ANDRÉ DA SILVA XAVIER

VIRUSES OF PHYTOPATHOGENS: ADAPTATION AND MODULATION OF PATHOGENESIS IN *Ralstonia* spp. AND *Alternaria alternata*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 31 de Agosto de 2016.

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BIOGRAFIA

ANDRÉ DA SILVA XAVIER, filho de José Barbosa Xavier e Lindaci Ferreira da Silva Xavier, nasceu em Vitória de Santo de Antão, Pernambuco, no dia 17 de Fevereiro de 1987. Em 2005 iniciou o curso de Engenharia Agronômica na Universidade Federal Rural de Pernambuco (UFRPE), Recife, Pernambuco, vindo a graduar-se em Julho de 2010. No mês seguinte, ingressou no Programa de Pós-graduação em Fitopatologia em nível de Mestrado na Universidade Federal de Viçosa (UFV), submetendo-se à defesa de dissertação em Julho de 2012. Em agosto de 2012, iniciou o curso de Doutorado em Fitopatologia na mesma instituição, submetendo-se à defesa de tese em 31 de agosto de 2016.

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ABSTRACT

XAVIER, André da Silva, D.Sc., Universidade Federal de Viçosa, September, 2016. **Viruses of phytopathogens: adaptation and modulation of pathogenesis in *Ralstonia* spp. and *Alternaria alternata***. Adviser: Francisco Murilo Zerbini Júnior. Co-adviser: Poliane Alfenas Zerbini.

Viruses of phytophatogens are present in the most diverse groups of hosts, infecting from Spiroplasma to nematode and playing an important role in the ecology of these hosts. Because of the potential as biological control agents, the discoveries about these viruses have increased which encourages the ecological management of plant diseases. In this context, this study aimed: isolate and characterize viruses that infect plant pathogenic fungi (1) and *Ralstonia* spp. in the Americas (2) and investigate antiviral defense mechanisms in *Ralstonia* spp. (3). In this thesis, three viruses were characterized at biological and molecular level. Two viruses infect bacteria, *Ralstonia* spp. and the third infect a fungus, *Alternaria alternata.* The bacterial virus displayed two propagation modes: pseudolysogenic for the RsIBR1 inovirus (*Inoviridae*) and lytic for the phiAP1 phikmvvirus (*Podoviridae*). RsIBR1 has been shown to dramatically change the phenotype of the host, while not causing cell lysis, converting the phytopathogenic *R. pseudosolanacearum* into a commensal bacterium. PhiAP1 was characterized as a new member of the genus *Phikmvvirus*, and the presence of exolisinas, in addition to the bactericidal properties and EPS degradation make this virus attractive for future proposals of the bacterial wilt management. In *A. alternata*, it was characterized Alternaria alternata parititivirus 1 (AtPV1), a new divergent mycovirus related to genus *Gammapartitivirus*. Its persistent lifestyle prevented to dissect completely the interaction, because in the absence of isogenic lineage, it is unclear whether the viral infection led to a conversion of a pathogen in a epiphytic or still if is responsible for controlling phenotypic plasticity expressed by the host. The presence of canonical CRISPR loci in *Ralstonia* spp. isolates suggested that, in these hosts, this system could compose the antiviral defense arsenal. However, here was demonstrated that in *R. solanacearum*, the CRISPR-Cas system is unable

to acquire new spacers or interfere with foreign DNA, even in a state of priming. The expression of the *Cas* genes was not detected; indicating that *Cas* operon apparently is a suppressed transcriptional unit. Two genes *h-ns*, expressed in *R. solanacearum* CFBP2957 are candidates to the putative transcriptional repression of CRISPR. The BIMs (Bacteriophages Insensitive Mutants) who escaped of infection by an alternative strategy to the CRISPR, were less competent, but still permissive to viral adsorption, and were able to survive under high inoculum pressure, which excludes the presence of abortive systems. Viral replication was also not detected and sequencing of two BIMs genome revealed the presence of particular mutations in genes encoding components of the secretory and motility pathways (such as Type II and Type IV), potentially involved in the antiviral resistance. Additional analyzes are being conducted to characterize in detail this defense strategy. In conclusion, the discovery and characterization of viruses infecting phytopathogens in Brazil expand the information on this diversity, little known in America, exposes the biotechnological potential of these natural enemies and reveals the important role of these viruses in the modulation and evolutionary course of their hosts

RESUMO

XAVIER, André da Silva, D.Sc., Universidade Federal de Viçosa, setembro de 2016. **Vírus de fitopatógenos: adaptação e modulação da patogênese em *Ralstonia* spp. e *Alternaria alternata***. Orientador: Francisco Murilo Zerbini Júnior. Coorientadora: Poliane Alfenas Zerbini.

Vírus que infectam fitopatógenos são encontrados nos mais diversificados grupos de hospedeiros, infectando desde spiroplasmas a nematóides e desempenhando papel relevante na ecologia desses hospedeiros. Graças ao potencial como agentes de controle biológico, as descobertas sobre esses vírus tem aumentado o que amplia as perspectivas do manejo ecológico de doenças de plantas. Neste contexto, este trabalho objetivou: Isolar e caracterizar vírus que infectam fungos fitopatogênicos (1) e os que infectam *Ralstonia* spp. no continente americano (2) e Investigar mecanismos de defesa antiviral in *Ralstonia* spp (3). Nesse trabalho, três vírus foram caracterizados a nível biológico e molecular. Dois deles infectam bactérias do complexo *Ralstonia* spp. e o terceiro infecta um fungo, *Alternaria alternata*. Os virus de bacterias isolados possuem dois modos de propagação distintos: pseudolisogênico, o inovírus RsIBR1 (*Inoviridae*) e lítico, o phikmvvirus phiAP1 (*Podoviridae*). RsIBR1 demonstrou ser capaz de alterar drasticamente o fenótipo do hospedeiro, convertendo a bactéria fitopatogênica *R. pseudosolanacearum* em uma comensal. PhiAP1 foi caracterizado como um novo membro do gênero *Phikmvvirus,* e a presença de exolisinas em adição as propriedades bactericida e de degradação de EPS o tornam atrativo para futuras propostas de manejo da murcha-bacteriana. Em *A. alternata,* foi caracterizado o Alternaria alternata parititivirus 1 (AtPV1), um novo micovírus divergente, relacionado ao gênero *Gammapartitivirus*. Seu estilo de vida persistente impediu dissecar por completo a interação, pois na ausência da linhagem isogênica, não foi possível confirmar se a infecção viral conduziu a uma conversão de um patógeno em um epifítico ou ainda se é responsável pelo controle da plasticidade fenotípica manifestada pelo hospedeiro. A presença de *loci* CRISPR canônicos entre isolados de *Ralstonia* spp. sugeriu que nesses hospedeiros esse sistema

pudesse compor o arsenal de defesa antiviral. No entanto, aqui foi demonstrado que em *R. solanacearum*, o sistema CRISPR é incapaz de adquirir novos *spacers* ou interferir com DNA invasor, mesmo em estado de *priming*. A expressão dos genes *Cas* não foi detectada, indicando que o operon *Cas* aparentemente é uma unidade transcricional reprimida. Dois genes *h-ns*, expressos em *R. solanacearum* CFBP2957 são candidatos na execução da provável repressão do CRISPR. Foram obtidos BIMs (Bacteriophages Insensitive Mutants) que escaparam da infecção por uma estratégia alternativa ao CRISPR. Os BIMs foram menos competentes, mas ainda permissivos a adsorção viral, e foram hábeis para sobreviver sob alta pressão de inóculo, o que exclui a presença de sistemas abortivos. A replicação viral também não foi detectada e o sequenciamento do genoma de dois BIMs revelou a presença de algumas mutações em genes que codificam proteínas componentes das maquinarias de secreção e motilidade bacteriana (Tipo II e Tipo IV), potencialmente envolvidas com o fenótipo da resistência. Análises adicionais estão sendo conduzidas para caracterizar em detalhes essa(s) estratégia(s) de defesa. Em conclusão, a descoberta e caracterização de vírus que infectam fitopatógenos no Brasil, além de ampliar as informações sobre essa diversidade, pouco conhecida na América, expõe o potencial biotecnológico desses inimigos naturais e revela o papel relevante desses vírus na modulação e curso evolutivo dos seus hospedeiros.

GENERAL INTRODUCTION

Viruses constitute the most diverse group among biological entities on the Earth and their ability to infect members of the three domains of life allows them to be found all environments (Penny et al., 2013). In addition to the important role in the balance of the biogeochemical cycles of the planet (Balogh et al., 2010; Doffkay et al., 2015; Fuhrman, 1999; Suttle, 2007), viruses that infect microorganisms have interested scientists as natural enemies of human and plant pathogens (Xie and Jiang, 2014; Zhang et al., 2015).

In phytopathogenic microorganisms, most viral infections have been reported in filamentous fungi and bacteria (Ghabrial et al., 2015; Okabe and Goto, 1963), although the presence of some viruses have been reported among oomycetes, nematodes and Mollicutes including *Phytophthora infestans* (Cai and Hillman, 2013), *Heterodera glycines* (Bekal et al., 2011) and *Spiroplasma citri* (Bébéar et al., 1996). Several viruses have been described infecting the species *R. solanacearum* and *R. pseudosolanacearum*, in Japan, Korea (Murugaiyan et al., 2011; Yamada et al., 2007) and recently in Thailand (Bhunchoth et al., 2015). In fungi, the presence of dsRNAs has clearly been an indicative of viral infections, as most viruses infecting such hosts have dsRNA genomes (Ghabrial et al., 2015). In *Alternaria* spp., including *A. alternata*, *A. brassicicola* and *A. longipes* different groups of viruses have been described and, in some cases, associated to a hypovirulence phenotype (Aoki et al., 2009; Lin et al., 2015; Shang et al., 2015).

The potential use of these viruses as therapeutic agents has been evaluated in several agricultural pathosystems, with a significant effect in management of bacterial diseases caused by *Xanthomonas* spp. (Balogh et al., 2010; Civerolo and Keil, 1969; Flaherty et al., 2000;

McNeil et al., 2001; Saccardi et al., 1993), *Erwinia* spp., *Pectobacterium* spp. (Ravensdale et al., 2007) *Ralstonia solanacearum* (Fujiwara et al., 2011), *Streptomyces* spp. (McKenna et al., 2001), and for fungal diseases caused by *Sclerotinia sclerotiorum* (Yu et al., 2013), *Fusarium graminearum* (Chu et al., 2002), *Rosellinia necatrix* (Chiba et al., 2009), *Cryphonectria parasitica* (Bryner et al., 2012) and *Botrytis cinerea* (Potgieter et al., 2013). In addition to the practical context in agroindustry, viral infection is a powerful force that shape host defense mechanisms, thus, fungi and bacteria have evolved mechanisms to evade of viral infection (Koskella and Brockhurst, 2014). Some of these mechanisms are innate, while others are adaptive, such as the CRISPR-Cas system of bacteria and RNA silencing in fungi (Dang et al., 2013; Horvath and Barrangou, 2010).

The latest findings reveal the diversity and potential of these viruses that besides modulating the host evolution can be protagonists in strategies in plant disease management (Balogh et al., 2010; Xie and Jiang, 2014). The objectives of this study were to isolate and characterize viruses infecting phytopathogenic microorganisms and investigate antiviral defense mechanism(s).

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CHAPTER 1

A NOVEL SPECIES IN *Phikmvvirus* INFECTING *Ralstonia* spp. DISPLAYS EPS-DEGRADING PROPERTIES

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A novel species in *Phikmvvirus* infecting *Ralstonia* spp. displays EPS-degrading properties

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Abstract

Bacterial wilt caused by *Ralstonia* spp., soil-borne Gram-negative bacteria, is considered one of the most important plant diseases in tropical and subtropical regions of the world. A large number of bacteriophages capable of lysing or physiologically reprograming cells of *Ralstonia* spp. have been reported in Asia. Despite the potential use of these organisms in the management of the bacterial wilt, information about viruses that infects *Ralstonia* spp. is nonexistent in the Americas. In this work we isolate a virus that infects *Ralstonia* spp. from a soil sample of Brazil. Microscopy and genomic analysis allowed us to classify the virus in the family *Podoviridae*. The genome is a linear double stranded DNA with 44.793 nt, G+C content of 61%, with direct terminal repeats of 416 bp and predicted to code 56 ORFs. The presence of a gene that encodes a RNA polymerase, phylogenetic analysis and a remarkable synteny with the genomes of *Pseudomonas virus phiKMV, Pseudomonas virus LKA1, Pseudomonas virus LKD16, Pantoea virus LIMEzero* and Ralstonia virus RSB3, allowed to classify the virus in the genus *Phikmvvirus*. In spite of relationship with Ralstonia virus RSB3 (60% identity), an Asian species, genomic and biological characteristics suggest that the virus isolated in Brazil belongs to a new species, tentatively named Ralstonia virus phiAP1. PhiAP1 has EPS-depolymerase activity and contains two putative virion-associated peptidoglycan hydrolases (VAPGHs), which reveals a robust mechanism of pathogenesis. Furthermore, phiAP1 infects specifically *Ralstonia solanacearum, R. pseudosolanacearum* and *R. syzygii*, causing cell lysis, not being able to infect other thirteen bacterial species tested, including phytopathogenic and commensal isolates. Together, these characteristics highlight the biotechnological potential of this virus for the management of bacterial wilt.

Introduction

In recent years, the discovery of new bacterial viruses with therapeutic potential, the characterization of the virome in different environments and the emergence of antibiotic resistant bacterial strain have led to a resurgent interest in viral therapy (Cassman et al., 2012; Dutilh, 2014; Klumpp et al., 2012). In the agricultural scenario, the characterization of new bacterial viruses has expanded the alternatives for the management of bacterial plant diseases following the increased demand for sustainable practices (Balogh et al., 2010; Frampton et al., 2012; Oliveira et al., 2015). Since the 1920s, ten years after the discovery of the first bacterial virus, the potential of these viruses as therapeutic agents in agriculture has been evaluated in several pathosystems, with success mentioned in the management of diseases caused by *Xanthomonas* spp. (Balogh et al., 2010; Civerolo and Keil, 1969; Flaherty et al., 2000; McNeil et al., 2001; Saccardi et al., 1993), *Erwinia* spp., *Pectobacterium* spp. (Ravensdale et al., 2007), *Ralstonia* spp. (Fujiwara et al., 2011) and *Streptomyces* spp. (McKenna et al., 2001).

Bacterial wilt is caused by the *Ralstonia solanacearum* complex (RSC) [(Smith, 1986; Yabuuchi et al., 1995)], (Mansfield et al., 2012), a heterogeneous group that contains three soil-borne Gram-negative species, *R. solanacearum R. pseudosolanacearum* and *R. syzygii* (Prior et al., 2016; Safni et al., 2014), that induce rapid and fatal wilting in host plants. *Ralstonia* spp. have a wide host range, infecting more than 200 species belonging to more than 50 botanical families, including economically important crops (Hayward, 1991, 2000). The high variability among isolates of *Ralstonia* spp., their broad host range and the persistence of these pathogens in the soil for long periods are characteristics that can explain the low efficiency of chemical and genetic control methods in the field (Allen et al., 2005; Wang et al., 2005; Yuliar et al., 2015).

Several bacteriophages classified in four families (*Podoviridae*, *Myoviridae*, *Siphoviridae* and *Inoviridae*) have been described infecting *Ralstonia* spp., all in the Asian continent (Japan, Korea and Thailand (Addy et al., 2012; Fujiwara et al., 2011; Kawasaki et al., 2016; Kawasaki et al., 2009; Ozawa et al., 2001; Thi et al., 2016; Toyoda et al., 1991; Yamada, 2012; Yamada et al., 2007), and some of them are proposed to be used in the management of bacterial wilt. We isolated and characterized a virus that infects *Ralstonia* spp. from a Brazilian soil sample. Genomic and biological characteristics suggest that the virus isolated is a new species of the family *Podoviridae*, subfamily *Autographivirinae,* genus *Phikmvvirus,* tentatively named Ralstonia virus phiAP1. The phiAP1 genome encodes an EPS-depolymerase and contains two putative virion-associated peptidoglycan hydrolases (VAPGHs) that suggest a biotechnological potential of this virus for the management of bacterial wilt.

Results

Isolation of virus and morphological characterization

A virus was isolated from a soil sample of a tomato field in Coimbra, Minas Gerais, Brazil, based on the ability to form large clear plaques on *Ralstonia solanacearum* RSB1. Interestingly, the sample was collected in a field where there has been no incidence of bacterial wilt for two years. Electron microscopy of purified virus showed that the particles have an icosahedral head of approximately 60 nm in diameter and a short non-contractile tail with 9 nm (Figure 1A). The viral DNA was extracted from purified virus and analyzed by Pulse Field Gel Electrophoresis. It was possible to identify a single DNA band of ~ 45 kbp (Figure 1B). These characteristics allowed us to classify the virus in the order *Caudovirales* and family *Podoviridae*.

Genome analysis and functional annotation

The complete genome of the virus was sequenced and *de novo* assembled with coverage of 50,230x and corresponds to a linear double stranded DNA of 44.793 bp including, the direct terminal repeats (DTRs) of 416 bp (Figure 2A), with a G+C content of 61%. Based on the sequence assembly an *in silico* restriction map of the putative complete sequence was predicted using the restriction enzymes *Kpn*I, *Dra*I, *Hind*III, *Cla*I, and *Spe*I. The viral DNA was cleaved independently with the same enzymes used in the prediction, and bands with the expected size were visualized after cleavage with *Kpn*I (2.4, 19.5, 22.8 kb), *Dra*I (22.9, 8.1, 13.7 kb), *Hind*III (1.1, 43.6 Kb) *Cla*I (8, 12.9, 11.4, 11.7, 0.64 kb) and *Spe*I (4.5, 40.2 kb) (Figure 2B). The short DNA fragments of 1.1 and ~0.6 kb obtained by *Hind*III and *Cla*I cleavage, correspond to the 3' and 5' ends of the phiAP1 genome, respectively (Figure 2B). This analysis allowed us to confirm the assembly of the viral genome.

A total of 56 putative open reading frames (ORFs) were identified in a unidirectional sense in the positive strand (Figure 3 and Table S1). Twelve of the predicted ORFs cannot be identified by similarity searches against proteins in the GenBank and UniProt databases, for some of them it was possible to detect transmembrane domains with high confidence (Table S3). The presence of an ORF that encodes the single subunit DNA-dependent RNA polymerase allowed us to classify the virus with members of the subfamily *Autographivirinae* of the family *Podoviridae*.

A total of five strong sigma70 promoters were found in the assembled genome. Four of these regulatory elements are located upstream of ORF 1, potentially controlling the expression of early genes, and the fifth is positioned in the middle-late interface genic blocks, upstream of ORF 36 (Figure 3 and Table S1), like in members of the genus *Phikmvvirus* (Adriaenssens et al.,

2011; Ceyssens et al., 2006; Drulis-Kawa et al., 2011; Eriksson, 2015). We were not able to find conserved sequence motifs that indicate specific promoters in the proximal region of the ORF that encodes a RNA polymerase. Two rho-independent transcription terminators were predicted, one downstream of ORF5 and the second downstream of ORF40, encoding the major capsid protein (Figure 3 and Table S1), a feature widely found in different groups of viruses classified in the order *Caudovirales*.

Pairwise comparisons of the complete genome sequence of the isolated virus with viruses formally classified by the International Committee on Taxonomy of Viruses (ICTV) in the subfamily *Autographivirinae* show the highest pairwise nucleotide identity with *Ralstonia*-infecting RSB3 (61.38%) and the species *Pseudomonas virus phiKMV* (*phiKMV*) (48.16%), and *Pseudomonas virus LKA1* (*LKA1*) (47.26%), all of them belonging to the genus *Phikmvvirus* (Table S1). Based on this analysis we propose that the virus isolated in this work is a new specie of the *Phikmvvirus* genus, tentatively named Ralstonia virus phiAP1 (phi, Phi*kmvvirus*; A, A*utographivirinae*; P, P*odoviridae*).

Analysis of structural proteins

To analize the phiAP1 virion-associated proteins a preparation of cesium chloride-purified particles was loaded in a 12% sodium dodecyl sulfate-polyacrylamide gel. About eleven structural proteins were expected according to the annotation of the genome (Figure 3, see Table S1). Of these, at least ten proteins with molecular weights ranging from 16 kDa to 138 kDa were resolved (Figure 4). The predicted proteins encodded by ORFs 39, 40 and 43 were excised for identification. Mass spectrometry analysis showed that the predominant band is the major capsid

protein (ORF40, ~37 kDa), as predicted. Similar results were observed for the structural proteins encoded by ORFs 38, 39, 41, 42, 44, 45 and 46.

Early, middle and late genomic blocks

The genomic architecture of phiAP1 is preserved, with the three functional genomic blocks/modules clearly distinguishable (Figure 5A), as expected for the viruses classified in *Phikmvvirus*. The first region (early genes) comprises the ORFs putatively involved in host adaptation and conversion (Class I genes), the second region (middle genes) encodes the components required for DNA metabolism and replication (Class II genes), while the third and last region (late genes) encodes virion associated and lysis proteins (Class III genes) (Figure 5A). Seventeen small ORFs (ORF01 to ORF17) make up the early genomic module. The majority are hypothetical proteins that share highest sequence identity with *Autographivirinae* members that infect *Ralstonia* spp. ORF11 encodes a putative PagP-like protein related to the one present in Burkholderia virus BcepIL02, classified in the genus *Bcep22virus*, not assigned to any subfamily in the family *Podoviridae*, and in Ralstonia phage RSL1, classified in the family *Myoviridae*. Furthermore, the presence of unique ORFs, such as ORF03, ORF04, ORF07, ORF13, ORF14 and ORF15, reinforce the variability of this early genes block (Figure 3).

The middle gene block has a greater syntenic and functional conservation when compared with others phikmvviruses, with exception of ORFs 21, 23, 24, 26, 27 and 28 (Figure 5A). Species-specific ORFs are present in the middle region of phikmvviruses, and same was observed in Ralstonia virus phiAP1, there are hypothetical conserved proteins, whereas others

containing canonical domains associated to DNA metabolism as MarR transcriptional regulator, Poly(A) polymerase and DNA polymerase III subunit epsilon (Figure 5A, Table S1).

The late gene block contains structural genes and the lysis cassette, and starts downstream of the RNA polymerase gene, with three small ORFs followed by the head-tail connector (Figure 5A). Of the three small ORFs, two (ORF36 and ORF37) show similarity to proteins of unknown function also present in Ralstonia virus RSB3 and *Pseudomonas virus LKA1*, situated upstream of the head-tail connector, in these species (Figure 5A and B).

In general, the conservation of the late module is even higher than that observed in the middle module. The order of the genes is preserved between phikmvviruses and Ralstonia virus phiAP1: Head-tail connector (gp08), Scaffolding (gp09), Major capsid (gp10), Tail tubular A (gp11), Tail tubular B (gp12), Internal virion proteins (gp13, 14 and 15), Tail spike(s) (gp16), TerS subunit (gp17), TerL subunit (gp18) and Lysis cassette (gp19) (Figure 5A). In contrast, some ORFs, despite their location and putative conserved function, show low amino acid sequence identity. Two tail fibers are encoded by the structural block, being a short and a long tail fiber (gp16; ORF43 and ORF47, respectively). (Figure 5A, black arrows).

