated genes would enable the plant to easily recognize the aggressor and inhibit its development and propagation through the general plant resistance response.

The *XAC*Δ*orf8* mutant (**Figure 6(a)**) was knocked-out for the gene *XAC*2243, which is responsible for encoding a protein with 86% identity (blastp analysis) to a *P. aeruginosa* dead box RNA helicase, which has recently characterized as being related to an indirectly up regulation of virulence type III secretion system [58]. This gene presents many orthologs with great similarity within the gamma/beta/alphaproteobacteria. However, among the bacteria of the genus *Xanthomonas*, an ortholog was only found in *X. axonopodis Xac*29-1, *X. citri* subsp. *citri* Aw12879, *X. campestris* pv. *campestris* B100 and *X. campestris* pv. *vesicatoria*, which could indicate a horizontal transfer of this gene.

The genes deleted in the mutants ΔXAC2344 (**Figure 6(b)**), ΔXAC3777 (**Figure 6(c)**), ΔXAC3815 (**Figure 6(d)**) and ΔXAC4321 (**Figure 6(e)**), encode for hypothetical genes. All genes had orthologs only in bacteria of the genus *Xanthomonas*. The gene *XAC*2344 has an YCII domain, which might indicate that this gene encodes for a protein with an enzymatic function, since it has highly conserved histidine and aspartate regions [52]. Studies on genetic expression analyses showed that this protein is induced in wild type strains of *Xac* immediately after the first 24 hours upon inoculation [14], showing that this protein plays a role in the pathogenic process. The gene XAC3777 has a nucleoplasmin domain, which is involved in the process of chromatin decondensation [59]. The deduced protein from gene XAC3815 has 3 domains (DUF3667, CitX, and zf-C3HC4_3), which are still not well characterized. Finally, the gene XAC4321 has no identified domains so far, thus making it impossible to infer any function of the protein