Viral RNA polymerase

BlastP analysis of the predicted viral DNA-dependent RNA polymerase (vDdRp, ORF34) showed a high identity with the RNA polymerase of RSB3 (70%) and members of the *Phikmvvirus* and *Kp34virus* genera, respectively, ranging from 32-42% (*Pantoea virus LIMElight*, 32%; *phiKMV,* 36%; *LKD16*, 36% and *LKA1,* 42%). The vDdRp is located at the end of the DNA metabolism and replication region (middle genes) and is related to T7 RNA

polymerase, with synteny and conservation among members of the sister groups *Phikmvvirus* and *Kp34virus*. In the vDdRp encoded in the phiAP1 genome it was possible to detect the presence of the essential catalytic residues Asp537, Lys631, Tyr639 and Asp812. Although there is considerable divergence in the recognition (residues 93 to 101) and specificity loops (residues 739 to 770), a high conservation between phiAP1 and RSB3 can be observed (Figure 5C).

Characterization of the lysis cassette

The lysis cassette of phiAP1 corresponds to the last cistrons of the late gene block and contains four ORFs encoding a putative holin (ORF50), endolysin (ORF51), i-spanin (formerly Rz protein) (ORF52) and o-spanin (formerly Rz1 protein) (ORF53), showing a typical configuration of lysis cassettes present in phikmvviruses and other viruses infecting Gram-negative bacteria. Comparative analyzes reveal the synteny of this small lysis block between phiAP1 and RSB3, the closest species, which also infects *Ralstonia* spp. (Figure 5B). As observed for other phikmvviruses, the genes in this operon display a remarkable degree of overlap (Figure 6A). In phiAP1 there is an expansion in the size of ORFs 49, 50, 52 and 53. The putative holin of phiAP1 (ORF50) and RSB3 have a single transmembrane domain (TMD) that characterize them in class III holins as in kp34viruses, showing a contrast in comparison to the classical topology (class II) of holins encoded by phikmvviruses (two TMDs with N-out and C-in topology) (Figure 6B). In addition to these characteristics, the phiAP1 holin is twice the size of holins of phiKMV-types (Figure 5A). The endolysin of phiAP1 is the second ORF of the lysis cassette, has 177 aa and as in other phikmvviruses contains a glycoside hydrolase domain, but is more related to bacterial glycosyl hydrolases of *Stenotrophomonas maltophilia*, *Comamonas testosteroni*, *Pseudoalteromonas translucida*, and *Rheinheimera* sp., as the endolysin encoded by

RSB3 virus. The catalytic triad (glutamic acid (E)-aspartic acid (D)-threonine (T)) found in endolysin is identical in order and position E-8aa-D-5aa-T with the T4 endolysin, and similar to phiKMV endolysins (E-9aa-D-6aa-T). In addition to a high hidrophobicity, the phiAP1 N-terminal endolysin, and contains a predicted strong peptide signal to outer membrane (OM), indicating involvement of the Sec machinery in the exportation of this endolysin.

Spanins were found downstream of the endolysin, both containing transmembrane helices at the N-terminal region and a long periplasmic domain at the C-terminal. The phiAP1 i-spanin (ORF52) is related to i-spanins present in the Xylella virus Prado (Prado), Pseudomonas virus phiKF77 (phiKF77), Pseudomonas virus LUZ19 (LUZ19), *phiKMV* and *LKD16*. The phiAP1 o-spanin shows relationship with o-spanins encodes by Enterobacteriaceae virus Sfv (Sfv) (*Myoviridae*) and Burkholderia virus JG068 (JG068) (*Phikmvvirus*), besides a large number of bacterial hypothetical proteins.

Structural genomic comparison, phylogeny and protein identity calculation

A combined analysis including structural genomic comparisons in Mauve and phylogenetic analysis using the full-lengh sequences of the twenty genomes of *Autographivirinae* members formally classified by the ICTV allowed group phiAP1 in the *Phikmvvirus* genus, close to RSB3 (Figure 7). There are remarkable differences in the genomic organization of RSB1-type, *Kp34virus*, and the *Phikmvvirus*, here supported by the tree topology, however called our attention the sharing of blocks 18 and 19 between phikmvviruses and RSB1-type viruses (Figure 7), since RSB1 is a distinct group supported in all analysis. A second phylogenetic analysis was done using concatenated sequences of four proteins conserved

in *Autographivirinae* members (DNA polymerase, RNA polymerase, Major capsid and TerL subunit). The phylogenetic tree showed similar topology to the complete genome tree and phiAP1 and RSB3 clearly group with members of genus *Phikmvvirus*, forming a subclade in the genus (Figure S1).

Proteomic in silico analysis

An *in silico* comparison of the proteome between phiAP1 and representative members of the subfamily *Autographivirinae* confirmed the grouping with phikmvviruses, with similarity values between 41-52% (Lavigne et al., 2007; Lavigne et al., 2012). For each taxon, hypothetical proteins (no hits in the database) were not counted in the calculation of similarity. When compared to RSB1-types, specifically with viruses that also infect *Ralstonia* spp., phiAP1 shows a remarkable proteomic similarity (50-52%). Maximum values were obtained in the comparison with RSB3 (79.5%), a sister species of phiAP1.

Identification of enzymatic domains in virion-associated proteins

In an attempt to locate a putative EPS-depolymerase in the phiAP1 genome, domains with enzymatic activity were scanned in proteins of the structural cluster (Class III). In ORF43, predicted as putative short tail fiber, we were able to identify a Rmlc-like cupin domain, present in dTDP-sugar isomerases. We were not able to identify enzymatic domains in the T7-likelong tail fiber, encoded by ORF47. In the structural proteins encoded by ORFs 45 and 46 we found a lysozyme-like domain and a lytic transglycosilase domain (Table S1), respectively. This indicates that these internal virion proteins are virion-associated peptidoglycan hydrolases

(VAPGHs), typical exolisins that attack peptidoglycan facilitating the injection of the nucleic acid in the early stages of infection. In phiAP1 the lysozyme-like domain is in the C-terminal region, specifically between the amino acids residues 750-900, and is present in synteny with the RSB3 genome (Figure 5B) and in phikmvviruses, not being found in kp34viruses. Interestingly, the lytic transglycosilase domain located in the N-terminal portion (between amino acids residues 28-215) of ORF46 cannot be found in either phikmvviruses or kp34viruses, although present in all *Autographivirinae* that infect *Ralstonia* spp.

Biological properties

The phiAP1 host range is broad, being able to infect 50 isolates from the species *R. pseudosolanacearum, R. solanacearum and R. syzygii* (Table 1). Brazilian isolates tested belong to the species *R. pseudosolanacearum* (phylotype I) and *R. solanacearum* (phylotype II) (Figure 8A). In addition to the broad host range, phiAP1 is a strongly virulent phage, able to promote an efficient lysis of the host cell in 24 hours (Figure 8B). Only 17% of the bacterial isolates tested showed resistance to phiAP1 (Figure 8A) and the resistance profile of these isolates was grouped into two categories. Isolates Rs424, Rs503, UW386, Rs64 and AMC76 were partially resistant while Rs425, CMR15, V14, V15 and CRMRS42 were considered completely resistant (Figure S2). The susceptibility/resistance profile was not correlated with bacterial species, geographical origin or host plant source of each bacterial isolate. PhiAP1 infects specifically *Ralstonia* spp. not being able to infect other bacterial species tested, including phytopathogenic and commensal species (Table 1). For some susceptible isolates strains, plaques containing a clear zone (Lysis +) surrounded by a turbid halo zone (Lysis -) were noted, suggesting that the EPS-depolymerase predicted in the phiAP1 genome is functional (Figure 8C). The increase of turbid halo zone was

observed at 48 hours post viral inoculation (Figure 8C). A variety of plaques morphotypes was detected among the susceptible isolates, including the size of the plaque and resistance to EPS-depolymerase activity, as showed by the bacterial isolate V8 (Figure 8D).

Discussion

In the present work, we describe a virus that infects *Ralstonia* spp. isolated from Brazil. Ralstonia virus phiAP1, was characterized as a member of the family *Podoviridae*. The phiAP1 genome have a GC content of 61% which is similar to the genome of *Ralstonia* spp. (64.5 to 66.9%) (Ailloud et al., 2015). Genomic and phylogenetic analyses allowed the classification of Ralstonia virus phiAP1 in the subfamily *Autographivirinae* of the family *Podoviridae*, clustered with *Phikmvvirus* members. PhiAP1 had a close relationship with Ralstonia virus RSB3 (RSB3), an Asian species that also infects *Ralstonia* spp. In *Phikmvvirus*, clearly phiAP1 and RSB3 compose a subgroup demarcated by discrepancies in relation to the arrangement of the conserved locally colinear blocks (LCBs), especially in the clusters of early genes and the tail fibers. The absence of conserved T7-like viral promoter sequences in phiAP1 and the abundance of sigma70 host promoters, especially in the region upstream to the early genes, suggest a higher dependency of the host RNA polymerase for transcription, even though a predicted viral DNA-dependent RNA polymerase (ORF34) was annoted. Similar results have been reported among phikmvviruses, suggesting that transcriptional control seems to be conserved among viruses of these group (Ceyssens et al., 2006).

In spite of the close relationship with RSB3, additional analyses revealed specific characteristics of phiAP1, as an EPS-degrading activity, putatively associated with ORF43, the short tail spike and the presence of unique putative ORFs. Despite the similar structure of the

lysis cassette, a remarkable protein divergence between the lytic systems of these two viruses, suggest that phiAP1 is one new species in the genus *Phikmvvirus*. Furthermore, the host range, between these two species is contrasting because RSB3 has a narrow host range infecting only a low number of *R. solanaceraum* isolates (Kawasaki et al., 2016) which phiAP1 has a broad host range, infecting isolates of *R. solanacearum, R. pseudosolanacearum and R. syzygii.* Although in low number, it was possible to find resistant isolates in the three species of *Ralstonia*. A detailed analysis of *Ralstonia* anti-viral defense mechanisms can help to better understand the resistance mechanisms used by *Ralstonia* spp. to combat viral infection, and help to develop new efficient technologies based on viruses for the control of bacterial wilt.

As described for the majority of viruses with properties to degrade EPS, the depolymerase activity in phiAP1 was putatively assigned to the tail-fiber protein, specifically to the short tail fiber (ORF43). EPS-depolymerase genes in viral genomes provide a sophisticated way to overcome the physical barrier to the bacterial cell surface receptors in the adsorption step (Roach et al., 2013; Scholl et al., 2005). In *Ralstonia* spp., it has been hypothesized that the pathogen needs EPS to form biofilms to induce disease and to survive desiccation or antibiosis in soil in the absence of the host plant, and furthermore protects the bacteria from plant host defenses, to hiding the surface features that could be detected by the host (Denny, 1995; Genin and Boucher, 2002). PhiAP1 EPS-depolymerase has a broad spectrum of activity against *Ralstonia* spp., being an interesting candidate to be used in biotechnological applications. In apple and pear transgenic plants expressing viral EPS-depolymerase, fire blight susceptibility (*Erwinia amylovora*) was significantly reduced (Malnoy et al., 2005) and this effect was likely due to removal of EPS and the exposing of the cells to host plant defenses (Kim et al., 2004; Malnoy et al., 2005).

In addition to this interesting EPS-depolymerase activity, phiAP1 contains two putative virion-associated peptidoglycan hydrolases (VAPGHs), not reported in the sister species, RSB3 (Kawasaki et al., 2016). Not all viruses requires VAPGH to inject DNA, the many do not encode any proteins known VAPGH. In phiAP1, these VAPGHs, , probably act in external hydrolysis of the peptidoglycan as "viral exolisins", after adsorption (Rodriguez-Rubio et al., 2013).

Interestingly, among the genes of the early block in phiAP1, we found a PagP-like gene. PagP enzymes contains the transmembrane beta (8,10)-barrel found in outer membrane (OM) proteins, which specific conditions, such as nutritional stress, attack lipopolysaccharide and modify the OM standard assembly, increasing access to small solutes (Evanics et al., 2006) However in no inducing conditions these PagP enzymes can negatively influence OM's properties (Jia et al., 2004; Murata et al., 2007). It is not obvious if holins can directly affect the OM, so it was hypothesized that PagP acts as a helper component of the lytic machinery, activating the function of SAR endolysin through the depolarization of the bacterial membrane. Additionally, the presence of MarR, a transcriptional repressor of some OM proteins, allocated between early and middle phiAP1 genes suggests a temporal control for the expression of PagP (Barbosa and Levy, 2000). Thus, the accumulation of the enzyme in the early stages of infection would be reduced, in agreement with our hypothesis on an enhancer lytic gene allocated in genomic context unfavorable in relation to the late genes. The same protein domain were found in some myoviruses infecting *Ralstonia* spp. (Barbosa and Levy, 2000) and in some isolates of the *Ralstonia* spp., suggesting horizontal gene transfer. However, this proposed mode of PagP-holin action controlled by SAR-endolysin needs to be experimentally validated. Finally, phiAP1 also codes two spanins (i-spanin and o-spanin genes) present in the lysis cassette, which is organized in operon-like display with a remarkable degree of overlap as in other phikmvviruses

(Briers et al., 2011; Ceyssens et al., 2006). Despite these spanins be required for lysis of the host cell only in stress conditions, as under high concentration of divalent cations, a large number of viruses carries these genes, suggesting that their role in the ecology of these viruses can be greater than demonstrated in vitro conditions (Summer et al., 2007).

Here, we describe phiAP1 a novel species in *Phikmvvirus* infecting *Ralstonia* spp. and its lytic arsenal reveals a robust mechanism of pathogenesis that highlights the biotechnological potential of viral peptides for the management of bacterial wilt.

Material and Methods

Bacterial isolates and culture conditions

Isolates of *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* were cultured in CPG medium containing casein (1g/L), peptone (10g/ L) and glucose (5g/L) (Horita and Tsuchiya, 2001) at 28°C with shaking at 250 rpm. The origin and a description of all isolates used in this work are listed in Table 1.

Soil samples and plaque assay

Soil samples were collected in tomato crop fields in Venda Nova do Imigrante, Espirito Santo, Brazil (V) and Coimbra, Minas Gerais, Brazil (C), and in an eucalyptus plantation in Mucuri, Bahia, Brazil (M). Seven samples were collected in each place (V1-7, M1-7 and C1-7), totaling 21 samples. The samples were collected in areas with incidence of bacterial wilt,

specifically from rhizospheric soil of symptomatic and asymptomatic plants, except a sample of Coimbra (C4) that was collected where the disease did not occur for more than two years. To detect the presence of bacteriophages, approximately one gram of soil was ressuspended in 2 ml of distilled water. The suspension was centrifuged at 8 000 *g* for 5 minutes and then filtered through a PVDF membrane filter, 0.22 µm (Millex-GV Syringe Filter, Millipore Merck). Aliquots of 100 µL of the filtrate obtained from each sample were subjected to standard soft agar overlay method (Adams, 1959) and *R. solanacearum* RSB1 was used as host (Table 1). A single lysis plaque was picked and amplified in the presence of *R. solanacearum* RSB1, in CPG medium. The culture was centrifuged, the supernatant filtered and isolated lysis plaques were obtained using standard soft agar overlay method. This procedure was repeated three times to ensure virus purity.

Viral propagation and purification

Single lysis plaques were picked and propagated in the presence of *R. pseudosolanacearum* GMI1000, in CPG medium. For viral propagation, 2 liters of a *R. pseudosolanacearum* GMI1000 culture (optical density at 600 nm of 0.2) was infected at a multiplicity of infection (MOI) of 0.1 and incubated until visible lysis. After incubation, 10% of chloroform were added and incubated with vigorous shaking at 250 rpm during 30 minutes at 37ºC. Cell debris was removed by centrifugation at 8 000 *g* at4ºC for 5 minutes. The supernatant was passed through a 0.22 µm membrane filter followed by an overnight precipitation with polyethylene glycol 6000 (5% wt/vol) and 0,5 M NaCl. The pellet was recovered by centrifugation at 15 000 *g* at4ºC for 30 minutes and ressuspended in SM buffer (50 mM Tris-HCl

at pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$ and 0.01% gelatin). Particles were purified by CsCl-gradient ultracentrifugation and dialyzed three times for 30 min against 300 volumes of phage buffer (Yamada et al., 2007).

Transmission electron microscopy

The phiAP1 purified particles were deposited on carbon-coated copper grids, negatively stained with 2% uranyl acetate and examined under a Zeiss EM 109 TEM electron microscope operating at 80 kV. Dimensions of phiAP1 particles were calculated from 25 particles.

Isolation and characterization of nucleic acids from virus particles

The total nucleic acid was isolated from purified virions by phenol-chloroform (Sambrook and Russell, 2001). To confirm the nature of the genome, the total nucleic acid was treated with RNAse A (Promega), DNAse I (Promega) and S1 nuclease (Promega) according to the manufacturer's recommendations. The viral genome size was estimated by Pulse Field Gel Electrophoresis (PFGE). For that the viral DNA was loaded on 1% agarose gel in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and ran at 6 V/cm at constant temperature of 14°C, with a switch interval of 0.5s (Tstart) and 5s (Tfinal) between two electric fields whose orientation differed at an angle of 120°C for 18 hours.

DNA sequencing and assembly

The phiAP1 genome was sequenced using a 454 GS-FLX Titanium sequencing plataform (Macrogen, Seul, South Korea). The reads were trimmed and quality filtered, and assembled into contigs by *de novo* assembly using three independent methods: DNAstar SeqMan II sequence analysis software, CLC Genomics Workbench version 6.5.1 (CLC bio, USA) and Geneious R8.1 (Biomatters Ltd., Auckland, New Zealand), followed by mapping analysis, adopting stringent parameters, in which 90% of each read had to cover the other read with 90% identity The assembled contigs were compared with sequences in GenBank using BLAST algorithms (Altschul et al., 1990; Altschul et al., 1997) and manually inspected to verify the expected genome size. The sequences on its extremities were mapped using the intensive method to map high quality reads in each extremity using CLC Genomics Workbench version 6.5.1 (CLC bio, USA), and Rob Edward's script for finding terminal repeats at http://salmonella.utmem.edu/cgi-bin/repeatfinder.cgi. An *in silico* restriction map of the putative complete sequence was obtained using Genius R8.1 (Biomatters Ltd., Auckland, New Zealand). Restriction enzymes that cleave near each terminal region of the genomic contig (5' and 3') and in other regions of the genome were selected, and used to cleave the phage DNA extracted from purified particles. The fragments were analyzed by agarose gel electrophoresis and compared with the *in silico* restriction map.

Open reading frames (ORFs) in the viral genome were annotated and analyzed using GeneMark.HMM (Besemer and Borodovsky, 1999) with a length threshold of 100 bp, PRODIGAL (Prokaryotic Dynamic Programming Genefinding Algorithm) (Hyatt et al., 2010) and ORF Finder (http:// www.ncbi.nlm. gov/gorf/gorf.html). Searches for conserved motifs in

aminoacids sequences were done using TMHMM (Sonnhammer et al., 1998), Phobius (Kall et al., 2004), Phyre (Kelley and Sternberg, 2009) and Batch Web CD-Search Tool (Marchler-Bauer et al., 2012) at http://www.ncbi.nlm.nih.gov /Structure/bwrpsb/bwrpsb.cgi. Putative promoters were identified using the Kodon sequence similarity search employing TTGACA (N15-18) TATAAT and allowing for a 2 bp mismatch and additionality by neural network promoter prediction (Reese, 2001). Rho-independent terminators were tentatively identified using ARNold (Gautheret and Lambert, 2001; Macke et al., 2001) at http://rna.igmors.u-psud.fr/toolbox/ arnold/index.php and the web tool FindTerm at http://linux1.softberry.com/berry.phtml.

The sequence reported in this manuscript has been deposited in the GenBank database under the accession number KY117485.

Genomic and Phylogenetic Analysis

For genomic and phylogenetic analysis, the dataset included sequences with greatest similarity with the genome assembled based on the BLAST results, and include twenty sequences of viruses classified in the subfamily *Autographivirinae*. Multiple sequence alignments were prepared using ClustalW (Thompson et al., 1994), and the alignment was manually inspected, with gaps excluded.

The collinearity analysis was done using Mauve (Darling et al., 2010) and comparative proteomic analysis was performed in CoreGenes (Kropinski et al., 2009; Mahadevan et al., 2009) and CLC Genomics Workbench version 6.5.1 (CLC bio, USA). For these analysis the phiAP1 genome was compared with twenty *Autographivirinae* genomes as formally classified by the International Committee on Taxonomy of Viruses (ICTV) and a pairwise distance matrix in

MEGA version 6 (Tamura et al., 2013) was calculated (Table S2). A dot plot comparison of the genome of phiAP1 and RSB3 was done using Gepard (Krumsiek et al., 2007). Multiple sequence alignments were prepared, as described, for concatenated amino acid sequences of four conserved viral proteins (DNA polymerase, RNA polymerase, Major capsid and TerL subunit). The dataset included all 57 sequences of *Autographivirinae* deposited in GenBank and pairwise comparisons were performed using SDT v. 1.0 (Muhire et al., 2013). Phylogenetic trees were constructed using Bayesian inference with MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003), with the model selected by MrModeltest v. 2.2 (Nylander, 2004) by the Akaike Information Criterion (AIC). The analyses were carried out running 20,000,000 generations discarding the first 10% of samples as burn-in. Trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree). The *Shewanella virus Spp001* (NC_023594.1) was selected as outgroup taxa.

SDS-PAGE and mass spectrometry of the phiAP1 structural proteins

The phiAP1 particles were suspended in SDS-PAGE loading buffer (Moak and Molineux, 2004) and boiled for 5 min before loading onto a 12% (w/v) polyacrylamide gel to characterize the virion-associated proteins. The profile of bands was visualized by staining with Coomassie Brilliant Blue R250. Individual protein bands were recovered from the gel and tripsinized as described by Welker et al (2007). The samples were desalted using C18 micro columns (Zip Tip, Millipore, Ireland) and protein identification was performed using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS with steel plate MTP Anchor Chip TM 600/384 TF, Bruker Daltonics). Peptide masses were

confronted with all putative protein sequences of phiAP1 and searched against the NCBI and SwissProt databases using the Mascot program (http://www.matrixscience.com) and MASCOT Peptide Mass Fingerprinting database search.

Host range

The phiAP1 host specificity was evaluated against a collection of isolates of *Ralstonia* spp. and other bacterial species, including phytopathogenic and commensal bacteria (Table 1) by plaque assay using standard soft agar overlay (Adams, 1959). The virus titer used was 10^6 PFU/mL. The presence of a lysis zone after incubation for 24 hours at 28°C was interpreted as sensitivity and the absence as resistance. To characterize the resistance spectrum expressed by some isolates a spot test was performed (Pantůček et al., 1998). Approximately 10 µL from six viral suspensions (10^{10}, 10^9, 10^8, 10^7, 10^6 and 10^5 PFU/mL) was spotted on cultures of each isolate immediately after plating on CPG soft agar overlay and incubated under the same conditions described before.

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Table 1. Bacterial isolates used in this study and Ralstonia virus phiAP1 host range

Isolates	Source[a]	Host	Origin[b]	Phyl-seq[c]	phiAP1 phikmvvirus	
					Plaque formation	EPS-depolymerase sensitivity
***Ralstonia pseudosolanacearum* native isolates**						
S388	Embrapa-CNPH	Bell pepper	AC/North	I/NS[d]	+	-
RS476	Embrapa-CNPH	Tomato	MA/Northeast	I/ND[e]	+	+
RS470	Embrapa-CNPH	Tomato	MA/Northeast	I/ND	+	+
RS18	Embrapa-CNPH	Tomato	AM/North	I/NS	+	+
RS424	Embrapa-CNPH	Tomato	AC/North	I/NS	-	-
RS425	Embrapa-CNPH	Tomato	AC/North	I/NS	-	-
RS508	Embrapa-CNPH	Tomato	BA/Northeast	I/ND	+	+
RS291	Embrapa-CNPH	Tomato	TO/North	I/18	+	-
CRMRS86	UFRPE	Tomato	PE/Northeast	I/18	+	+
CRMRS42	UFRPE	Bell pepper	PE/Northeast	I/18	-	-
CRMRS97	UFRPE	Tomato	PE/Northeast	I/18	+	+
***R. solanacearum* native isolates**						
RS333	Embrapa-CNPH	Tomato	TO/North	IIA/6	+	+
RS263	Embrapa-CNPH	Potato	SP/Southeast	IIA/ND	+	-
RS278	Embrapa-CNPH	Tomato	RS/South	IIB/NS	+	+
RS99	Embrapa-CNPH	Potato	PR/South	IIB/NS	+	+
RS467	Embrapa-CNPH	Tomato	MG/Southeast	IIA/ND	+	-
RS246	Embrapa-CNPH	Potato	MG/Southeast	IIB/NS	+	+
RS503	Embrapa-CNPH	Eggplant	CE/Northeast	IIA/ND	-	-
RS167	Embrapa-CNPH	Tomato	RO/North	IIA/NS	+	+
RS235	Embrapa-CNPH	Tomato	PA/North	IIA/41	+	+
RS68	Embrapa-CNPH	Potato	BA/Northeast	IIB/1	+	+
RS300	Embrapa-CNPH	Tomato	MG/Southeast	IIB/NS	+	-
RS342	Embrapa-CNPH	Tomato	AM/North	IIA/6	+	-
RS64	Embrapa-CNPH	Tomato	PE/Northeast	IIA/50	-	-
RS480	Embrapa-CNPH	Geranium	SP/Southeast	IIB/NS	+	+
RSCOI	In this study	Tomato	MG/Southeast	II/ND	+	+
RSB1	In this study	Eucalypt	BA/Northeast	II/ND	+	+
RSB2	In this study	Eucalypt	BA/Northeast	II/ND	+	+
B11	UFRPE	Banana	AM/North	IIA/24	+	-
AMC22	UFV[1]	Eucalypt	AP/North	IIA/NS	+	+
AMC76	UFV[1]	Eucalypt	AP/North	IIA/41	-	-

***Ralstonia* sp. native isolates (PP)**[f]						
RS17738	AGR	Tobacco	RS/South	ND	+	-
RS5191	AGR	Tobacco	RS/South	ND	+	-
V1	INPA	Tomato	AM/North	ND	+	+
V3	INPA	Tomato	AM/North	ND	+	+
V4	INPA	Tomato	AM/North	ND	+	-
V5	INPA	Tomato	AM/North	ND	+	+
V6	INPA	Tomato	AM/North	ND	+	+
V14	INPA	Tomato	AM/North	ND	-	-
V15	INPA	Tomato	AM/North	ND	-	-
V17	INPA	Tomato	AM/North	ND	+	-
V20	INPA	Tomato	AM/North	ND	+	-
V26	INPA	Bell pepper	AM/North	ND	+	+
V30	INPA	Bell pepper	AM/North	ND	+	+
V35	INPA	Capsicum	AM/North	ND	+	-
V39	INPA	Eggplant	AM/North	ND	+	+
V41	INPA	Eggplant	AM/North	ND	+	-
V43	INPA	Scarlet eggplant	AM/North	ND	+	+
V45	INPA	Cucumber	AM/North	ND	+	+
V55	INPA	Tomato	AM/North	ND	+	+
R. pseudosolanacearum, ***R. solanacearum*** and ***R. syzygii* subsp. *indonesiensis* type-isolates**						
IBSBF2576	IBSBF	Eucalypt	SC/South	IIA/NS	+	+
IBSBF2568	IBSBF	Eucalypt	SC/South	IIA/NS	+	-
IBSBF624	IBSBF	Eucalypt	PA/North	IIB/26	+	+
IBSBF292	IBSBF	Tomato	United States	IIA/7	+	+
GMI1000	UW	Tomato	French Guyana	I/18	+	+
K60^{T}	UW	Tomato	United States	IIA/7	+	-
CFBP2957	UW	Tomato	Martinique	IIA/36	+	+
CMR15	UW	Tomato	Cameroon	III/29	-	-
UW386	Embrapa-CNPH	Tomato	Nigeria	III	-	-
Psi07	UW	Tomato	Indonesia	IV/10	+	+
Others species						
Pectobacterium brasiliensis	UFV[2]	Tomato	Brazil	-	-	-
P. carotovorum subsp. *carotovorum*	UFRPE	Tomato	Brazil	-	-	-
Pseudomonas syringae pv. *glycinea*	UFV[2]	Soybean	Brazil	-	-	-
P. syringae pv. *tabaci*	UFV[2]	Tobacco	Brazil	-	-	-
P. syringae pv. *garcae*	UFV[2]	Coffee	Brazil	-	-	-
Erwinia psidii	UFV[1]	Eucalypt	Brazil	-	-	-
Clavibacter michiganensis subsp. *michiganensis*	UFV[2]	Tomato	Brazil	-	-	-
Xanthomonas campestris pv. *campestris*	UFRPE	Cabbage	Brazil	-	-	-
X. campestris pv. *viticola*	UFRPE	Grapevine	Brazil	-	-	-

X. vesicatoria	UFV[2]	Bell pepper	Brazil	-	-	-
Streptomyces scabies	UFRPE	Potato	Brazil	-	-	-
Bacillus subtillis	UFV[3]	-	Brazil	-	-	-
B. cereus	UFV[3]	-	Brazil	-	-	-
P. putida	UFV[3]	-	Brazil	-	-	-
P. fluorescens	UFV[3]	-	Brazil	-	-	-

[a]Source of the isolates: EMBRAPA-CNPH, Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisas em Hortaliças, Distrito Federal, Brazil. UFRPE, Universidade Federal Rural de Pernambuco. Recife, Brazil.UFV[1], Laboratório de Patologia Florestal Molecular, Universidade Federal de Viçosa, Viçosa, Brazil. AGR, Agronômica-Laboratório de Diagnóstico Fitossanitário, Porto Alegre, Brazil. INPA, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil. IBSBF, Instituto Biológico, São Paulo, Brazil. UW, University of Wisconsin-Madison, Madison, USA. UFV[2], Laboratório de Bacteriologia da Universidade Federal de Viçosa**,** Viçosa, Brazil. UFV[3], Departamento de Microbiologia, Universidade Federal de Viçosa**,** Vicosa, Brazil. [b]Origin of the isolates: AC=Acre State; AM=Amazonas State; AP=Amapá State; BA=Bahia State; CE=Ceara State; MA= Maranhao State; MG=Minas Gerais State; PA=Pará State; PE=Pernambuco State; PR=Paraná State; RO=Rondônia State, RS=Rio Grande do Sul State; SC=Santa Catarina State; SP=São Paulo State; TO=Tocantins State. [c]Phylotype-sequevar. [d]NS=novel sequevar . [e]ND=Non determined. [f]PP= Plant pathogen.

Supplemental Table S1. Functional genomic annotation of Ralstonia virus phiAP1

Coding sequence	Predicted protein	Genomic coordinates	Strand	Protein lenght (aa)	Mol. Mass (Da)	Protein pI	Homologs (a) & Motifs	E-value
ORF01	Conserved hypothetical protein	1601..2344	+	247	26635.5	8.37	YP_008853889.1 hypothetical protein [Ralstonia phage RSB3]	3E-60
ORF02	Conserved hypothetical protein	2521..2802	+	93	10221.5	6.89	BAP15808.1 hypothetical protein [Ralstonia phage RSJ2]	8E-32
ORF03	Hypothetical membrane protein	2799..2906	+	35	3667.4	8.36	protein motifs: one transmembrane domain discovered using TMHMM, Phobius and Phyre2	
ORF04	Hypothetical protein	2914..3315	+	133	15363.4	7.68	-	-
ORF05	Conserved hypothetical protein	3412..3669	+	85	9309.7	10.60	BAP34899.1 hypothetical protein [Ralstonia phage RSJ5]	2E-21
ORF06	Conserved hypothetical protein	3672..3866	+	64	7204.2	10.24	YP_008853894.1 hypothetical protein [Ralstonia phage RSB3]	0.005
ORF07	Hypothetical protein	3870..4013	+	47	5490.9	5.54	-	-
ORF08	Conserved hypothetical protein	4016..4408	+	130	14010.0	4.93	BAP34900.1 hypothetical protein [Ralstonia phage RSJ5]	7E-21
ORF09	Conserved hypothetical protein	4405..4740	+	111	12689.11	5.25	YP_002213694 hypothetical protein RSB1_gp05 [Ralstonia phage RSB1]	2E-06
ORF10	Conserved hypothetical protein	4737..5132	+	131	15049.4	8.66	BAP15816.1 hypothetical protein [Ralstonia phage RSJ2]	2E-11
ORF11	Putative PagP-like protein	5129..5629	+	166	17868.2	8.47	YP_0029226941 PagP-like outer membrane protein [Burkholderia phage BcepIL02]	6E-13
ORF12	Conserved hypothetical protein	5643..5846	+	67	7233.8	5.42	BAP15817.1 hypothetical protein [Ralstonia phage RSJ2]	0.006
ORF13	Hypothetical protein	5848..6042	+	64	7043.1	7.76	-	-
ORF14	Hypothetical protein	6039..6362	+	107	11970.4	9.22	-	-
ORF15	Hypothetical protein	6362..6799	+	145	15987.4	10.73	-	-
ORF16	Conserved hypothetical protein	6796..7242	+	148	16279.1	5.61	YP_008853902.1 hypothetical protein [Ralstonia phage RSB3]	3E-45
ORF17	Conserved hypothetical	7275..7796	+	173	17901.4	4.79	YP_008853903.1 hypothetical	1E-29

	protein						protein [Ralstonia phage RSB3]	
ORF18	DNA primase	8031..8897	+	288	32569.1	8.73	YP_008853904.1 putative DNA primase [Ralstonia phage RSB3]	6E-68
ORF19	DNA helicase	8857..10134	+	425	46722.1	5.57	YP_008853905.1 putative DNA helicase [Ralstonia phage RSB3]	0
ORF20	Putative DNA binding transcriptional regulator MarR family	10135..10470	+	111	12444.4	7.01	YP_008853906.1 hypothetical protein [Ralstonia phage RSB3] & WP_031513969.1 MarR family transcriptional regulator [Streptomyces sp. NRRL F-5123]	1E-12;0.015
ORF21	Conserved hypothetical protein	10467..10772	+	101	11334.1	8.98	YP_008853907.1 hypothetical protein [Ralstonia phage RSB3]	9E-05
ORF22	DNA ligase	10759..11619	+	286	32247.6	5.73	YP_008853908.1 putative DNA ligase [Ralstonia phage RSB3]	6E-119
ORF23	Hypothetical protein	11616..11810	+	64	7305.3	9.05	-	-
ORF24	Hypothetical protein	11807..12016	+	69	7699.6	6.30	-	-
ORF25	DNA polymerase	12016..14508	+	830	95589.4	5.60	YP_008853911.1 putative DNA polymerase [Ralstonia phage RSB3]	0
ORF26	Hypothetical protein	14505..14704	+	69	7794.8	9.69	-	-
ORF27	Conserved hypothetical protein	14711..15403	+	230	25324.8	6.55	BAP15830.1 hypothetical protein [Ralstonia phage RSJ2]	2E-34
ORF28	Conserved hypothetical protein	15446..16411	+	321	33123.8	5.82	YP_008853913.1 hypothetical protein [Ralstonia phage RSB3]	4E-106
ORF29	DNA exonuclease	16500..17396	+	298	33291.1	4.93	YP_008853914.1 putative exonuclease [Ralstonia phage RSB3]	2E-136
ORF30	DNA endonuclease	17520..17921	+	133	14837.1	9.91	YP_008853915.1 putative endonuclease [Ralstonia phage RSB3]	6E-48
ORF31	Putative poly(A) polymerase	17918..18469	+	183	20072.7	4.38	YP_008853916.1 hypothetical protein [Ralstonia phage RSB3] & WP_041165858.1 poly(A) polymerase [Chlamydia trachomatis]	9E-11; 0.002

ORF32	DNA polymerase III epsilon subunit	18479..19282	+	267	31131.8	9.47	YP_002213711 hypothetical protein RSB1_gp22 [Ralstonia phage RSB1] & YP_007238105.1 putative DNA polymerase III epsilon subunit [Xanthomonas phage CP1]	5E-114;6E-57
ORF33	Conserved hypothetical protein	19493..19672	+	59	6534.4	5.70	YP_008853918.1 hypothetical protein [Ralstonia phage RSB3]	2E-11
ORF34	RNA polymerase	19673..22105	+	810	91356.8	6.54	YP_008853919.1 putative phage RNA polymerase [Ralstonia phage RSB3]	0
ORF35	Hypothetical protein	22337..22531	+	64	7110.1	7.77	-	-
ORF36	Conserved hypothetical protein	22591..22968	+	125	14645.1	9.04	YP_008853920.1 hypothetical protein [Ralstonia phage RSB3]	2E-24
ORF37	Structural protein	22969..23283	+	104	10848.0	5.34	YP_008853921.1 hypothetical protein [Ralstonia phage RSB3] & YP_001522881.1 putative structural protein [Enterobacteria phage LKA1]	1E-07; 0.001
ORF38	Head-tail connector protein	23293..24888	+	531	58370.9	5.44	YP_008853922 putative head-tail connector protein [Ralstonia phage RSB3]	0
ORF39	Scaffolding protein	24898..25857	+	319	33110.8	5.99	YP_008853923.1 putative scaffolding protein [Ralstonia phage RSB3]	2E-83
ORF40	Major capsid protein	25910..26947	+	345	37750.4	5.49	YP_008853924.1 putative capsid protein [Ralstonia phage RSB3]	0
ORF41	Tail tubular A	27003..27563	+	186	21245.1	6.60	YP_008853925.1 putative tail tubular protein A [Ralstonia phage RSB3]	2E-77
ORF42	Tail tubular B	27567..29921	+	784	85300.9	5.25	YP_008853926.1 putative tail tubular protein B [Ralstonia phage RSB3]	0
ORF43	Putative short tail fiber	29924..30376	+	150	16223.3	5.44	YP_008853927.1 hypothetical protein [Ralstonia phage RSB3]	3E-35

ORF44	Internal virion protein A	30373..31104	+	243	24678.3	6.58	YP_008853928.1 putative internal virion protein [Ralstonia phage RSB3]	5E-23
ORF45	Internal virion protein	31114..33861	+	915	98966.2	6.77	YP_008853929.1 hypothetical protein [Ralstonia phage RSB3] & YP_001522888.1 putative C-terminus lysozyme motif internal virion protein [Enterobacteria phage LKA1]	0;1E-14
ORF46	Internal virion protein	33864..37685	+	1273	137987.7	6.52	YP_008853930.1 putative internal core protein [Ralstonia phage RSB3]	0
ORF47	Long tail fiber	37688..40303	+	871	90036.8	6.04	YP_008853931.1 putative tail fiber protein [Ralstonia phage RSB3]	3E-77
ORF48	TerS subunit	40313..40603	+	96	10584.9	5.31	YP_008853933.1 putative DNA maturase A [Ralstonia phage RSB3]	2E-39
ORF49	TerL subunit	40610..42442	+	610	67839.0	6.02	YP_008853934.1 putative DNA maturase [Ralstonia phage RSB3]	0
ORF50	Holin	42346..42804	+	152	16604.7	9.16	YP_009146417.1 hypothetical protein ECBP5_0046 [Escherichia phage ECBP5]; protein motifs: one transmembrane domain discovered using TMHMM and Phobius	0.07
ORF51	Endolysin	42614..43129	+	171	18334.3	9.28	YP_008853935.1 putative lysozyme [Ralstonia phage RSB3]	5E-55
ORF52	i-spanin	43126..43494	+	122	12811.5	9.61	YP_008853936.1 hypothetical protein [Ralstonia phage RSB3] & YP_002727869.1 putative Rz protein [Pseudomonas phage phikF77]	2E-09;0.066
ORF53	O-spanin	43382..43660	+	92	9768.3	7.97	WP_034628614.1 hypothetical protein [Desulfovibrio africanus]	7E-06;0.02

							& WP_023209980.1 Rz1 lytic protein from bacteriophage origin [Salmonella enterica]	6
ORF54	Hypothetical protein	43657..43884	+	75	7736.6	4.72	-	-
ORF55	Hypothetical protein	43901..44074	+	57	6000.9	6.04	-	-
ORF56	Hypothetical protein	44080..44256	+	58	6109.9	6.03	-	-

Supplemental Table S2. Pairwise sequence comparison of Ralstonia virus phiAP1 and other viruses in the subfamily *Autographivirinae*

Viruses name	NCBI acession	Genome size (bp)	Identity (%)	Host	Origin	Reference
Ralstonia virus RSB1	NC_011201.1	43,079	41.11	*Ralstonia* spp.	Japan	Kawasaki et al., 2009
Ralstonia virus RSB2	NC_023736.1	40,411	39.79	*Ralstonia* spp.	Japan	Kawasaki et al., 2016
Ralstonia virus RSB3	NC_022917.1	44,578	61.38	*Ralstonia* spp.	Japan	Kawasaki et al., 2016
Ralstonia virus RSJ2	NC_028988.1	44,360	42.84	*Ralstonia* spp.	Thailand	Kawasaki et al., 2016
Ralstonia virus RSJ5	NC_029007.1	43,745	42.68	*Ralstonia* spp.	Thailand	Kawasaki et al., 2016
Kluyvera virus Kvp1	NC_011534.1	39,472	37.11	*Kluyvera cryocrescens*	Canada	Lingohr et al., 2008
Escherichia virus T7	NC_001604.1	39,937	36.63	*Escherichia coli*	United States	Dunn and Studier, 1983
Pseudomonas virus gh1	NC_004665.1	37,359	38.71	*Pseudomonas putida*	Canada	Kovalyova and Kropinski, 2003
Pantoea virus LIMElight	NC_019454.1	44,546	42.91	*Pantoea agglomerans*	Belgium	Adriaenssens et al., 2011
Pseudomonas virus phiKMV	NC_005045.1	42,519	48.16	*P. aeruginosa*	Russia	Lavigne et al., 2003
Pseudomonas virus LKA1	NC_009936.1	41,593	47.26	*P. aeruginosa*	Belgium	Ceyssens et al., 2006
Klebsiella virus KP34	NC_013649.2	43,809	43.05	*Klebsiella pneumoniae*	Poland	Drulis-Kawa et al., 2011
Klebsiella virus SU503	NC_028816.1	43,809	42.94	*K. pneumoniae*	Sweden	Eriksson et al., 2015
Klebsiella virus F19	NC_023567.2	43,766	43.07	*K. pneumoniae*	China	Eriksson et al., 2015
Erwinia virus Era103	NC_009014.1	45,445	36.47	*Erwinia amylovora*	United States	Vandenbergh and Vidaver, 1985
Salmonella virus SP6	NC_004831.2	43,769	36.38	*Salmonella enterica*	United States	Dobbins et al., 2004
Escherichia virus K1E	NC_007637.1	45,251	35.66	*E. coli*	United States	Stummeyer et al., 2006
Synechococcus virus P60	NC_003390.2	46,675	31.83	*Synechococcus* sp.	Georgia	Chen and Lu, 2002
Prochlorococcus virus PSSP7	NC_006882.2	45,176	32.48	*Prochlorococcus* sp.	United States	Sullivan et al., 2005
Synechococcus virus Syn5	NC_009531.1	46,214	36.3	*Synechococcus* sp.	United States	Wilson et al., 1993
Shewanella virus Spp001	NC_023594.1	54,789	34.83	*Shewanella putrefacies*	China	Han et al., 2014

Figure Legends

Figure 1. (A) Electron micrograph of particles of Ralstonia virus phiAP1 negatively stained with 0.5% uranyl acetate. The scale bar indicates 50 nm. **(B)** Pulsed-field gel electrophoresis analysis of full-length phiAP1 genomic dsDNA (as indicated). Lanes 1 and 3 include the PFGE markers, λ mix mono cut and λ phage dsDNA NEB, respectively. Ladder sizes are indicated in each gel.

Figure 2. (A) In silico restriction map of the Ralstonia virus phiAP1 genome **(B)** Restriction profile agarose gel electrophoresis using selected in silico enzymes. DNA Ladder, 1kb Plus DNA ladder (Invitrogen). The genome ends are indicated with the expected size in bp and the asterisk indicates a weak band of ~0.6kb in *Cla*I cleavage.

Figure 3. Genomic organization of Ralstonia virus phiAP1. Each arrow represents a putative ORF, and the numbering refers to Suppl. Table S1. The three functional modules: class I, class II, and class III are represented with different colors. Early genes, class I (white), Middle genes, class II (green), and Late genes, class III (gray and yellow), including structural and lysis genes respectively. The gene that encodes a RNA polymerase is indicated as a black arrow.

Figure 4. SDS-PAGE analysis of Ralstonia virus phiAP1 virion-associated proteins. Beside the gel (right) are the respective ORF names for each spot (Suppl. Table S1), consistent with molecular mass (KDa) and mass spectrometry analysis. Lane 1, reference ladder (kDa; BioRad). The asterisk indicates the viral proteins confirmed by mass spectrometry analysis.

Figure 5. Genomic architecture in members of genus *Phikmvvirus*. **(A)** Comparison of the Ralstonia virus phiAP1 genome with *Pseudomonas* viruses phiKMV, LKA1, *Pantoea* viruses LIMEzero and LIMElight. The predicted open reading frames are indicated by arrows. ORFs of unknown function are in white, predicted DNA metabolism ORFs in green, structural ORFs in gray, and lysis ORFs in yellow. Functionally equivalent ORFs are marked with the same number. Host promoters are indicated by bent arrows with triangular points and factor-independent terminators are shown with loop structures. The divergent tail region is indicated by black arrows. **(B)** Comparative genomic map between phiAP1 and RSB3. The protein pairwise identity was calculated using Geneious R8 package for each gene product and the color matrix indicates the similarity between them (right). **(C)** Alignment of the recognition and specificity loops of the RNA polymerase in members of *Phikmvvirus*. Filled stars indicate identical amino acid residues among the species of the genus, unfilled stars indicate identical amino acid residues also among the species of the genus, with exception of LKA1.