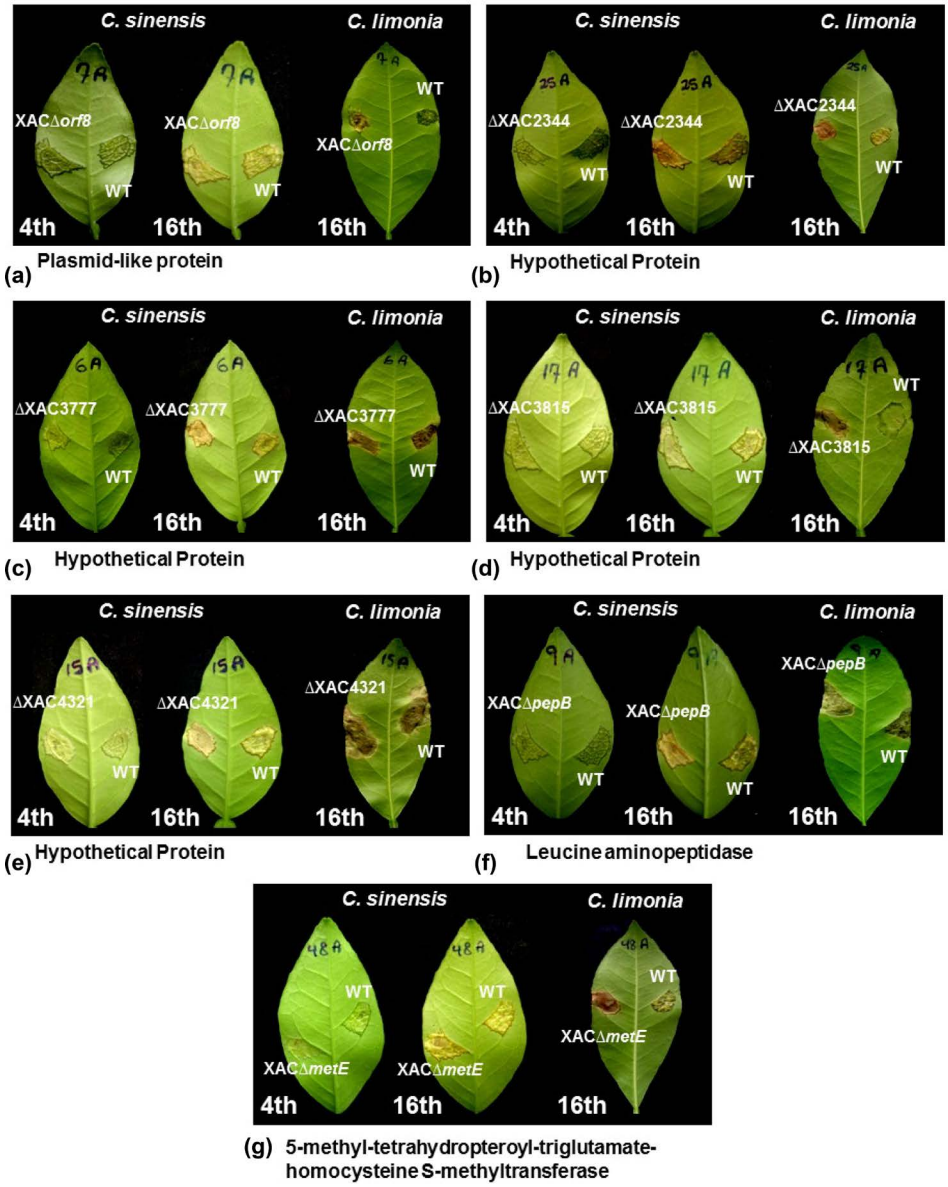

Figure 6. Mutants that led to increased necrosis in *Citrus sinensis* and *Citrus limonia*. In *C. sinensis* virulence symptoms at days 4 and 16 after infection are presented, while in *C. limonia* only the virulence phenotype at the 16 days after infection is presented.

encoded by this gene. In *X. vesicatoria* this gene was annotated as a putative secreted protein.

The *XAC*Δ*pepB* mutant (**Figure 6(f)**) was knocked-out for the gene *XAC*3987, which is responsible for encoding leucine aminopeptidase, an enzyme that catalyzes the removal of leucine residues from the N-terminus of proteins [60]. Similarity blastp analysis demonstrated that all representatives of the genera *Xylella*, *Xanthomonas*, *Stenotrophomonas* and *Pseudoxanthomonas* have an ortholog for this gene.

The *XAC*Δ*metE* mutant (**Figure 6(g)**) was knocked-out for the gene *XAC*0336, which is responsible for encoding the 5-methyl-tetrahydropteroyltriglutamate-homocysteine S-methyltransferase, a protein involved in cysteine and methionine amino acid metabolism, acting as a methyltransferase in the conversion of L-homocysteine into L-methionine

[20]. It was shown that this gene is conserved in a large number of gamma/beta-proteobacteria. In a recent study with *Ralstonia solanacearum*, it was observed that both the MetR, a classic repressor of genes involved in methionine synthesis, and HrpG, a regulator associated with the expression of T3SS gene effectors, are capable of modulating the expression of MetE [61], which highlights the importance of this gene in processes of plant adaptation and virulence induction.

3.6. Analysis of Mutated Orthologous Genes in Other Genomes of the Family Xanthomonadaceae

In an attempt to understand the possible origin of these genes in the *Xac* genomes, and the possible presence of homologs in other bacteria of the same family, the presence or absence of orthologs was assessed by sequence comparison. Another thirteen different species of bacteria of the genus *Xanthomonas* were included in this analysis, four species of the genus *Stenotrophomonas*, five of the genus *Xylella* and two of *Pseudoxanthomonas* (see list and abbreviations in the methodology section), all with their respective genomes deposited in the Kegg (**Figure 7**). As a result, four sets of genes were identified in a cladogram generated from a presence/absence matrix.

From the first set consisted of six mutants, which genes were characterized as being unique to bacteria strains of the genus *Xanthomonas* that infect citrus (13-*XAC*Δ*aslB*, 16-*XAC*Δ*helD*, 1-ΔXAC0068, 14-*XAC*Δ*cynX*, 10-ΔXAC1491 and 20-ΔXAC3278), three of which show a complete absence of symptoms when inoculated into both hosts analyzed, 6, 1 and 20. Mutant 20 still stands out in this set for being one of the 3 mutants whose genes are unique to the species associated with citrus, and as for mutants 13 and 10, which are inserted in into a putative horizontal transfer islands. This was the only gene associated with the species that infect citrus, which was also found in *X. axonopodis* pv. *citrumelo* F1. The gene *cynX* was found only in *Xac* and *Xao*, and XAC1491 only in *Xac* and *Xci*.