Figure 6. The Ralstonia virus phiAP1 Lysis cassette is structurally conserved, but contains a distinct holin gene. **(A)** Lysis cassete comparison between phiAP1 and Ralstonia virus RSB3 and **(B)** among the *Phikmvvirus* members. The ORFs are drawn to scale (boxes) and the colors of each gene indicate: (black) unrelated genes to lysis; (gray) genes encoding proteins with at least one TMD, potentially holins; (white) endolysin genes and (green) o-spanin (light) and i-spanin (dark). Topological models to putative holins are shown in right.

Figure 7. Phylogenetic tree of genomes of *Autographivirinae* members (left) was calculated by Bayesian MCMC coalescent analysis. The posterior probability values (PP) calculated using the best trees found by MrBayes are shown beside each node. The outgroup taxon is the *Shewanella virus Spp001*. The colored squares in the schematic view of genomes (right) correspond to the conserved locally collinear blocks (LCBs) predicted by Mauve. The

numbers and colors indicate the LCBs that are shared between the viruses. The distinct genera of subfamily *Autographivirinae* are shaded with the respective colors *T7virus* (yellow), *Sp6virus* (light green), *Phikmvvirus* (purple), *Kp34virus* (dark green). In addition, the Unassigned (pink) and the RSB1-types (white) were included.

Figure 8. Ralstonia virus phiAP1 is a broad host range phikmvvirus which contains EPS-depolymerase activity. **(A)** Susceptibility or resistance during the phiAP1 infection and geographical origin of Brazil native strains. **(B)** Strong lytic activity on GMI1000 isolate growth 24 hours post inoculation with phiAP1. (**C)** EPS-depolymerase activity during phiAP1 infection of isolate V43. (D) Plaques phenotypic variation during phiAP1 infection in *Ralstonia* spp. Isolates between phiAP1 and *Ralstonia* spp. strains.

Supplemental Figure S1. Phylogenetic tree of amino acid sequences using concatenated data of four conserved proteins (Major capsid, DNA polymerase, RNA polymerase and TerL subunit) among the members of subfamily *Autographivirinae* was calculated by Bayesian MCMC coalescent analysis and the posterior probability values (PP) calculated using the best trees found by MrBayes are shown beside each node. The outgroup taxon is the *Shewanella virus Spp001*.

Supplemental Figure S2. Resistance antiviral profile in *Ralstonia* spp. isolates inoculated with Ralstonia virus phiAP1. The controls are represented by symbols (+) and (-) and correspond to the extremely susceptible *R. pseudosolanacearum* GMI1000 and *Xanthomonas campestris* pv. *viticola* (non host), respectively. The different viral titer used in the infection assay are indicated in the plate drawn (right) in PFU/mL.

Figure 1

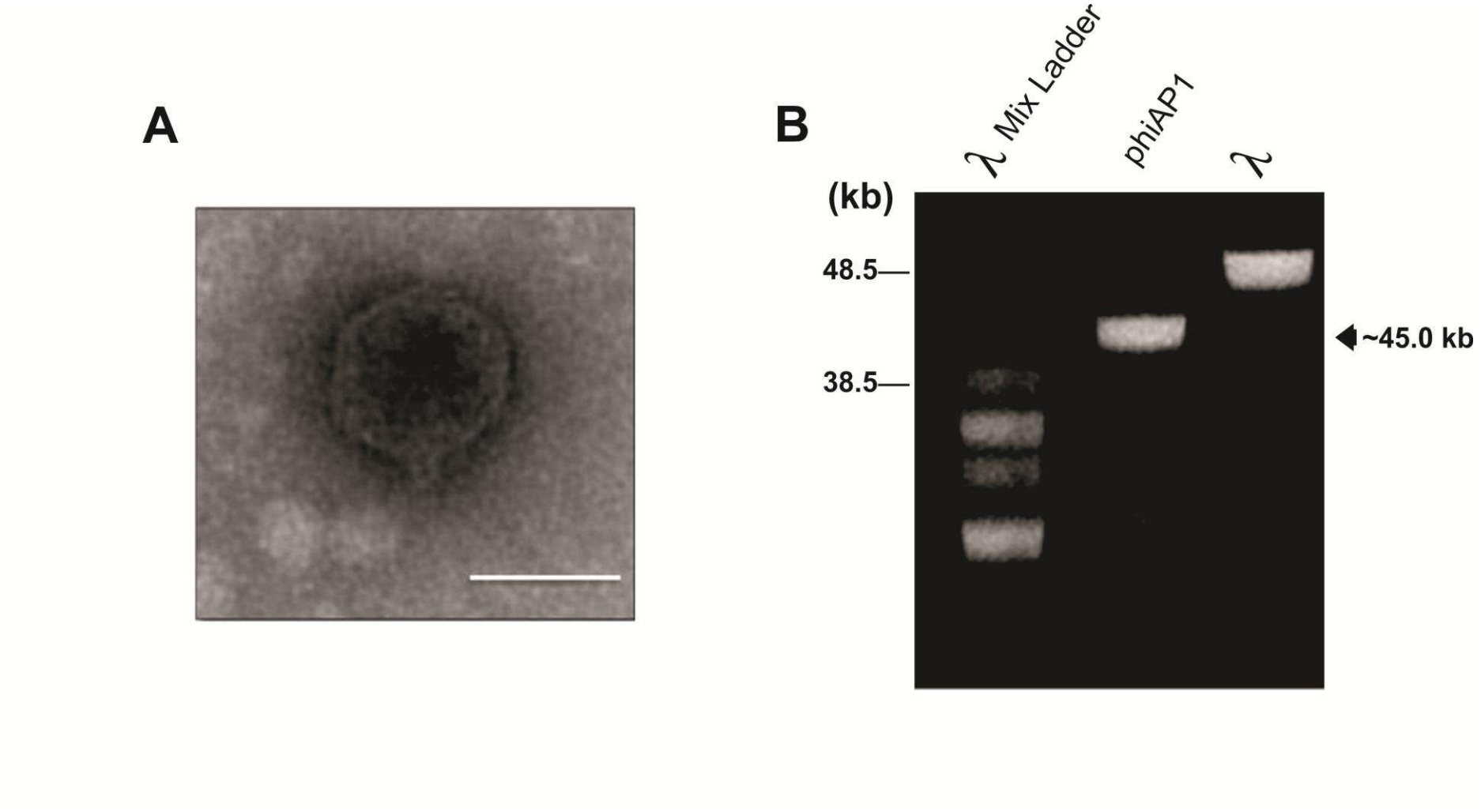


Figure 2

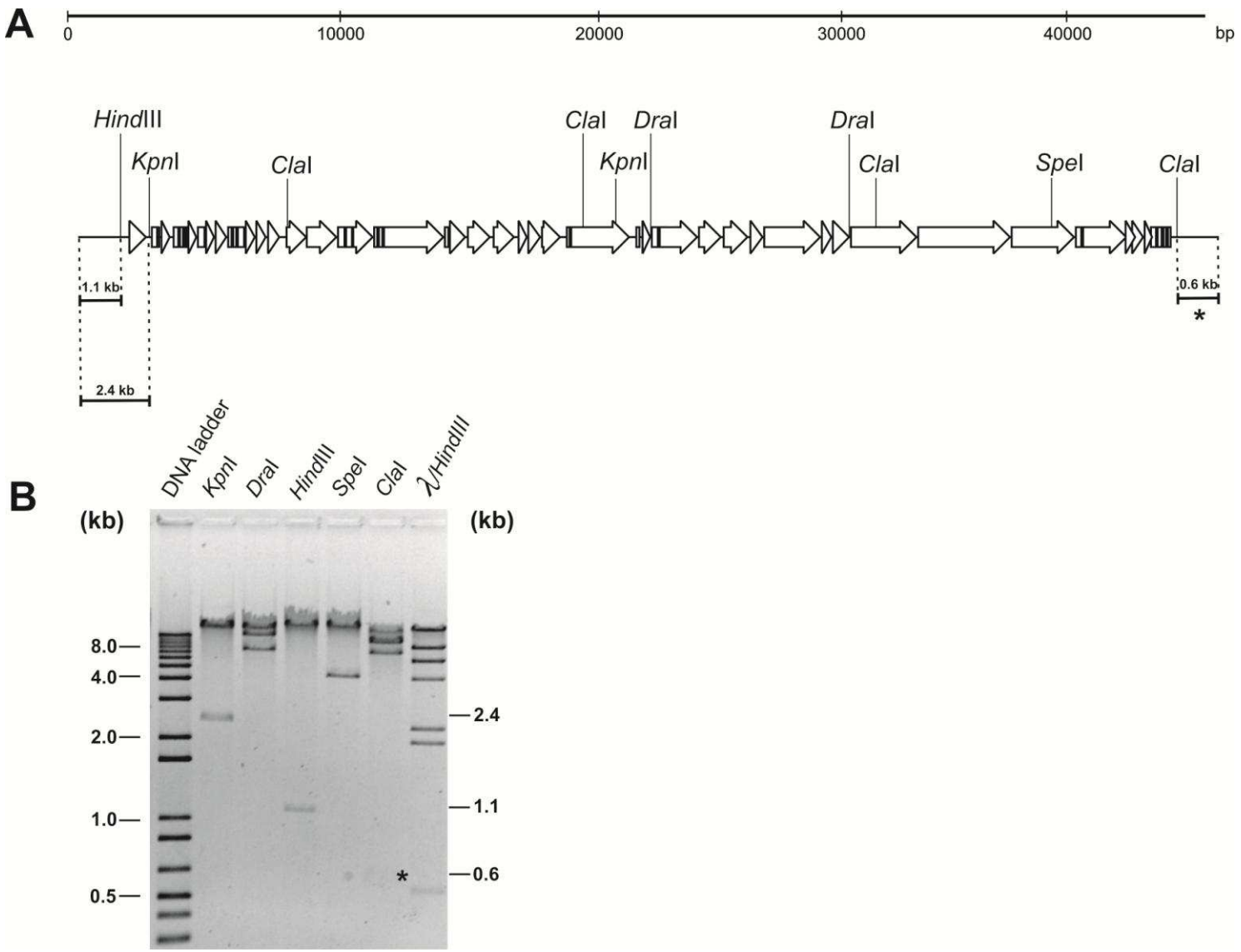

A
0
10000
20000
30000
40000
bp
HindIII
KpnI
ClaI
ClaI
DraI
KpnI
DraI
ClaI
SpeI
ClaI
1.1 kb
2.4 kb
0.6 kb
*
B
DNA ladder
KpnI
DraI
HindIII
SpeI
ClaI
λ/HindIII
(kb)
(kb)
8.0
4.0
2.0
1.0
0.5
2.4
1.1
0.6
*

Figure 3

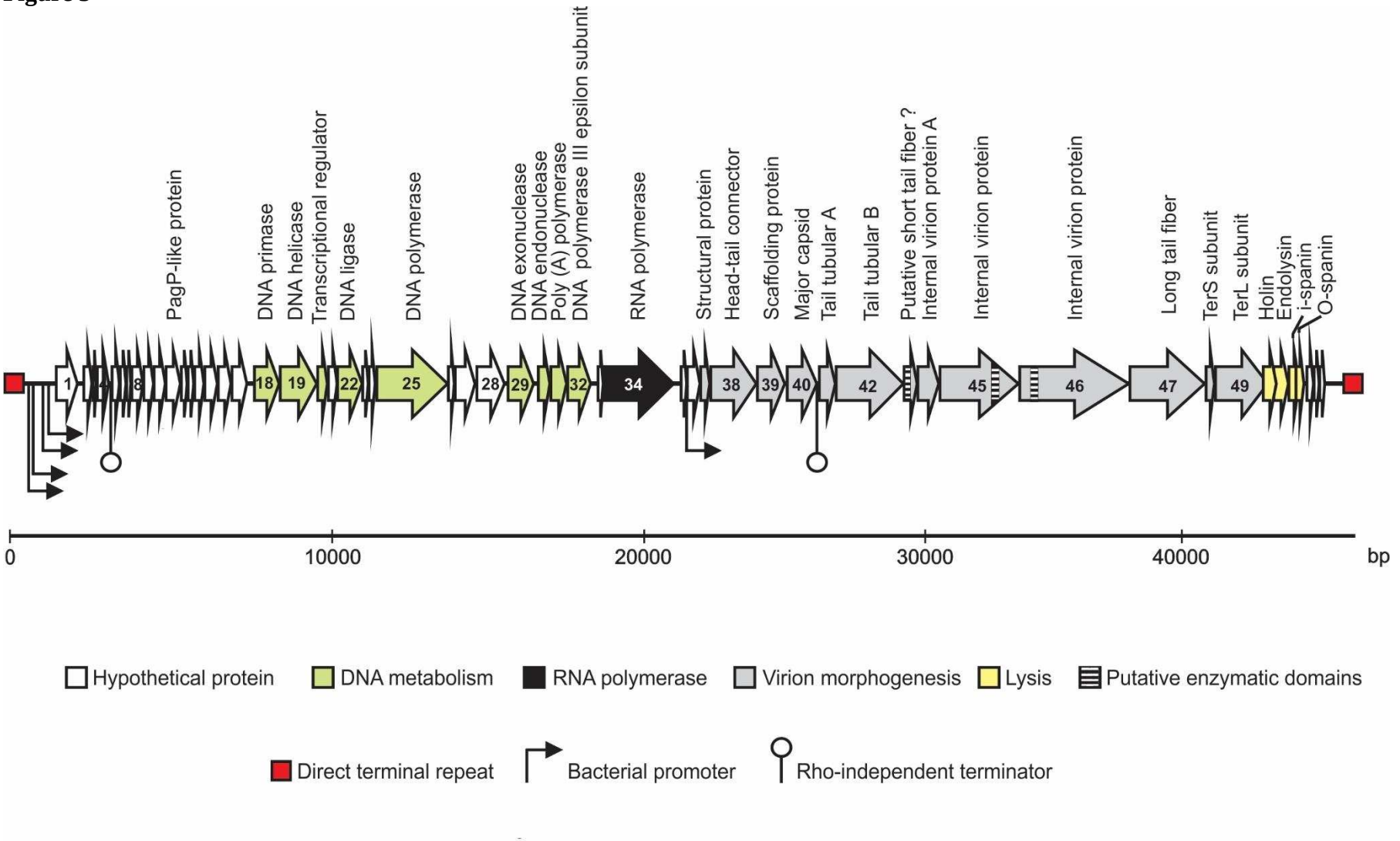

PagP-like protein
DNA primase
DNA helicase
Transcriptional regulator
DNA ligase
DNA polymerase
DNA exonuclease
DNA endonuclease
Poly (A) polymerase
DNA polymerase III epsilon subunit
RNA polymerase
Structural protein
Head-tail connector
Scaffolding protein
Major capsid
Tail tubular A
Tail tubular B
Putative short tail fiber ?
Internal virion protein A
Internal virion protein
Internal virion protein
Long tail fiber
TerS subunit
TerL subunit
Holin
Endolysin
i-spanin
O-spanin
1
4
8
18
19
22
25
28
29
32
34
38
39
40
42
45
46
47
49
0
10000
20000
30000
40000
bp
Hypothetical protein
DNA metabolism
RNA polymerase
Virion morphogenesis
Lysis
Putative enzymatic domains
Direct terminal repeat
Bacterial promoter
Rho-independent terminator

Figure 4

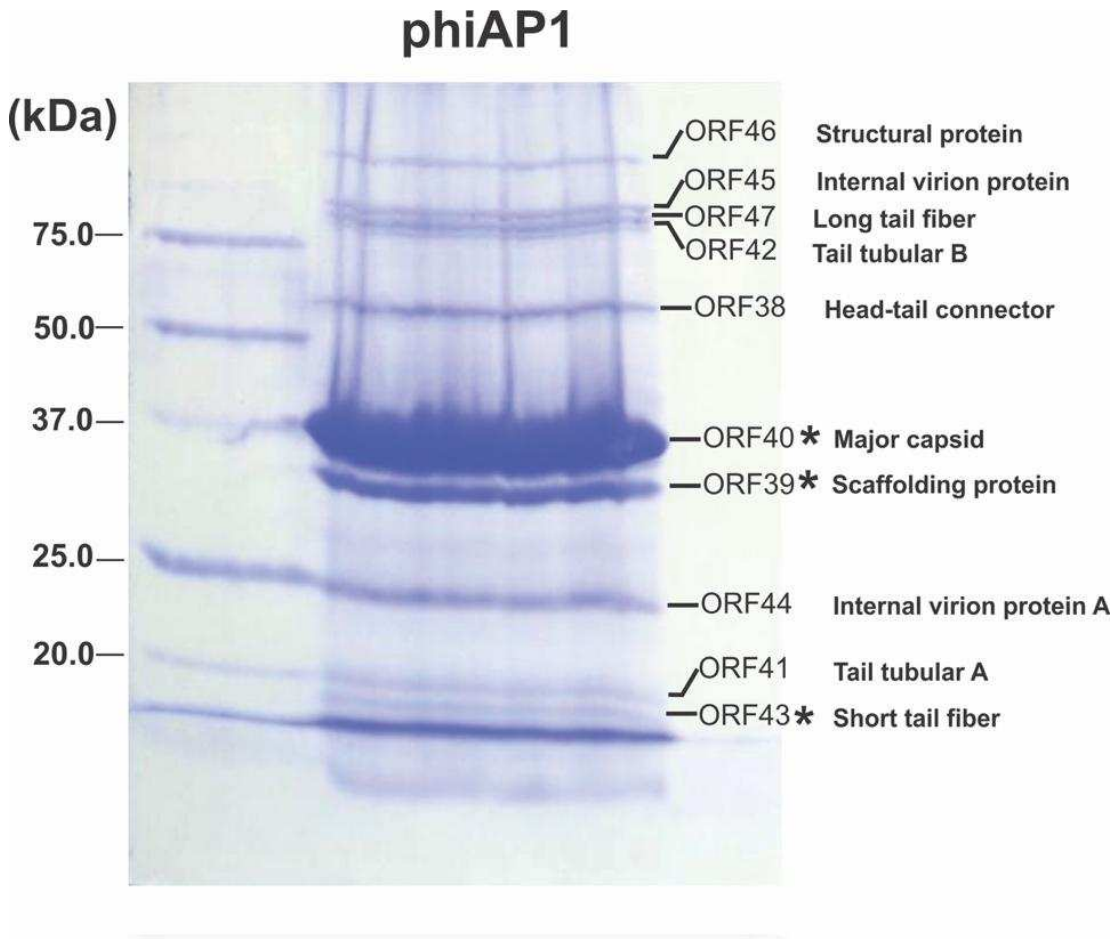

phiAP1
(kDa)
75.0
50.0
37.0
25.0
20.0
ORF46
Structural protein
ORF45
Internal virion protein
ORF47
Long tail fiber
ORF42
Tail tubular B
ORF38
Head-tail connector
ORF40*
Major capsid
ORF39*
Scaffolding protein
ORF44
Internal virion protein A
ORF41
Tail tubular A
ORF43*
Short tail fiber

Figure 5

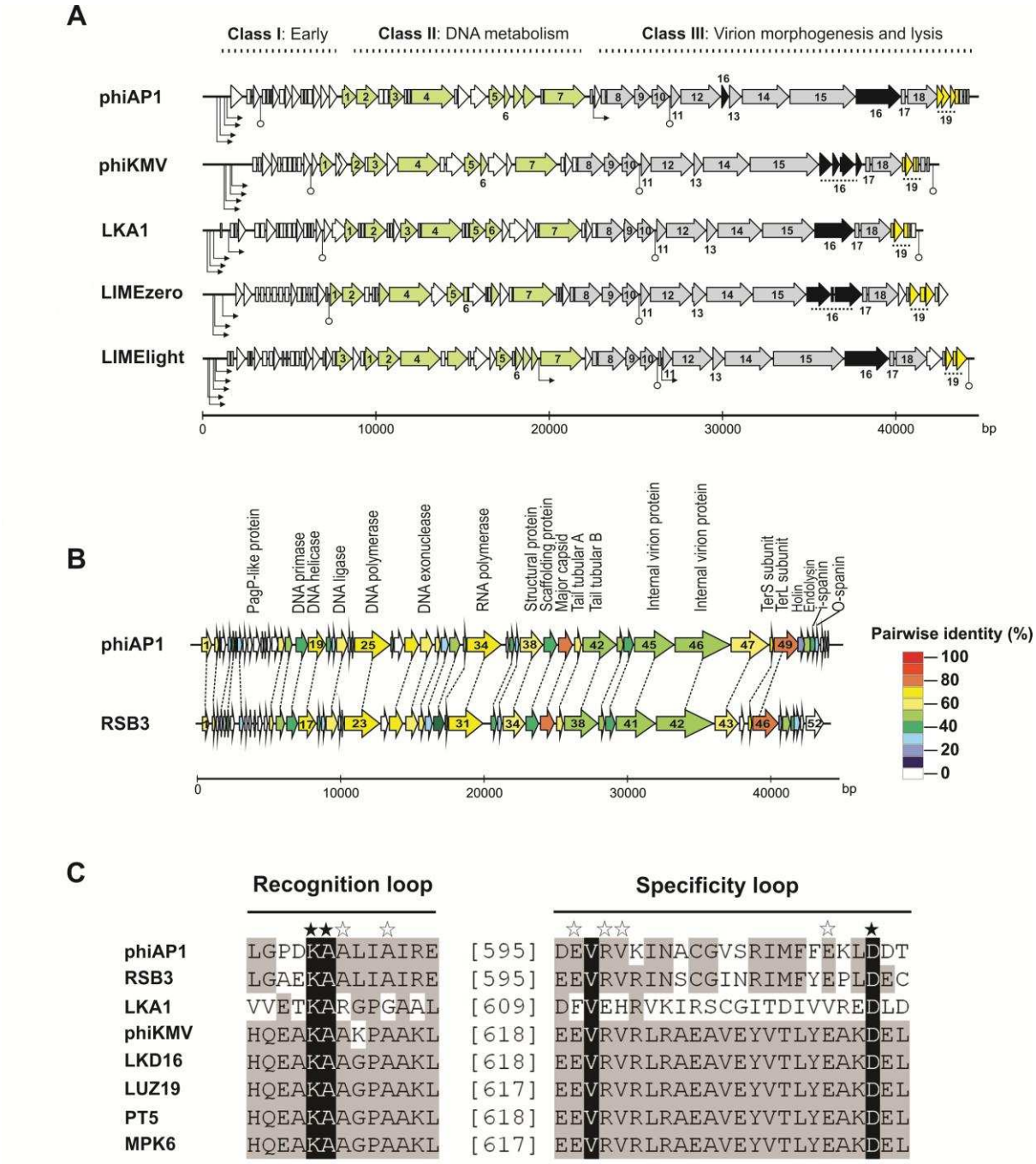

A
Class I: Early
Class II: DNA metabolism
Class III: Virion morphogenesis and lysis
phiAP1
phiKMV
LKA1
LIMEzero
LIMElight
0
10000
20000
30000
40000
bp
B
PagP-like protein
DNA primase
DNA helicase
DNA ligase
DNA polymerase
DNA exonuclease
RNA polymerase
Structural protein
Scaffolding protein
Major capsid
Tail tubular A
Tail tubular B
Internal virion protein
Internal virion protein
TerS subunit
TerL subunit
Holin
Endolysin
I-spanin
O-spanin
phiAP1
RSB3
Pairwise identity (%)
100
80
60
40
20
0
C
Recognition loop
Specificity loop
phiAP1 LGPDKAALIAIRE [595] DEVRVKINACGVSRIMFFEKLDDT
RSB3 LGAEKAALIAIRE [595] EEVRVRINSCGINRIMFYEPLDEC
LKA1 VVETKARGPGAAL [609] DFVEHRVKIRSCGITDIVVREDLD
phiKMV HQEAKAAKPAAKL [618] EEVRVRLRAEAVEYVTLYEAKDEL
LKD16 HQEAKAAGPAAKL [618] EEVRVRLRAEAVEYVTLYEAKDEL
LUZ19 HQEAKAAGPAAKL [617] EEVRVRLRAEAVEYVTLYEAKDEL
PT5 HQEAKAAGPAAKL [618] EEVRVRLRAEAVEYVTLYEAKDEL
MPK6 HQEAKAAGPAAKL [617] EEVRVRLRAEAVEYVTLYEAKDEL