In the second set, 7 other mutants were grouped together (23-Δ*XAC*3815, 26-Δ*XAC*4321, 5-Δ*XAC*0732, 9-*XAC*Δ*yagT*, 22-ΔXAC3777, 2-*XAC*Δ*xylR* and 19-*XAC*Δ*guml*). This set draws our attention, since their respective knocked-out genes were characterized as being unique to the genomes of bacteria of the genus *Xanthomonas*, except for mutants 23 and 19, whose genes were also found in bacteria of the genus *Pseudoxanthomonas*. Also in this set, it is important to highlight that subgroups can be observed, since there is the absence of genes shared by specific species, as it is the case for the genes knocked-out in mutants 23, 26 and 5, absent in bacteria of the strain *X. campestris*, and in mutants 9 and 22, absent in bacteria of the strain *X. oryzae*. In this set of knocked-out genes, the only ortholog present in *X. albilineans* is the *XAC*4321 gene.

The third set of 6 knocked-out genes were also highlighted (21-ΔXAC3368, 6-*XAC*Δ*rnk*, 3-*XAC*Δ*metE*, 17-ΔXAC2344, 11-ΔXAC1531 and 7-ΔXAC1008). In this case, it is due to the fact that they coexist only in bacteria of the genera *Xanthomonas* and *Stenotrophomonas*, except for the genes related to mutants 11, 7 and 6, which also coexist in bacteria of the genus *Pseudoxanthomonas*. Only the genes associated to mutants 21 and 6 were not found in the genomes of *X. albilineans* and *X. oryzae* pv. *oryzicola* BLS256.