Figure 6

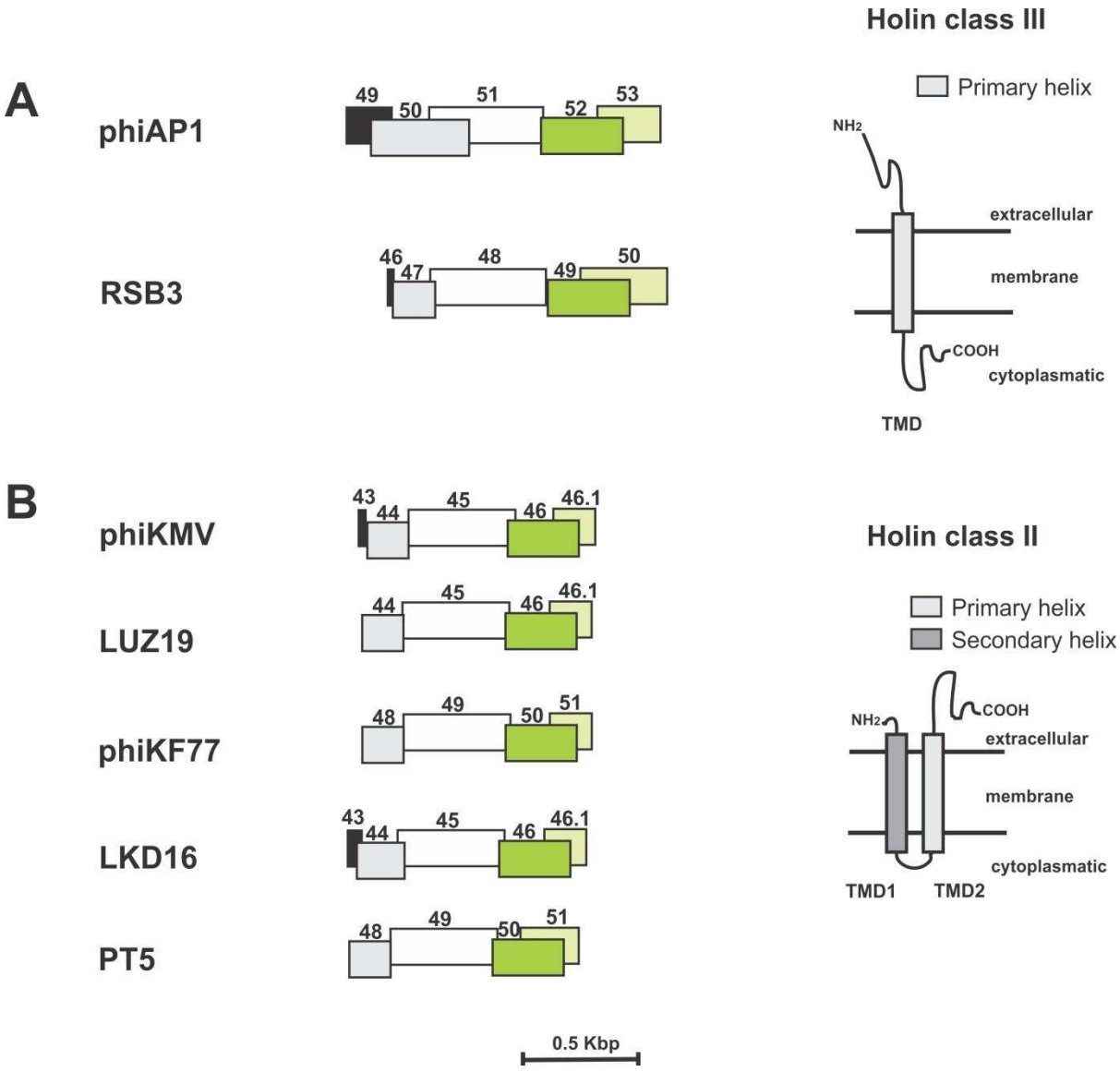

A
phiAP1
49
50
51
52
53
RSB3
46
47
48
49
50
Holin class III
Primary helix
NH2
extracellular
membrane
COOH
cytoplasmatic
TMD
B
phiKMV
43
44
45
46
46.1
LUZ19
44
45
46
46.1
phiKF77
48
49
50
51
LKD16
43
44
45
46
46.1
PT5
48
49
50
51
0.5 Kbp
Holin class II
Primary helix
Secondary helix
NH2
COOH
extracellular
membrane
cytoplasmatic
TMD1
TMD2

Figure 7

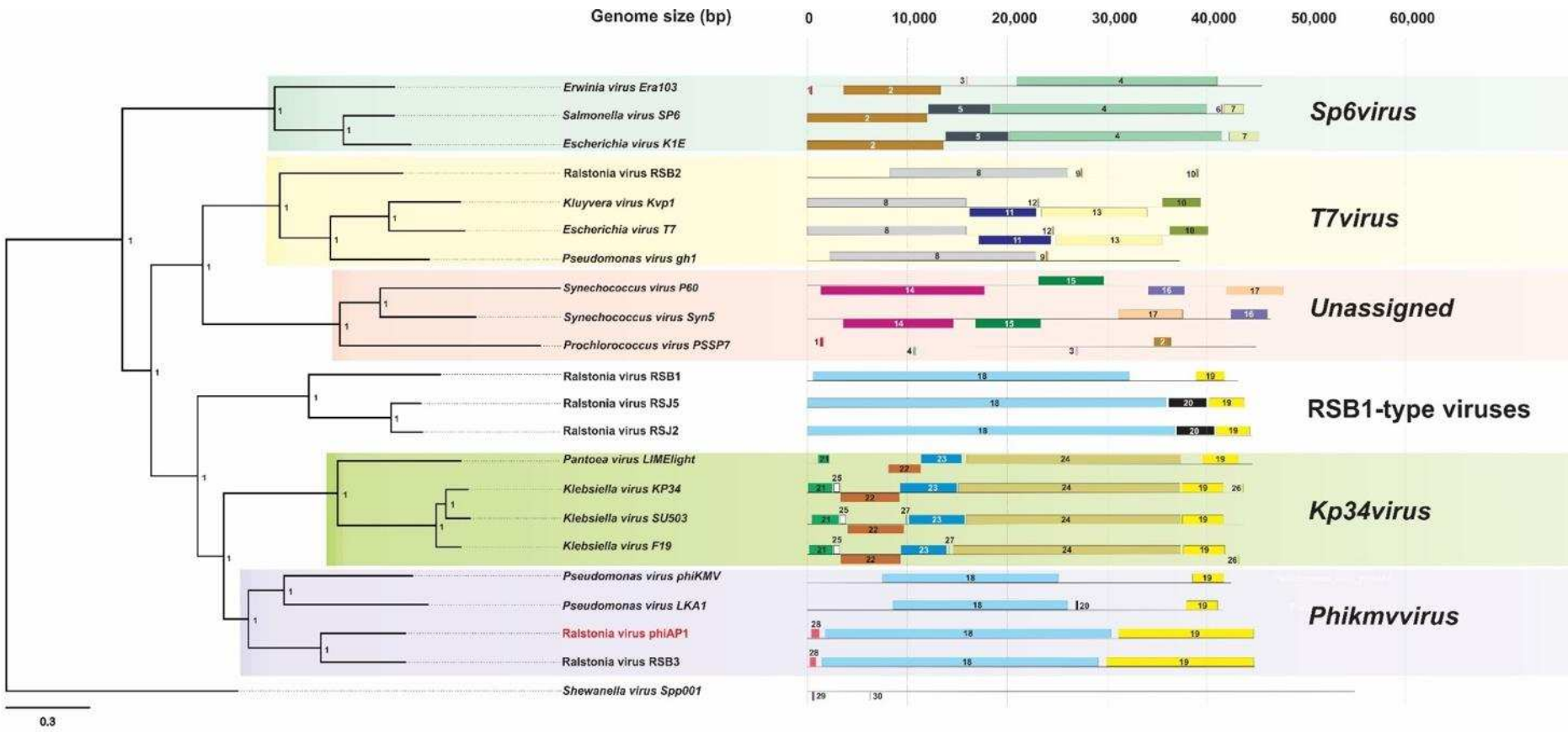
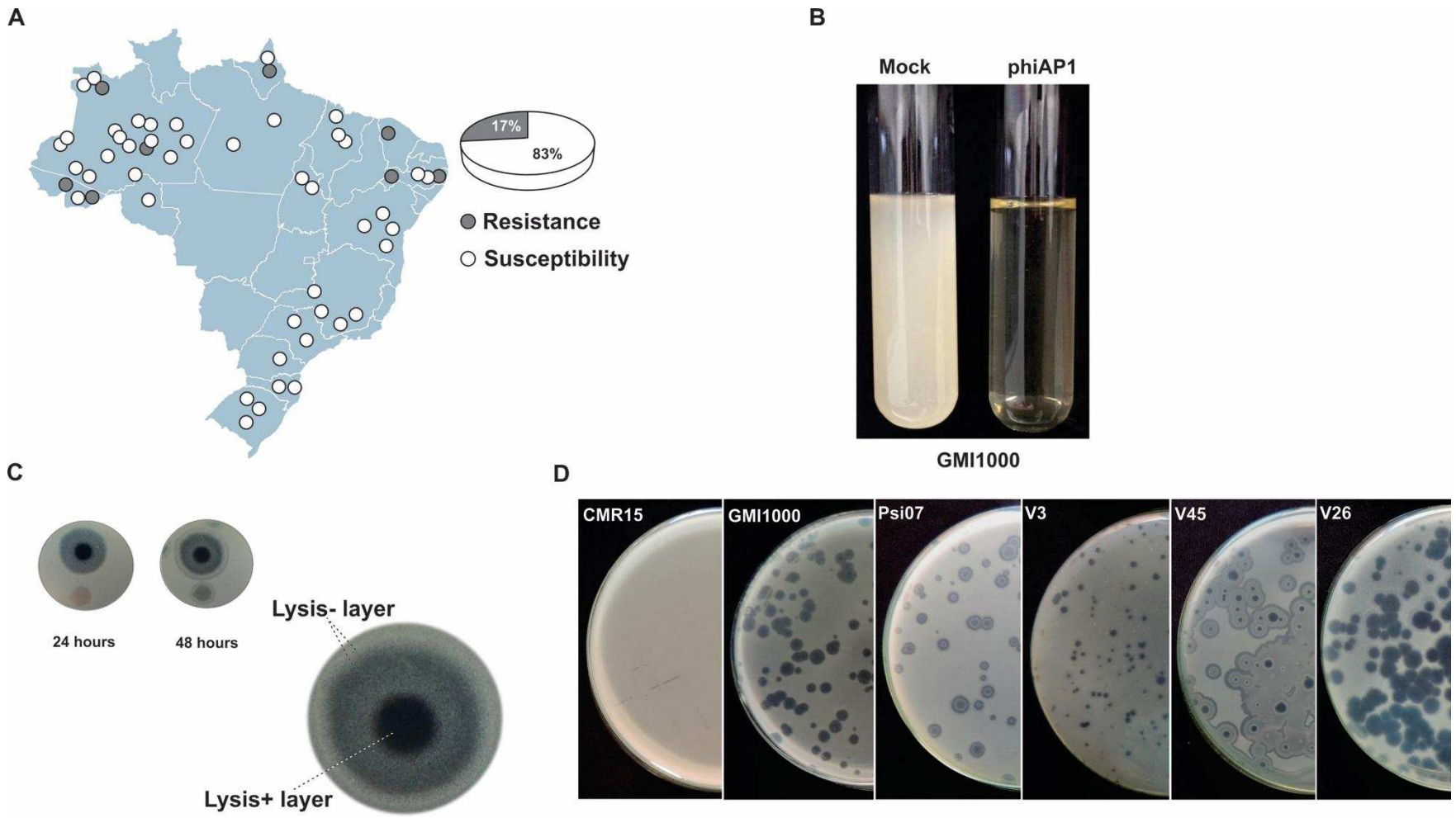
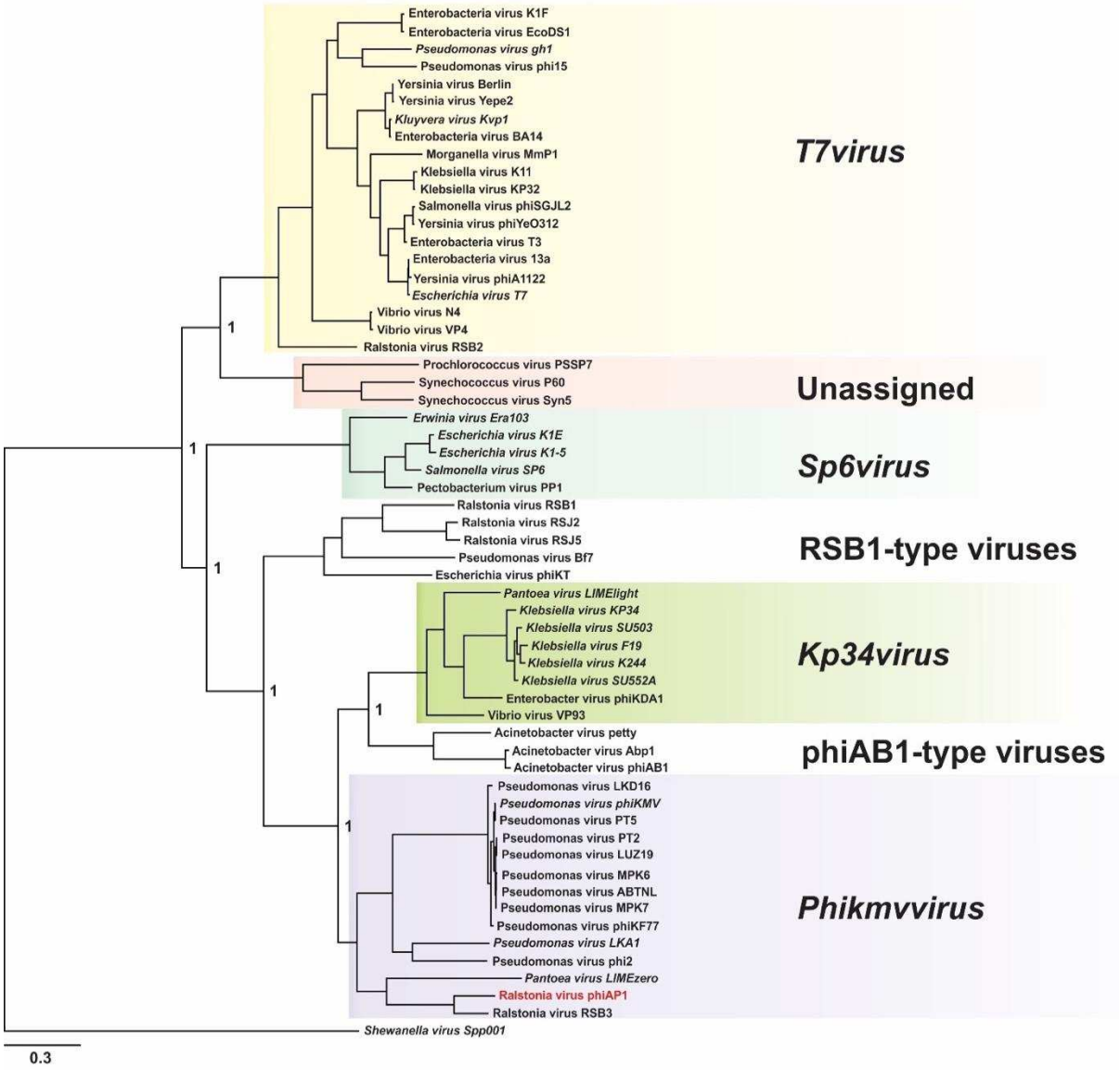

Genome size (bp)
0
10,000
20,000
30,000
40,000
50,000
60,000
Erwinia virus Era103
Salmonella virus SP6
Escherichia virus K1E
Sp6virus
Ralstonia virus RSB2
Kluyvera virus Kvp1
Escherichia virus T7
Pseudomonas virus gh1
T7virus
Synechococcus virus P60
Synechococcus virus Syn5
Prochlorococcus virus PSSP7
Unassigned
Ralstonia virus RSB1
Ralstonia virus RSJ5
Ralstonia virus RSJ2
RSB1-type viruses
Pantoea virus LIMElight
Klebsiella virus KP34
Klebsiella virus SU503
Klebsiella virus F19
Kp34virus
Pseudomonas virus phiKMV
Pseudomonas virus LKA1
Ralstonia virus phiAP1
Ralstonia virus RSB3
Phikmvvirus
Shewanella virus Spp001
0.3

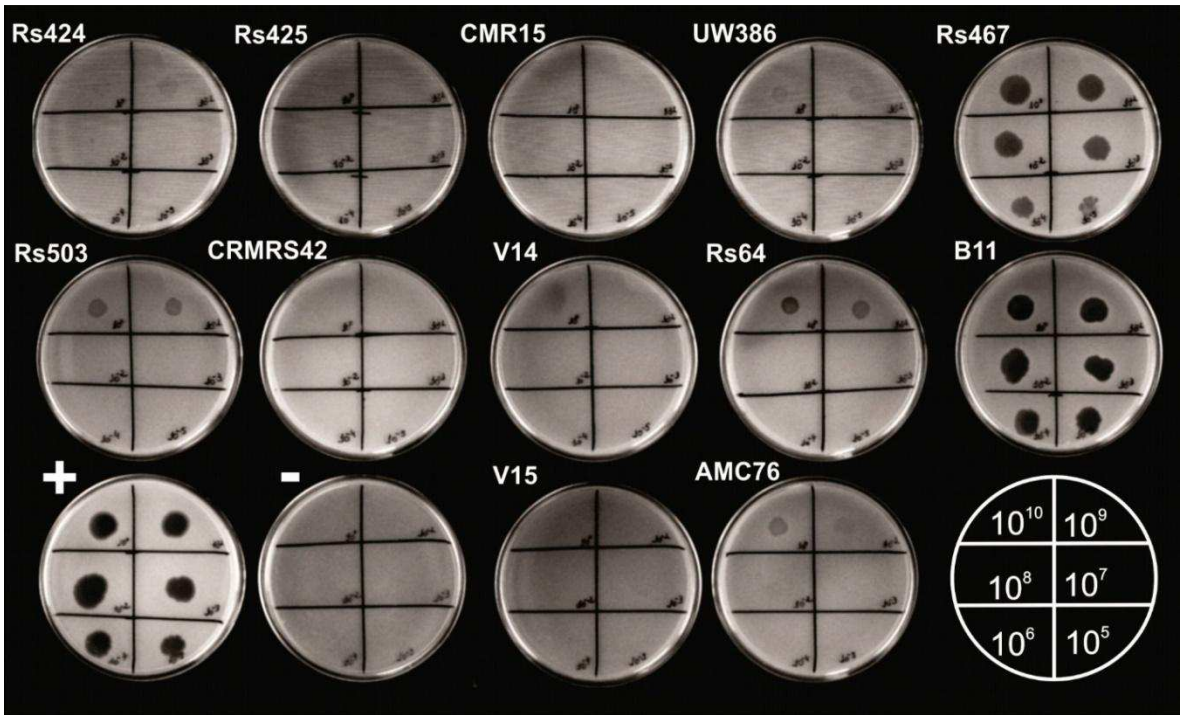
Figure 8


A
17%
83%
Resistance
Susceptibility
B
Mock
phiAP1
GMI1000
C
24 hours
48 hours
Lysis- layer
Lysis+ layer
D
CMR15
GMI1000
Psi07
V3
V45
V26

Supplemental Figure S1



Supplemental Figure S2



CHAPTER 2

Ralstonia solanacearum CRISPR-CAS SYSTEM IS AN IMMUNOCOMPROMISED UNIT UNDER IN VITRO CONDITIONS

Xavier, A.S., Rousseau, G.M, Tremblay, D.M, Melo, A.G, Moineau, S. and Alfenas-Zerbini, P. *Ralstonia solanacearum* CRISPR-Cas system is an immunocompromised unit under in vitro conditions. ***Frontiers in Microbiology***, will be submitted.

***Ralstonia solanacearum* CRISPR-Cas system is an immunocompromised unit under in vitro conditions**

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Short title: Inactivity of *Ralstonia solanacearum* CRISPR-Cas system

Contents category: Bacterial immunity

Key words: Adaptive immunity, transcriptional control, bacteria-virus interaction

Number of words in the main text (including figure legends): 8,435

Number of tables: 6

Number of figures: 8

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPRs) comprise an array of short DNA repeat sequences that are alternated by unique spacer sequences and, typically, are flanked by CRISPR-associated (*Cas*) genes. In combination with a suite of Cas proteins, they act as an adaptive immune system against viruses and other foreign DNA. This study aimed to investigate the occurrence of CRISPR-Cas system in the plant pathogenic *Ralstonia* spp. and to characterize them structurally and functionally. The CRISPR-Cas system was found in 31% of *Ralstonia* spp. isolates. Subtypes I-E and II-C were found, being the I-E the most common. The presence of the same CRISPR subtypes (I-E and II-C) in distinct phylotypes (currently different species) suggests the acquisition of the system by the common ancestor of all species before of migration of ancestors and segregation of Gondwana. Additionally, cas1 phylogeny (I-E subtype), agrees with the evolution of the *Ralstonia* complex, showing perfect geographical segregation of phylotypes, which supports the hypothesis of the ancient acquisition before the segregation of the species. BIMs (Bacteriophages Insensitive Mutants) can be isolated in cultures of isolates CFBP2957 and $K60^T$ post viral infection, however the presence of CRISPR loci does not explain the phenotype of resistance, because no acquisition of spacers was detected in BIMs. The functionality of the interference step was also tested in the CFBP2957, using a spacer-PAM delivery system. The results showed that the CRISPR-Cas in this isolate is either inactive or in a conditional repression state against foreign nucleic acid, which corroborates with the constitutive expression of *h-ns* genes.

Introduction

The plant pathogenic bacteria *Ralstonia* spp. are Gram-negative bacteria and belong to a complex with considerable genetic diversity (Fegan and Prior, 2005). These pathogens have wide host range, infecting more than 200 species belonging to more than 50 botanical families, including economically important crops (Denny, 2007; Hayward, 1991, 2000). Despite the heterogeneity of the group, these species induce rapid and fatal wilting symptoms in host plants. Recent genomic and proteomic approaches support the division of this complex into three species, *R. solanacearum* (phylotypes IIA and IIB), *R. pseudosolanacearum* (phylotypes I and III) and *R. syzygii* (phylotype IV) (Prior et al., 2016), in agreement with the hierarchical classification into four phylotypes (Fegan and Prior, 2005).

In nature, bacteria and viruses are involved in continuous cycles of co-evolution, in which resistant hosts emerge and genotypic composition of populations effectively change. In this dynamic scenario, anti-viral mechanisms have a key role in regulating bacterial populations (Koskella and Brockhurst, 2014). Bacteria use a wide range of strategies to evade viral infection, including innate defense responses, or adaptive immunity, in particular the CRISPR-Cas system (Marraffini, 2015). In *Ralstonia* spp. natural resistance to viral infection has been observed in the population and the variability in resistance phenotypes suggests the involvement of more than one defense mechanism (Kawasaki et al., 2016; Xavier et al., 2016 (Chapter 1).

CRISPR loci and CRISPR-associated (*Cas*) genes code for an inheritable immune adaptive system that provide sequence-specific protection against foreign DNA, including viruses, plasmids and selfish genetic elements, and, in some cases, have RNAs as target (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). These systems are widely distributed

in the genome of archaea [90%] and bacteria [50%] (Grissa et al., 2007a) and their diversification allows group them to be grouped into five types and subdivided into 16 subtypes according to differences in *Cas* genes content, and in the interference and adaptation modules (Makarova et al., 2015).

A generic defense CRISPR unit consists of a CRISPR array comprising short palindromic repeats interspersed by hypervariable short DNA sequences, referred as spacers, bordered by the CRISPR-associated (*Cas*) genes. Three distinct steps control CRISPR mediated immunity phenotype: adaptation, expression and interference (Magadán et al., 2012). During adaptation, fragments of foreign DNA, known as protospacers, are incorporated into the CRISPR array and constitute the memory of the prokariotic immune system. Later, spacers and repeats constituting the CRISPR arrays are expressed as a long precursor RNA which is processed into small CRISPR RNAs, that guide the CAS proteins involved in the degradation of cognate DNA (interference step) (Makarova et al., 2015; Van der Oost et al., 2009; Wiedenheft et al., 2012).

The subtype I-E CRISPR-Cas system found in a large number of bacteria including *E. coli*, *Salmonella* spp. and *Streptomyces* spp. has been extensively studied (Fabre et al., 2012; Guo et al., 2011; Haft et al., 2005; Kiro et al., 2013; Shariat et al., 2015) and functional characterization has revealed a system "immunocompromised" in its native state (Guo et al., 2011; Kiro et al., 2013; Medina-Aparicio et al., 2011; Pul et al., 2010; Westra et al., 2010). Despite the widespread involvement in adaptive immunity, some studies have suggested a distinct and interesting role for CRISPR-Cas systems in some species, such as biofilm and pathogenicity regulation (Westra et al., 2014). Here, we demonstrate the presence of canonical CRISPR loci in *Ralstonia* spp. and provide a comparative analysis of CRISPR diversity across *Ralstonia* spp. Functional analysis of the adaptation and interference activity showed that the

Ralstonia solanacearum CRISPR-Cas is inactive or in a conditional repression state against foreign nucleic acid, is in agreement with the constitutive expression of *h-ns* genes

Results

The analysis using CRISPRs tools conducted with 51 genomes of *Ralstonia* spp. isolates, including non-plant pathogens and species belonging to the complex associated with wilt disease revealed the presence of canonical CRISPR loci of I and II types, classified in I-E and II-C subtypes (Figure 1). The CRISPR-Cas system was found in apparently intact conditions (CRISPR loci and Operon Cas) only in 31% of the sequences analyzed (16 sequences). Thirteen of the sixteen isolates have the subtype I-E and only three have the II-C subtype (Table 1). Ten isolates of *R. solanacearum* (phylotype II), two of *R. pseudosolanacearum* (with one in the phylotype I and another in the phylotype III), and one of *R. syzygii* subsp. *celebesensis* (BDB 229, formely Blood Disease Bacterium, phylotype IV) showed the subtype I-E. The remaining three isolates have the II-C subtype, being one *R. syzygii* subsp. *syzygii* (phylotype IV), and the other two isolates, *R. solanacearum* (phylotype II) (Table 1). CRISPR loci were not found in non-pathogenic species of *Ralstonia*.

Despite of the conserved architecture of CRISPR loci among *Ralstonia* spp. isolates, we observed a variation in the size ranging from 4-80 spacers/array (Figure 1) and number of CRISPR array, around 1-3 arrays/chromosome (Table 1). The CRISPR subtype I-E locus is conserved in all 13 isolates, flanked upstream by an operon containing decarboxylases and dowsntream by a gene encoding a argininosuccinate lyase (Figure 1A), the same characteristics are not shared among isolates containing II-C subtype (Figure 1B). The pairwise identity analysis of the Cas proteins from subtype I-E (Cas3, Cse1, Cse2, Cas7, Cas5, Cas6, Cas2 and Cas1) of

the isolate of *R. solanacearum* CFBP2957 with the other isolates showed a high degree of conservation (88-100%) (Table 2). The Cas proteins of the subtype II-C (Cas9, Cas1 and Cas2) were more conserved, showing 94-100% identity (Supplemental Table S4). The I-E subtype was the most frequent and the maintenance of this subtype in distinct lineages that comprise the *Ralstonia* spp. complex, suggests an activity in the life cycle of these hosts, so we decided to focus our analysis on this subtype.

The phylogenetic reconstruction using the Cas1 protein of the *Ralstonia* spp. and some species containing the CRISPR subtypes I-A, I-B, I-C, I-E and I-F positioned all thirteen *Ralstonia* spp. isolates in the I-E subtype clade (Figure 2), supporting our previous analysis. The phylogenetic analysis with only *Ralstonia* spp. Cas1 protein of I-E subtype, showed a perfect congruence with the phylogenetic tree of *Ralstonia* spp. using the amino acid sequence of the protein of core genome, Egl (Castillo and Greenberg, 2007), suggesting an ancient acquisition of the CRISPR locus subtype I-E, before the segregation of the *Ralstonia* species (Supplemental Figure S1).

In isolate CFBP2957, the CRISPR-Cas system contain a *Cas* operon around ~ 9.5 Kb flanked by two CRISPR arrays, a smaller one with 7 repeat-spacer units (CRISPR1, ~ 0.5 Kb) and the other one containing 59 repeat-spacer units (CRISPR2, ~ 4 Kb), indicating that CRISPR2 is the array with higher activity (Figure 3A). The analysis of the leader sequence in CRISPR1 and CRISPR2 revealed typical leader sequences with high A/T, non-coding sequences, conserved within located on one side of the CRISPR units (Jansen et al., 2002). More detailed analyzes to characterize the repeats and spacers in all isolates are underway, but preliminary analysis indicates that they have a profile similar to that observed for CFBP2957.

The spacers content reveals the diversity for foreign DNA in R. solanacearum CFBP2957

To investigate the immune function of CRISPR-Cas system in *R. solanacearum* CFBP2957, we predicted the putative origin of spacers in arrays. Only 5% of the spacers from protospacers present in plasmids or temperate virus/provirus (Figure 3B). Match with viral sequences in CRISPR1 was detected for spacer 6 (Mycobacterium virus crimD, 5 SNPs, 27/32nt, 85% identity) and in CRISPR2 for spacers 36 (Pseudomonas virus JBD44, 4 SNPs, 28/32nt, 90% identity) and 49 (Ralstonia virus phiRSA1, 1 SNP 31/32nt, 97% identity). 95% of the spacers are from unknown protospacers.

R. solanacearum CRISPR repeats are competent to form secondary structure

The CFBP2957 CRISPR arrays contain typical palindromic repeats of 29 bp (Figure 3C), which are interspaced by regular spacers of 32 bp. CRISPR loci are transcribed as a single-stranded RNA molecule starting from the CRISPR leader (Peng et al., 2003; Viswanathan et al., 2007), thus potentially the pairing of consecutive repeats could give greater stability for the primary transcript to be processed (Horvath et al., 2008). The putative secondary structure of the primary transcript of six consecutive CRISPR2 repeats showed a typical conformation presented by these long RNAs (Figure 3D), suggesting that the CRISPR locus subtype I-E is functional.

The CRISPR system is not functional against phiAP1.

To test if the *R. solanacearum* CRISPR system is active against the phikmvvirus Ralstonia virusphiAP1, we isolate BIMs (Bacteriophages Insensitives Mutants) of *R. solanacearum* isolates CFBP2957 and $K60^T$ that carry the CRISPR subtype I-E containing two arrays (Figure 4A). We were able to isolate the mutants 72 hours post inoculation (Figure 4B). For each wild-type parental strain (WT), a total of thirty BIMs were analyzed to confirm the

resistance phenotype by spot test. It was possible to observe extreme resistance despite the high viral titer (Figure 4C). The BIMs were permissive to viral adsorption, survive at high inoculum pressure, excluding the occurrence of Abi infection. Viral replication was not detected in BIMs (Xavier et al., 2016 (Chapter 3)). We tested spacer acquisition hypothesis, assuming that the CRISPR adaptation was responsible for viral immunity in these BIMs. PCR screening to detect integrations of new spacers next to leader region, showed that apparently there was no spacer acquisition in $K60^T$-derived (Figure 4D) or CFPB2957-derived BIMs (Figure 4E). The sequencing of the PCR products confirmed that there was not acquisition of spacers in the BIMs. The CRISPR subtype I-E is one of the most studied subtypes where the integrations of new spacers occur next to leader region, which is consistent with our PCR screening strategy. However, an unusual activity in the CRISPR arrays of some BIMs of *Streptococcus thermophilus* has been documented (Mills et al., 2010). Thus, to ensure that throughout all the two CRISPR arrays there are not occurring integration, the complete CRISPR arrays of all CFBP2957-derived BIMs were amplified and the PCR analysis showed that there was no spacer acquisition (Figure 5). To confirm the PCR result, the arrays of 15 BIMs, randomly chosen, were sequenced and the sequence analysis confirmed the PCR results, showing that BIMs arrays remained as in CFBP2957 WT. These results showed that the acquisition step is not active in *R. solanacearum* CFBP2957.

Absence of DNA interference reveals an immunocompromised unit to the R. solanacearum CRISPR-Cas system

To verify if the CRISPR-mediated interference step is active in *R. solanacearum* CFBP2957, we used a protospacer-delivery system into *R. solanacearum* cells, simulating the invasion of a cognate foreign DNA. This approach simulates a scenario of 'priming' for an

operant immune system and allows to access the CRISPR activity by transformation efficiency. Two protospacers (36 and 49) whose sequences match viral genomes were chosen (Figure 6A). Because some sequence motifs at the 3' end of the spacer make some protospacers preferred targets for interference (Yosef et al., 2013), we decided to clone around 8 nucleotides downstream from the protospacer, in addition to 8 nucleotides upstream from it, include the PAM (Protospacer Adjacent Motif) motif, in this case, an AGG (Figure 6B). The values of CFU/ug DNA obtained indicated that the transformation with plasmids that contain the spacer 36 (pPsp36) or 49 (pPsp49) was as efficient as that of the empty vector (pEmpty), being both on average 90%, showing that the interference is in CFBP2957 impaired (Figure 6C and 6D).

The Cas operon is not expressed and the h-ns genes are active in R. solanacearum CFBP2957

In *Enterobacteriaceae*, the phenotype of CRISPR inactivity is associated to regulation by the antagonist H-NS (Medina-Aparicio et al., 2011; Pul et al., 2010) In *E. coli* K12, it has been shown that the transcription of the Cas operon is repressed by H-NS (Pul et al., 2010). So, to test if in *R. solanacearum* the phenotype of CRISPR inactivity can be explained by the expression of the *Cas* operon repressors, the expression of CRISPR-associated (*Cas*) genes was analyzed using RNA isolated from from virus-infected and non-infected (mock) CFBP2957 cultures. We noted that regardless of viral presence in culture, the expression of *Cas* genes was not detected (Figure 7). As *Ralstonia* spp. contains multiple copies of genes encoding nucleoid-associated proteins, such as H-NS, (Dorman, 2004), we decided to investigate their expression as well. Of the three genes (*h-ns* 1-3) present in CFBP2957, two (*h-ns1* and *h-ns3*) are transcriptionally active (Figure 7). These results suggested that CRISPR activity in *R. solanaceraum* CFBP2957 can be modulated by the expression of the *h-ns* genes.

Discussion

In plant pathogenic *Ralstonia* spp., the subtype I-E CRISPR-Cas system was found at higher frequency in comparison to II-C subtype (Table 1). This same subtype is present in *E. coli* K12 (Kunin et al., 2007) and in many proteobacteria (Haft et al., 2005). Here, the presence of the same CRISPR subtypes (IE and II-C) in distinct phylotypes (currently different species), including BDB 229 and R24, ecotypes endemic of Indonesia (Remenant et al., 2011) suggests the acquisition of this immune system by common ancestor in the putative center of origin of *Ralstonia* spp. (Indonesia), before fragmentation of Gondwana, according to the demographic history and probable migration of the *Ralstonia* common ancestor (Wicker et al., 2012). The Cas1 protein (I-E subtype) showed an excellent phylogenic signal, because the topology of the phylogenetic tree, shows the perfect geographical segregation of phylotypes, as well as the phylogeny using a protein of core genome, Egl (Castillo and Greenberg, 2007). These evidences support the hypothesis of the ancient acquisition of the CRISPR locus and indicate that the CRISPR-cas system is as old as the species of the complex.

Although the *R. solanacearum* CFBP2957 CRISPR-Cas system contains the intact minimum elements for immunity, the expression of *Cas* genes was not detected in vitro, which can explain the abolished protective phenotype against foreign DNA. The nucleoid-associated proteins of bacteria, including heat-stable nucleoid-structuring (H-NS) protein, are proteins that participate of the main DNA transactions and their influence on transcription modulates the global gene-expression profile of the cell (Dorman, 2004). As in *Ralstonia* spp., bacteria such as *Streptomyces* spp., *E. coli* and *Salmonella* spp. isolates harbor the subtype I-E CRISPR-Cas system, a subtype which has been shown not to be able to adapt or interfere in its native state (Guo et al., 2011; Medina-Aparicio et al., 2011; Pul et al., 2010; Westra et al., 2014), with

exception of *Streptomyces avermitilis* (Qiu et al., 2016). In both species the *Cas* genes derepression is sufficient for restoring CRISPR-mediated immunity (Swarts et al., 2012).

In addition to two transcriptionally active copies of *h-ns*, *Ralstonia* spp. contains several transcription factors, as well as LeuO, belongs to the LysR family of transcription factors, that also includes PhcA, a global regulator of pathogenesis in *Ralstonia* spp. (Brumbley et al., 1993; Stoebel et al., 2008). The presence and transcriptional activity of these regulators suggest that CRISPR immunity in *R. solanacearum* CFBP2957 can also be under the control of these proteins. Besides regulation by H-NS and LeuO in *Enterobacteriaceae*, environmental signals or stress can also interfere with the *Cas* gene expression in various species (Koskenniemi et al., 2011; Laakso et al., 2011; Melnikow et al., 2008; Rodriguez et al., 2011).

It has been shown that in the absence of viral infections the spacers can be easily lost owing to the deletion during bacterial genome evolution (Kuo and Ochman, 2009). The cost of maintaining the CRISPR systems is high (Weinberger et al., 2012), so, the maintenance of these systems in a constitutive inactive state should be challenging for the species. Interestingly, the expression of some CRISPR elements can be conditioned to environmental stimuli as in *Salmonella enterica* serovar Typhi and *Campylobacter jejuni* which had the expression of the cas genes activated when its were isolated from the blood circulation system and during intestinal passage respectively (Sheikh et al., 2011; Jerome et al., 2011).

Alternatively, can be proposed that, as in human pathogens, the expression profile of the *Cas* genes of *Ralstonia* spp. in the environmental may be different, supporting a vestigial activity for this system, which is being kept for millions of years in these phytophatogenic bacteria (Storey, 1995; Wicker et al., 2012). In addition to involvement in viral defense, CRISPR-Cas

systems in some species appears to be involved in the regulation of expression of virulence genes and biofilm formation (Westra et al., 2014), suggesting an alternative function for the CRISPR *R. solanacearum*, if the differential expression of the operon *Cas* not change the CRISPR immunity phenotype.

Knockout studies of the *hn-s* genes in *R. solanacearum* CFBP2957 is currently being conducted in an attempt to reprogram the CRISPR activity, to assess its mode of regulation and its still functionality.

Material and Methods

Bacterial isolates, virus and growth conditions

R. solanacearum isolates, CFBP2957, $K60^T$ and their derivative lines were cultured in CPG medium containing casein (1g/L), peptone (10g/L) and glucose (5g/L) (Horita and Tsuchiya, 2001) at 28°C with shaking at 250 rpm. Ralstonia virus phiAP1, a phikmvvirus recently characterized was propagated on CFBP2957 and $K60^T$ as described (Xavier et al., 2016 (Chapter 1)). NEB® 5-alpha Competent *E. coli* was grown at 37°C using Luria-Bertani (LB) (Miller, 1992).

CRISPR bioinformatics analysis

A total of 48 genomes of phytopathogenic *Ralstonia* spp., and three genomes of the non-pathogenic species *R. mannitolilytica*, *R. eutropha* and *R. pickettii*, including full-length or draft versions, were analyzed. The complete genome sequences or contigs (for the drafts) were

downloaded from the NCBI (National Center for Biotechnology Information) Genomes database (http://www.ncbi.nlm.nih. gov/genome/browse) and are listed in Table 1.

To find CRISPR arrays and *Cas* operons, , we used CRISPR database (Grissa et al., 2007b) (http://crispr.i2bc.paris-saclay.fr), CRISPI (http://crispi.genouest.org) (Rousseau et al., 2009), CRISPRfinder software tools (http://crispr.u-psud.fr/Server) (Grissa et al., 2007a), CRISPR Recognition Tool CRT (Bland et al., 2007), besides manual inspection in Geneious R8.1 (Biomatters Ltd., Auckland, New Zealand) to detect the loci's context. Secondary structure predictions were obtained by using the Mfold 3.2 program (Zuker, 2003). Sequence consensus motifs were visualized by using WebLogo (http:// weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004). The spacer content was analyzed and the potential protospacers were classified in three categories using CRISPRTarget (Biswas et al., 2013), adopting the parameters defined by Shariat et al. (2015): spacers with potential protospacer matches to fewer than six SNPs (or ≥27/32nt matching). Pairwise comparisons of amino acid sequences of the Cas proteins (Supplemental table S1) were performed with Geneious R8.1 (Biomatters Ltd., Auckland, New Zealand) and the alignments were performed using the MAFFT algorithm (Edgar, 2004)

Phylogeny

The phylogeny of Cas1 proteins of *Rasltonia* spp. were analyzed together with Cas1 proteins from bacterial species that contain different CRISPR subtypes, including I-A, I-B, I-C, I-E and I-F (Supplemental Table S2). Additionally, phylogeny only Cas1 proteins of *Rasltonia* spp. and a phylogeny of the amino acid sequence of the gene that codes the protein Egl (Fegan and Prior, 2005; Prior and Fegan, 2004) were constructed. The sequences were aligned with

ClustalX2 and maximum likelihood tree was constructed in the MEGA 7.0 program using Jones-Taylor-Thornton (JTT) evolutionary model (Kumar et al., 2016). Phylogenetic trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Isolation of Bacteriophage Insensitive Mutants (BIMs) and Spot assay

Bacteriophage insensitive mutants **(**BIMs) were obtained by challenging the virus-sensitive *Ralstonia solanacearum* isolates CFBP2957 and $K60^T$ with the phikmvvirus phiAP1, as described (Deveau et al., 2008) with some modifications. *R. solanacearum* isolates were grown in CPG broth to an OD_{600} of 0.2 at 28 °C. A 0, 25 mL aliquot was mixed with 100 uL of purified phiAP1 (10^{10} PFU/mL). After a 15 min incubation, the mixture was embedded in 3 mL of 0.45 % low melting point CPG agar and poured onto a 1,5% CPG bottom agar. The plates were incubated at 28°C for 96 hours. Thirty colonies resistant derived from each parental virus-sensitive strain were selected. Resistant colonies were picked and after three successive replications on CPG agar, single colonies were preserved and confirmed for resistance phenotype through de spot test (Pantůček et al., 1998) using 10µL from viral suspension (10^{10}, 10^9, 10^8, 10^7, 10^6 and 10^5 PFU/mL).

DNA isolation

The genomic DNA from BIMs and the respective viruses-sensitive parental isolates were extracted as described by Garneau et al., (2010), except the lysozyme step, that was not

preformed. The purity of the DNA and concentration were estimated with a NanoDrop and all samples were diluted in PCR-grade water to a final concentration of 20ng/µl.

CRISPR array amplification and sequencing

To investigate if the complete resistance phenotype on BIMs is linked to new spacers acquisition in CFBP2957 or $K60^T$ CRISPR arrays, primers were designed to detect the CRISPR1 and CRISPR2 arrays. Initially, a set of primers were designed based on the sequence of CRISPR arrays found on chromosome CFBP2957 [PRJEA50685] and $K60^T$ [PRJEB8309] isolates for subsequent PCR reaction (Supplemental Table S3). The PCR reaction was performed using 20ng/µL of the genomic DNA according to standard protocols and the PCR products were analyzed in a 2,5% agarose gel stained with EZ-Vision Three (Amresco, Solon, OH), and visualized under UV light. PCR products were sequenced (Plateforme de Séquençage et de Génotypage des Génomes at the CHUL/CHUQ Research Center). The sequences were trimmed and analyzed using Geneious R.8 (Biomatters Ltd., Auckland, New Zealand).