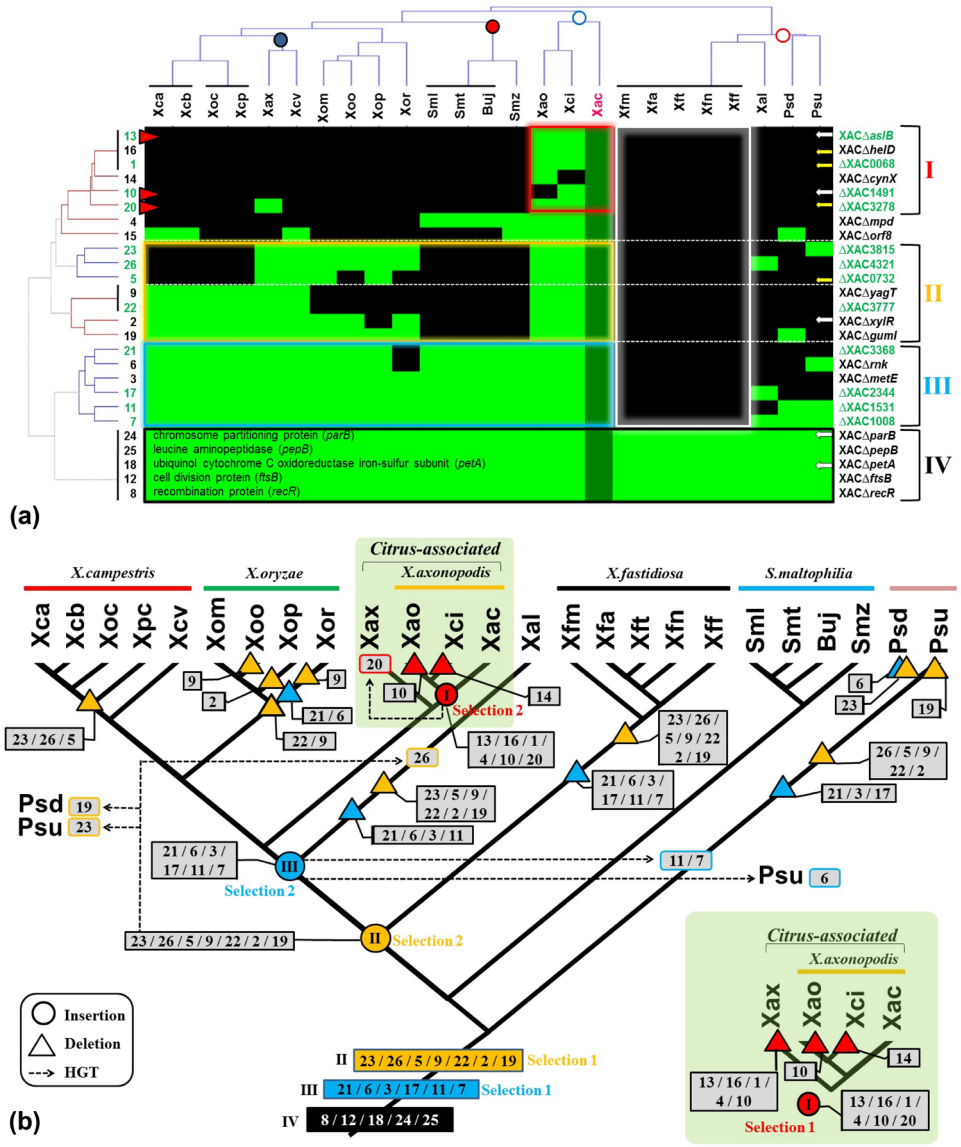

Figure 7. Presence and absence analysis of mutated genes in other bacteria of the family *Xanthomonadaceae*. The green color shows the presence of orthologous genes in their respective XAC mutants analyzed in this study and named according to **Figure 1(c)**. The organisms are presented in a horizontal cladogram based on a gene matrix of presence and absence enabling the creation of the cladogram. The phylogeny of the strains based on conserved gene sequences (gyrB or 16S-rDNA) and maintained by the displacement of two clads are highlighted by full circles, which should be allocated in the positions of empty circles of the same color. The red triangles highlight the genes inserted in the genomic islands. The yellow arrows indicate nonpathogenic mutants in both hosts analyzed, whereas white arrows show a total loss of virulence only in *Citrus limonia*. Red outlines highlight specific genes in strains associated with citrus. Yellow outlines, specific genes in bacteria of the genus *Xanthomonas*, however, absent in some specific strains set. The numbers from 1 to 4 on the right side of the design highlight determined sets of genes discussed in the text. Blue outlines, specific genes of bacteria of the genus *Xanthomonas* and in some cases, present also in *Pseudoxanthomonas*. White outlines, total absence of genes in *Xylella*.

Contrary to what has been described for the three sets analyzed so far, none of these related genes was found in the genomes of bacteria of the genus *Xylella*. This event

might have a direct relationship with the alleged massive genome reduction that occurred in the species of this genus [62]. However, it can also result from the acquisition of other genera in the genomes.

Finally, a fourth set of genes was found consensually in the genomes of all species (24-*XAC*Δ*parB*, 25-*XAC*Δ*pepB*, 18-*XAC*Δ*petA*, 12-*XAC*Δ*ftsB*and 8-*XAC*Δ*recR*). The products of these genes are associated with fundamental cellular processes such as DNA replication and repair, cell growth and development. Although essential to the maintenance of cellular survival, their absence did not cause any reduction in their growth rates *in vitro.*

4. Conclusion

The data presented in this study enabled to associate twenty-six *Xac* genes, where eleven were hypothetical, with adaptation events and pathogenicity in compatible hosts, e.g. *C. sinensis* and *C. limonia*. Six of these genes were characterized as being unique to species infecting citrus, including *X. citri* subsp. *citri* Aw12879, *X. axonopodis Xac*29-1 and *X. axonopodis* pv. *citrumelonis* F1. Three of the six citrus species associated with genes are inserted into putative horizontal transfer islands. Other seven knocked out genes are specific to the genus *Xanthomonas*. Therefore, this *in vivo* study of a new repertoire of genes related to pathogenicity and virulence contributes to a better understanding of the biology of *Xac*-citrus interaction, allowing its extrapolation to other pathosystems and suggesting new targets to further studies on plant-pathogen disease.

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