Protospacers cloning

The protospacers 36 and 49 (match with viral sequence) of the CFBP2957 CRISPR loci were cloned in vector pUFJ10 (Gabriel et al., 2006). We cloned the 8 nucleotides upstream (containg Protospacer Adjacent Motif, PAM) and 8 nucleotides downstream (containg probable enhancer motifs) of protospacer present in each respective target genome. Primers were designed with restriction sites for *Eco*RI and *Xba*I (Supplemental Table S3) compatible with the multiple

cloning sites of pUFJ10. Plasmid DNA isolation was done using Qiagen Maxi-Prep kit as recommended by the manufacturer. DNA manipulations were performed according to standard techniques (Sambrook and Russell, 2001). Heat shock transformations using NEB® 5-alpha Competent *E. coli* (High Efficiency) were performed according manufacturers' protocols and the putative clones were confirmed by PCR. Two clones were confirmed after the sequencing, pPsp36 (protospacer 36 cloned into pUFJ10 plasmid) and pPsp49 (protospacer 49 cloned into pUFJ10 plasmid).

DNA interference assay

To verify if CRISPR interference step is active in *R. solanacearum* we transformed *R. solanacearum* CFBP2957 with 1µg of plasmid DNA via electroporation as described (Allen et al., 1991) using the construct pPsp36 (protospacer 36 cloned pUFJ10 plasmid) and pPsp49 (protospacer 49 cloned pUFJ10 plasmid) and pUFJ10 (pEmpty) as negative control. The transformation experiments were performed with three replicates for each treatment and repeated twice.

Cas and h-ns genes expression

Total RNA from *R. solanacearum* CFBP2957 was isolated from samples collected from cultures with OD_{600} = 0.2 (Duplessis et al., 2005) using TRIzol Reagent (Invitrogen). Two groups of samples were analyzed, not inoculated cultures (mock) and cultures 60 min after inoculation with Ralstonia virus phiAP1. The pellet obtained from approximately 25 mL of culture was suspended in 1 ml TRIzol Reagent and transferred into a 2-mL tube containing 250 mg of glass beads (106 uM, Sigma). The mixture was vortexed with a Mini-Beadbeater-8 cell

(BioSpec Products) four times for 2 min. The RNA samples were treated with 20U DNAseI (Invitrogen) for 60 min at 37°C in presence of 80U RNaseOUT (Invitrogen). The cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. PCR reactions were conducted using specific primers for *Cas* and *h-ns* genes (Supplemental Table S3). The absence of DNA in the RNA samples treated with DNAseI treated was tested by PCR.

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Figure legends

Figure 1. Overview of the two types of CRISPR-Cas systems found in *Ralstonia* spp. **(A)** CRISPR organization loci in I-E subtype and **(B)** II-C subtype. The ORFs (arrows) and the CRISPR arrays (black traces) are drawn to scale. Conserved ORFs in the CRISPR flank region (white). Adaptation and interference modules are in yellow and gray **(A)** or green **(B)** respectively.

Figure 2. Phylogeny of Cas1 proteins of bacterial species that contains different CRISPR subtypes including I-A, I-B, I-C, I-E and I-F. The maximum likelihood tree was constructed in the MEGA 7.0 program using Jones-Taylor-Thornton (JTT) evolutionary model. Triangles denote multiple collapsed branches. Individual genes are labelled with taxa names. Bootstrap values are indicated as percentage points; values below 50% are not shown.

Figure 3. *R. solanacearum* CFBP2957 CRISPR-Cas system. **(A)** Characterization of CRISPR arrays and *Cas* operon. **(B)** Classification of potential protospacers made in three categories by adopting the following parameters: spacers with potential protospacer matches to fewer than six SNPs (or ≥27/32nt matching). **(C)** The repeats conservation was visualized with the Weblogo program (http:// weblogo.berkeley.edu/logo.cgi) for each CRISPR array. **(D)** Putative structure obtained by pairing of six consecutive CRISPR2 repeats using MFOLD.

Figure 4. BIMs isolation and CRISPR adaptation analysis. **(A)** PCR design to screening of the spacers acquisition in $K60^T$ and CFBP2957 isolates. **(B)** Isolation of BIMs derived from virus-sensitive $K60^T$ isolate after Ralstonia virus phiAP1 infection. **(C)** $K60^T$ and CFBP2957-derived BIMs exhibit complete resistance in spot test, independent of viral titer. **(D)** PCR screening to

detect novel spacer acquisition in CRISPR1 (CR1) and CRISPR2 (CR2) for $K60^T$-derived BIMs or **(E)** CFBP2957-derived BIMs. Ctrl-, PCR-grade H_2O.

Figure 5. *R. solanacearum* CFBP2957 CRISPR arrays. **(A)** Schemes for conventional amplification of the CRISPR 1 and overlapping primers for full-length amplification of CRISPR 2. **(B)** PCR for detection of the spacer acquisition in thirty CFBP2957-derived BIMs according to the strategies showed in **(A)** for the arrays 1 and 2.

Figure 6. CRISPR Interference in *R. solanacearum* CFBP2957. **(A)** Spacers 36 and 49 belong to CRISPR array 2 and their sequences match with viral protospacers. **(B)** Characterization and cloning of the protospacers to validate the cognate DNA delivery system. **(C)** Transformation of the *R. solanacearum* CFBP2957 by electroporation using plasmids that contain protospacers. Samples were plated with or without dilution. **(D)** Comparison of transformation efficiency. pEmpty, pUFJ10 without protospacers (negative control), pPsp36 and pPsp49, pUFJ10 containing the protospacers 36 or 49, respectively. Bars in the graphs are presented as mean values from two independent experiments ± 1 SD.

Figure 7. *Cas* and *h-ns* genes expression profile before and after viral challenge. **(A)** RT-PCR using RNA extracted from virus-infected CFBP2957 or mock culture after DNAseI treatment **(B)** Negative control of PCR reaction using RNA after DNAseI treatment. **(C)** Positive control of PCR reaction using genomic DNA from CFBP2957 strain. The primers used in these reactions are listed in the Supplemental Table S3.

Supplemental Figure S1. Topological comparison between phylogenetic trees Cas1 (flexive genome) and Egl (core genome) proteins. For the phylogeny amino acid sequences of proteins Cas1 or Egl of thirteen *Ralstonia* spp. isolates, listed in Supplemental Table S2, were used. The

maximum likelihood trees were constructed in the MEGA 7.0 program using Jones-Taylor-Thornton (JTT) evolutionary model. Bootstrap values are indicated as percentage points; values below 50% are not shown.

Table 1: Presence of CRISPR loci in *Ralstonia* spp. chromosome and description of isolates used in this study.

Isolates	CRISPR loci	*Cas* genes	Subtype	Phylotype	Host	Origin	Genome sequence data	Reference	Accsession number
Ralstonia solanacearum									
UY031	No	No	-	IIB	Potato	Uruguay	complete genome	Guarischi-Souza, et al. 2016	PRJNA278086
Po82	2	Yes	I-E	IIB	Potato	Mexico	complete genome	Xu et al. 2011	PRJNA66837
UW179	2	Yes	I-E	IIB	Banana	N/D[a]	draft	Ailloud et al. 2015	PRJEB7432
Molk2	No	No	_	IIB	Banana	Philippinnes	complete genome	Guidot et al. 2009	PRJNA32085
IBSBF1503	2	Yes	I-E	IIB	Cucumber	Brazil	complete genome	Ailloud et al.2015	PRJEB7433
K60T	2	Yes	I-E	IIA	Tomato	United states	complete genome	Remenant et al.2012	PRJEB8309
IPO1609	No	No	_	IIB	Potato	Netherlands	complete genome	Guidot et al. 2009	PRJNA32087
IBSBF1900	3	Yes	I-E	IIA	Banana	Brazil	complete genome	Cellier et al. 2015	PRJEB8309
CFBP7014	1	Yes	II-C	IIB	*Anthurium andreanum*	Trinindad	complete genome	Cellier et al. 2015	PRJEB8309
CIP417	No	No	_	IIB	Banana	Philippines	complete genome	Ailloud et al.2015	PRJEB7427
UW551	No	No	_	IIB	Geranium	Kenya	draft	Gabriel et al. 2006	PRJNA275889
UW163	2	Yes	I-E	IIB	Plantain	Peru	complete genome	Ailloud et al.2015	PRJNA297400
UW181	No	No	_	IIA	Plantain	Venezuela	complete genome	Cellier et al. 2015	PRJEB8309
P673	No	No	_	IIB	*Epipremnum aureum*	Costa Rica	complete genome	Bocsanczy et al. 2014	PRJNA230111
NCPPB909	No	No	_	IIB	Potato	Egypt	draft	Yuan et al.2015	PRJNA241341
CFBP1416	No	No	_	IIB	Plantain	Costa Rica	complete genome	Ailloud et al.2015	PRJEB7434
CFBP6783	2	Yes	I-E	IIB	*Heliconia caribea*	French west islands	complete genome	Ailloud et al.2015	PRJEB7432
B50	2	Yes	I-E	IIA	Banana	Peru	complete genome	Ailloud et al.2015	PRJEB7421
Grenada91	No	No	_	IIA	Banana	Grenada	complete genome	Ailloud et al.2015	PRJEB7428
CIP120	2	Yes	I-E	IIA	Potato	Peru	complete genome	Bocsanczy et al. 2014	NZ_JXAY00000000
RS2	No	No	_	IIB	Potato	Bolivia	draft	Zou et al. 2016	PRJNA261373
NCPPB282	No	No	_	IIB	Potato	Colombia	complete genome	University of Exeter	PRJNA259636
POPS2	No	No	_	IIB	Potato	China	draft	Clarke et al.2015	PRJNA259638

23-10BR	1	Yes	II-C	IIB	Potato	Brazil	complete genome	Clarke, R et al.2015	PRJNA259632
CFIA906	No	No	_	IIB	Potato	India	draft	Yuan, K et al.2015	PRJNA241399
R. pseudosolanacearum									
UW757	No	No	_No	I	Tomato	Guatemala	complete genome	Weibel et al. 2016	PRJNA286126
CFBP2957	2	Yes	I-E	IIA	Tomato	French west islands	complete genome	Remenant et al. 2010	PRJEA50685
CMR15	No	No	_No	III	Tomato	Cameroon	complete genome	Remenant et al. 2010	PRJEA50681
FQY_4	No	No	_No	I	Bacterial wilt nursery	China	complete genome	Cao et al. 2013	PRJNA182081
GMI1000	No	No	_No	I	Tomato	French Guyane	complete genome	Salanoubat et al. 2002	PRJNA148633
Rs09161	2	Yes	I-E	I	Eggplant	India	complete genome	Ramesh et al. 2014	PRJNA217471
Rs10244	No	No	_	I	Chili pepper	India	complete genome	Ramesh et al. 2014	PRJNA236788
FJAT-1458	No	No	_	I	Healthy tomato	China	draft	Zheng et al	PRJNA80749
Y45	No	No	_	I	Tobacco	China	complete genome	Li et al. 2011	PRJNA71179
CFBP3059	2	YES	I-E	III	Eggplant	Burkina Faso	draft	Guinard et al. 2016	PRJEB11298
SD54	No	No	_	I	Ginger	China	complete genome	Shan et al .2013	PRJNA220227
FJAT-91	No	No	_	I	Tomato	China	draft	Zou et al. 2016	PRJNA80751
Rs-T02	No	No	_	I	Tomato	China	draft	Zou et al. 2016	PRJNA298373
CFBP7058	No	No	_	I	*Solanum nigrum*	Cameroon	draft	Guinard et al. 2016	LN899820.1
PSS4	No	No	_	I	Tomato	Taiwan	draft	Guinard et al. 2016	PRJEB11298
RD13.01	No	No	_	I	Eggplant	Reunion Island	draft	Guinard et al. 2016	LN899822.1
CIV23	No	No	_	I	Eggplant	Ivory Coast	draft	Guinard et al. 2016	LN899823
CIR011-208	No	No	_	I	Eggplant	French Guyana	draft	Guinard et al. 2016	LN899824
TD13.01	No	No	_	I	Eggplant	Reunion Island	draft	Guinard et al. 2016	PRJEB11298
TF31.08	No	No	_	I	Eggplant	Reunion Island	draft	Guinard et al. 2016	PRJEB11298
TO10	No	No	_	I	Tomato	Thailand	draft	Guinardet al. 2016	PRJEB11298
YC45	No	No	_	I	*Rhizoma kaempferiae*	China	complete genome	She et al. 2015	PRJNA286156
R. syzygii									
PSI07 (*subsp. indonesiensis)*	No	No	_	IV	Tomato	Indonesia	complete genome	Remenant et al. 2010	PRJEA50683
R229 (*subsp. celebesensis)*	2	Yes	I-E	IV	Banana and Plantain	Indonesia	complete genome	Remenant et al. 2011	PRJNA53877

R24 (*subsp. syzygii)*	1	Yes	II-C	IV	Clove tree	Indonesia	complete genome	Remenant, B et al.2011	PRJNA53879
Non-plant pathogenic *Ralstonia* species									
R. mannitolilytica	No	No	_	_		_	complete genome	Suzuki, M et al. 2015	PRJDB3529
R. eutropha	No	No	_	_		_	draft	Pohlmann, A et al. 2006	AM260479 (Chr1); AM260480 (Chr2)
R. pickettii	No	No	_	_		_	complete genome	Ohtsubo, Y et al.2013	CP006667 (Chr1); CP006668 (Chr2); CP006669 (Chr3)

[a]Non determined

Table 2. Cas proteins comparison among *Ralstonia* spp. isolates that contain subtype I-E CRISPR-Cas system

Isolates	% Identity of Subtype I-E CRISPR-Cas Proteins to CFBP2957							
	Cas3	Cse1	Cse2	Cas7	Cas5	Cas6	Cas1	Cas2
Ralstonia solanacearum								
IBSBF1503 (II)[a]	95	93	93	96	96	97	99	100
IBSBF1900 (II)	97	96	92	97	100	98	100	98
B50 (II)	97	96	92	97	100	98	100	98
CIP120 (II)	99	97	94	97	100	100	100	98
UW163 (II)	94	93	93	96	96	97	99	100
UW179 (II)	94	93	93	96	96	97	99	100
Po82 (II)	94	93	93	96	96	97	99	100
$K60^T$ (II)	96	95	92	96	97	95	100	98
CFBP6783 (II)	95	93	93	96	96	97	99	100
R. pseudosolanacearum								
CFBP3059 (III)	88	92	88	94	94	91	97	96
Rs09161 (I)	88	90	87	94	94	94	96	96
R. syzygii subsp. celebesensis								
R229 (IV)	89	92	88	94	94	93	89	98

[a]Phylotypes

Supplemental Table S1. Cas proteins NCBI accession numbers for thirteen *Ralstonia* spp. isolates that contain subtype I-E CRISPR-Cas system used to calculate the amino acid identity

Isolates	Proteins							
	Cas3	Cas1	Cas2	Cas5	Cas6	Cas7	Cse1	Cse2
Ralstonia solanacearum								
B50 (II)[a]	WP_043945478.1	WP_043908263.1	WP_003272386.1	WP_013205431.1	WP_052477488.1	WP_043945482.1	WP_043945479.1	WP_043945480.1
CFBP2957 (II)	WP_013205427.1	WP_043908263.1	WP_003272386.1	WP_013205431.1	WP_013205432.1	WP_013205430.1	WP_013205428.1	WP_013205429.1
CFBP6783 (II)	WP_042548986.1	WP_042548989.1	WP_014616412.1	WP_014616409.1	WP_014616410.1	WP_014616408.1	WP_042548987.1	WP_014616407.1
IBSBF1503 (II)	WP_042548986.1	WP_042548989.1	WP_014616412.	WP_014616409.1	WP_014616410.1	WP_014616408.1	WP_042548987.1	WP_014616407.1
IBSBF1900 (II)	WP_043945478.1	WP_043908263.1	WP_003272386.1	WP_013205431.1	WP_052477488.1	WP_043945482.1	WP_043945479.1	WP_043945480.1
K60^{T} (II)	CCF98722.1	CCF98728.1	CCF98729.1	CCF98726.1	CCF98727.1	CCF98725.1	CCF98723.1	CCF98724.1
Po82 (II)	WP_014616405.1	WP_042548989.1	WP_014616412.1	WP_014616409.1	WP_014616410.1	WP_014616408.1	WP_042548987.1	WP_014616407.1
UW163 (II)	WP_014616405.1	WP_042548989.1	WP_014616412.1	WP_014616409.1	WP_014616410.1	WP_014616408.1	WP_042548987.1	WP_014616407.1
UW179 (II)	WP_014616405.1	WP_042548989.1	WP_014616412.1	WP_014616409.1	WP_014616410.1	WP_014616408.1	WP_042548987.1	WP_014616407.1
CIP120 (II)	WP_064297492.1	WP_043908263.1	WP_003272386.1	WP_013205431.1	WP_013205432.1	WP_043945482.1	WP_064297493.1	WP_064297494.1
R. pseudosolanacearum								
CFBP3059 (III)	OAI80987.1	OAI81002.1	OAI80993.1	OAI80991.1	WP_013205432.1	OAI80990.1	OAI80988.1	OAI80989.1
Rs09161 (I)	WP_028853491.1	WP_049832837.1	WP_028853484.1	WP_028853487.1	WP_028853486.1	WP_028853488.1	WP_028853490.1	WP_028853489.1
R. syzygii celebesensis								
R229 (IV)	CCA82578.1	CCA82572.1	CCA82572.1	CCA82574.1	CCA82573.1	CCA82575.1	CCA82577.1	CCA82576.1

[a]Phylotypes

Supplemental Table S2. Cas1 proteins NCBI accession numbers for fifty-six taxa that contain distinct CRISPR-Cas subtypes and Egl protein NCBI accession numbers for thirteen *Ralstonia* spp. isolates

Species	Proteins		CRISPR subtype
	Cas1	Egl	
Propionibacterium freudenreichii	CEG98845.1		I-E
Methylomicrobium album BG8	EIC29364.1		I-E
Streptomyces sp.	016470313.1		I-E
Anaerococcus prevotii ACS-065- V-Col13	EGC81789.1		I-E
Saccharomonospora azurea NA-128	EHY91198.1		I-E
Thiorhodovibrio sp. 970	EIC19430.1		I-E
Escherichia coli K12	WP_001514501.1		I-E
Selenomonas sp. oral taxon 137 str. F0430	EFR41044.1		I-C
Selenomonas flueggei ATCC 43531	EEQ48796.1		I-C
Eubacterium siraeum DSM 15702	EDS00209.1		I-C
Bacteroides sp. 3_1_33FAA	EEZ20543.1		I-C
Paenibacillus sp. oral taxon 786 str. D14	EES74004.1		I-C
Bifidobacterium breve ACS-071- V-Sch8b	AEF27489.1		I-C
Filifactor alocis ATCC 35896	EFE27914.1		I-C
Streptococcus parasanguinis ATCC 15912	AEH55431.1		I-C
Clostridium sp. HGF2	EFR37095.1		I-C
Acidovorax avenae ATCC 19860	ADX47764.1		I-C
Xanthomonas citri subsp. *citri* A306	AJD70426.1		I-C
Neisseria sp. oral taxon 014 str. F0314	EFI23590.1		I-F
Escherichia coli D9	EGJ07919.1		I-F
Acinetobacter baumannii ATCC 19606	EEX04033.1		I-F
Oxalobacter formigenes OXCC13	EEO29472.1		I-F
Escherichia coli IHE3034	ADE88357.1		I-F
Enterobacter cancerogenus ATCC 35316	EFC58005.1		I-F
Yersinia pestis KIM D27	EFA49547.1		I-F
Halomonas titanicae BH1	ELY20639.1		I-F
Aggregatibacter actinomycetemcomitans	WP_005594204.1		I-F
Pasteurella dagmatis ATCC 43325	EEX50297.1		I-F

Anaerococcus vaginalis ATCC 51170	EEU11828.1		I-B
Calditerrivibrio nitroreducens DSM 19672	ADR19822.1		I-B
Mucilaginibacter paludis DSM 18603	EHQ30582.1		I-B
Caldithrix abyssi DSM 13497	EHO40940.1		I-B
Marinithermus hydrothermalis DSM 14884	AEB12795.1		I-B
Odoribacter splanchnicus DSM 20712	ADY32335.1		I-B
Prevotella multisaccharivorax DSM 17128	EGN57515.1		I-B
Haliscomenobacter hydrossis DSM 1100	AEE48389.1		I-B
Syntrophothermus lipocalidus DSM 12680	ADI02107.1		I-B
Hydrogenobaculum sp. 3684	AEF19554.1		I-B
Aeropyrum pernix	WP_010866253.1		I-A
Aeropyrum camini	WP_022541529.1		I-A
Ignisphaera aggregans	WP_013303388.1		I-A
Vulcanisaeta distributa	WP_013336798.1		I-A
Hyperthermus butylicus	WP_011821827.1		I-A
Ralstonia pseudosolanacearum CFBP3059	WP_064048809.1	OAI79578.1	I-E
R. pseudosolanacearum Rs09161	WP_049832837.1	WP_028852736.1	I-E
R. solanacearum UW163	WP_042548989.1	WP_014617644.1	I-E
R. solanacearum UW179	WP_042548989.1	WP_014617644.1	I-E
R. solanacearum IBSBF1503	WP_042548989.1	WP_014617644.1	I-E
R. solanacearum IBSBF1900	WP_043908263.1	WP_043945195.1	I-E
R. solanacearum CFBP6783	WP_042548989.1	WP_014617644.1	I-E
R. solanacearum B50	WP_043908263.1	WP_043945195.1	I-E
R. solanacearum CIP120	WP_043908263.1	WP_042590418.1	I-E
R. solanacearum CFBP2957	CBJ42362.1	WP_042590418.1	I-E
R. solanacearum K60^{T}	WP_43892480.1	CCF96805.1	I-E
R. solanacearum Po82	WP_042548989.1	014617644.1	I-E
R. syzygii subsp. *celebesensis* BDB 229	CCA82572.1	CCA82743.1	I-E

Supplemental Table S3. Primers used in this study

Target	Primer name	Sequence 5' - 3'	Amplicon size
Isolate K60^T			
CRISPR1	CR1KF	AAG GTG GGG TAC TTC GTC GAC TGC	304 bp
	CR1KR	ATG CGA GAG GCT CCA TTG ATT TGG	
CRISPR2	CR2KF	GAA TTC CAG ACA CTG GGC GCC AAC	341 bp
	CR2KR	TAC ACC TGG GCG TCG AAG TAA TAC	
Isolate CFBP2957			
CRISPR1	CR1CF	CTT CGA GAC CGA TAT CCA CGG	300 bp
	CR1CR	CAA CGA CGG AAT GAT GCG TGC C	
CRISPR2	CR2CF	TAC GAA TTC CAG ACA CTG GGC	430 bp
	CR2CR	TCG TTA CCA TAC GAG GCA GAT GG	
CRISPR1	CR1F	GGA TCA AGG GTT TGC GGT AAG	429 bp
	CR1R	TTG TTG ATG CAG CGG TTC ATC C	
CRISPR2	CR2_1F	TGA CAA CGC GAA TAA TCG GTA CG	1013bp
	CR2_1R	GAA GAC GCT CAG TGT GCT CTA CG	
	CR2_2F	CTG AGG CGA GTG ACC CAC GTAC	1191 bp
	CR2_2R	ACG ACC GCG CTG GTG AAG AAG C	
	CR2_3F	TGC TGG ATC TGA TGG ATA GTG C	942 bp
	CR2_3R	GCC AGC TTG ATC GGT TGA TCG	
	CR2_4F	TGA TTG GCA GCA AGA GCG AG	919 bp

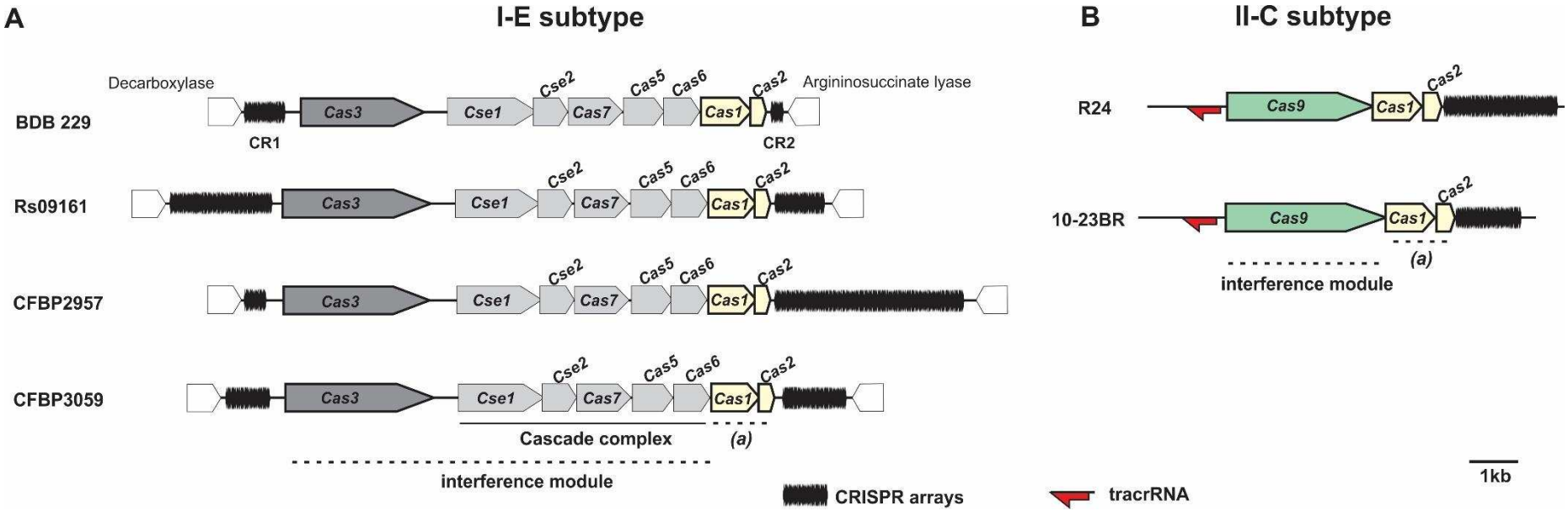
	CR2_4R	TCA AGC TGG CAC TAG TGT GTA GC	
Protospacer 36	Sp36***Eco*RI**F2	**GAATTCGAATTCC**GACGAGGCGTTCATCGTTCGGGCGCTGGCCTGCTTTGAC	52 bp
	Sp36***Xba*I**R2	**TCTAGATCTAGA**CGGTGTTCGTCAAAGCAGGCCAGCGCCCGAACGATGAACG	
Protospacer 49	Sp49***Eco*RI**F2	**GAATTCGAATTCC**GGCGAGGCCTACCTGGTCGGCCTAGCCGTGACCGACAAC	52 bp
	Sp49***Xba*I**R2	**TCTAGATCTAGAC**CGGCAAGGTTGTCGGTCACGGCTAGGCCGACCAGGTAGG	
pUFJ10 MCS	T7 foward	TAATACGACTCACTATAGGG	420 bp
	M13 reverse	CAGGAAACAGCTATGAC	
Cas3	Cas32F	CATGCATTGGATGTCGCAGCTTGC	618 bp
	Cas32R	AGACAAGTCTTGCGG ATGCACGC	504 bp
Cse2	Cse2F	AGTGGGTACGAAGCTGGTGGCAGG	
	Cse2R	CGTGGCTCGGTTTGACGGTGGTATG	420 bp
Cas7	Cas7F	CAACTGCATGCACTGGTGTCCTATC	
	Cas7R	CAGCGCGTTCAACTCCTCTTCCG	
Cas6	Cas6F	ACTACTTCTCCAGGGTGCGGCTG	490 bp
	Cas6R	TAG CCG CTG GTC AGG ACG CAT TCG	
Cas1-Cas2	OPcasF75’	ACA TTG CCG ATC TGT TCA AGT TTG	385 bp
	OPcasF63’	AAC CAG ATG GCG AGC CGT C	
Cas2	Cas2F	TCG TGG TCG TCA CCG AAG ATG TCC	265 bp
	Cas2R	TGA AAG GCC ACG AGC TGC AGT CC	
h-ns1	H-NSMF2	ACA AGT CCG TCA AAC CGT GGC CG	181 bp

	H-NSMR2	GTC ACG GTT CTT GCC GAT CCA GG	
h-ns2	H-NSLF	GTT CAG AAG ATC TCT GAA CTG CAG C	275 bp
	H-NSLR	AAA TCT TCC ATT TTC TTG CCG G	
h-ns3	H-NSSF	CTG CTT GCC CAG AAG GCA AAG CTC G	212 bp
	H-NSSR	GTC TTC GGA TCG CGG TAC TTG GG	

Supplemental Table S4. Cas proteins comparison among *Ralstonia* spp. isolates that contain subtype II-C CRISPR-Cas system

Isolates	**% Identity of subtype II-C CRISPR-Cas Proteins**		
	Cas9	Cas1	Cas2
Ralstonia solanacearum			
23-10BR	99	100	100
CFBP7014	99	100	100
R. syzygii subsp. syzygii			
R24	94	97	97

Figure 1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Cas5
Cas6
Cas1
Cas2
CR1
CR2
Cascade complex
(a)
interference module
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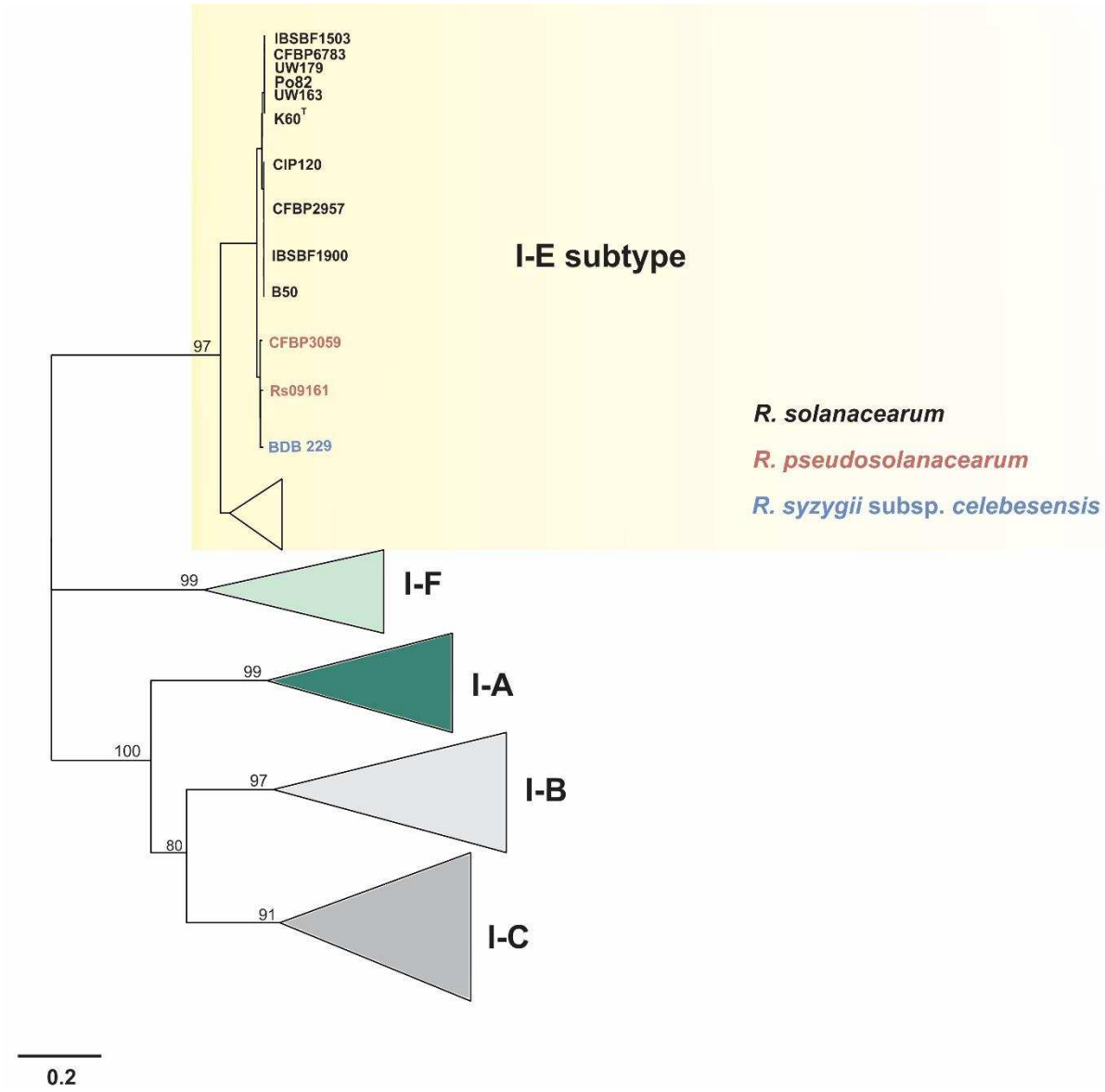
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BDB 229
I-E subtype
R. solanacearum
R. pseudosolanacearum
R. syzygii subsp. celebesensis
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I-A
I-B
I-C
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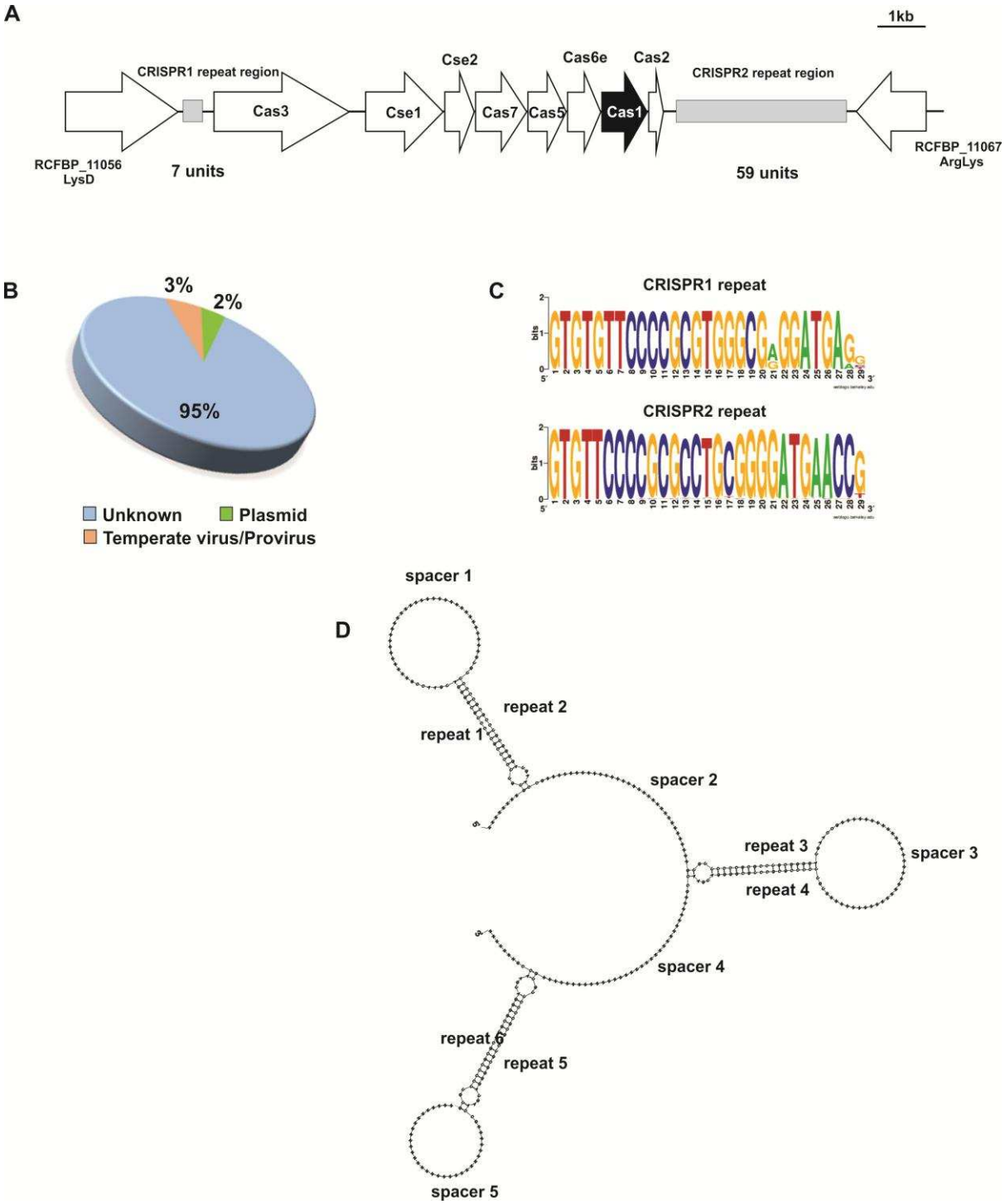
Figure 3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Cse1
Cse2
Cas7
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Cas2
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LysD
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59 units
RCFBP_11067
ArgLys
B
3%
2%
95%
Unknown
Plasmid
Temperate virus/Provirus
C
CRISPR1 repeat
CRISPR2 repeat
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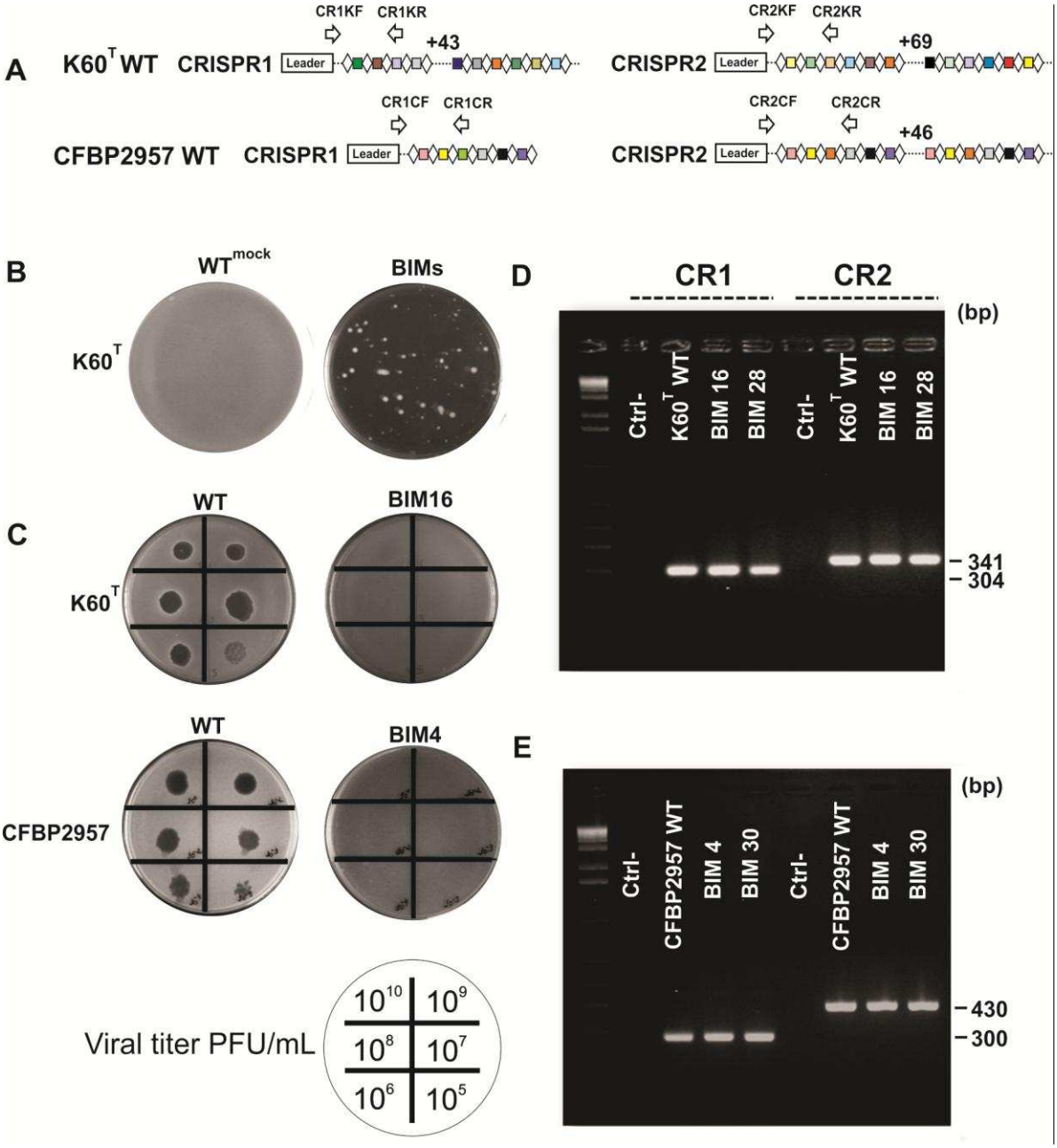
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+69
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CR1CF
CR1CR
CRISPR2
CR2CF
CR2CR
+46
B
WTmock
BIMs
K60T
C
WT
BIM16
K60T
WT
BIM4
CFBP2957
Viral titer PFU/mL
10^10
10^9
10^8
10^7
10^6
10^5
D
CR1
CR2
(bp)
Ctrl-
K60T WT
BIM 16
BIM 28
341
304
E
(bp)
Ctrl-
CFBP2957 WT
BIM 4
BIM 30
430
300

Figure 5

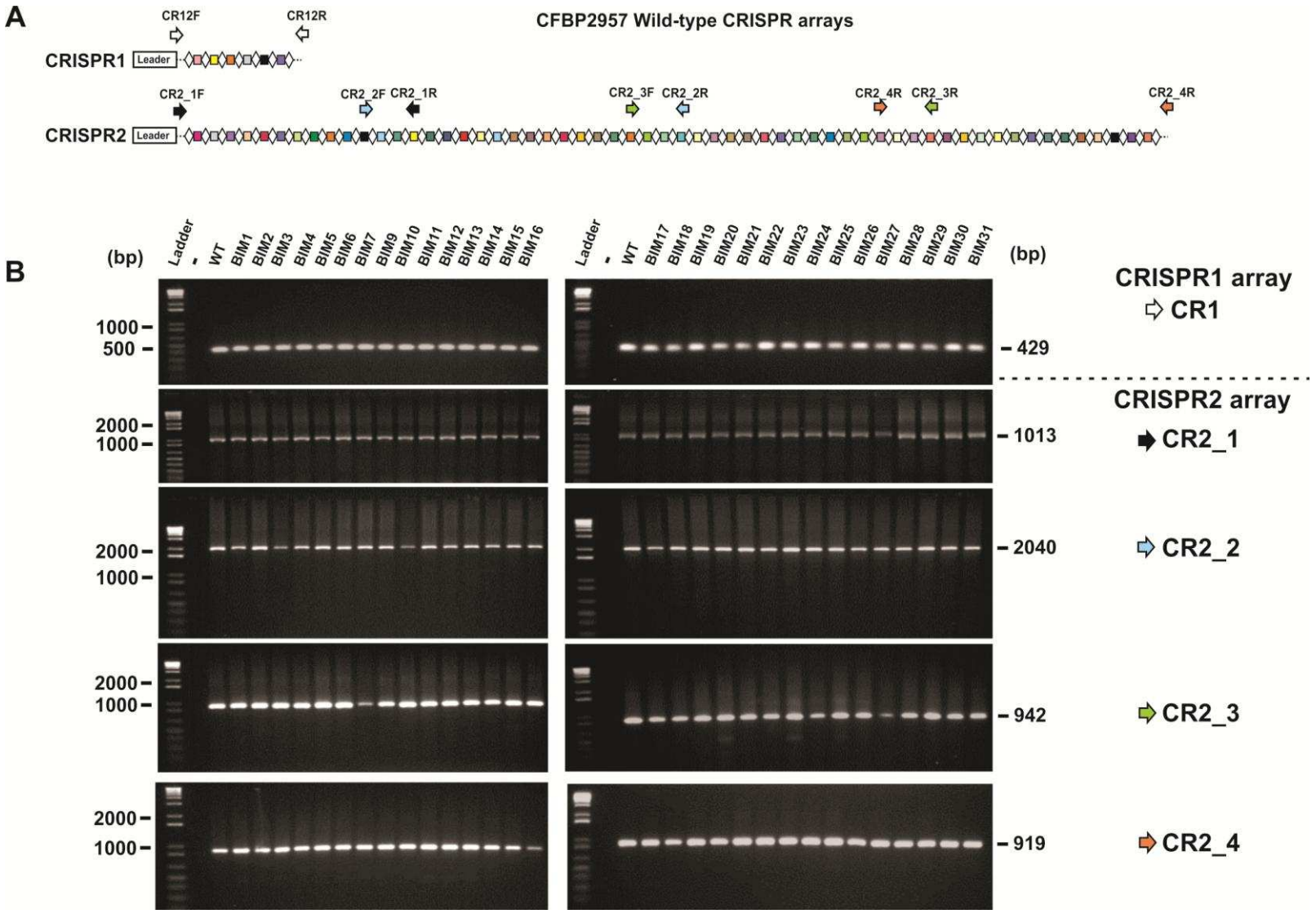

A
CFBP2957 Wild-type CRISPR arrays
CR12F
CR12R
CRISPR1
Leader
CR2_1F
CR2_2F
CR2_1R
CR2_3F
CR2_2R
CR2_4R
CR2_3R
CR2_4R
CRISPR2
Leader
B
(bp)
Ladder
-
WT
BIM1
BIM2
BIM3
BIM4
BIM5
BIM6
BIM7
BIM9
BIM10
BIM11
BIM12
BIM13
BIM14
BIM15
BIM16
Ladder
-
WT
BIM17
BIM18
BIM19
BIM20
BIM21
BIM22
BIM23
BIM24
BIM25
BIM26
BIM27
BIM28
BIM29
BIM30
BIM31
(bp)
1000
500
429
2000
1000
1013
2000
1000
2040
2000
1000
942
2000
1000
919
CRISPR1 array
CR1
CRISPR2 array
CR2_1
CR2_2
CR2_3
CR2_4

Figure 6

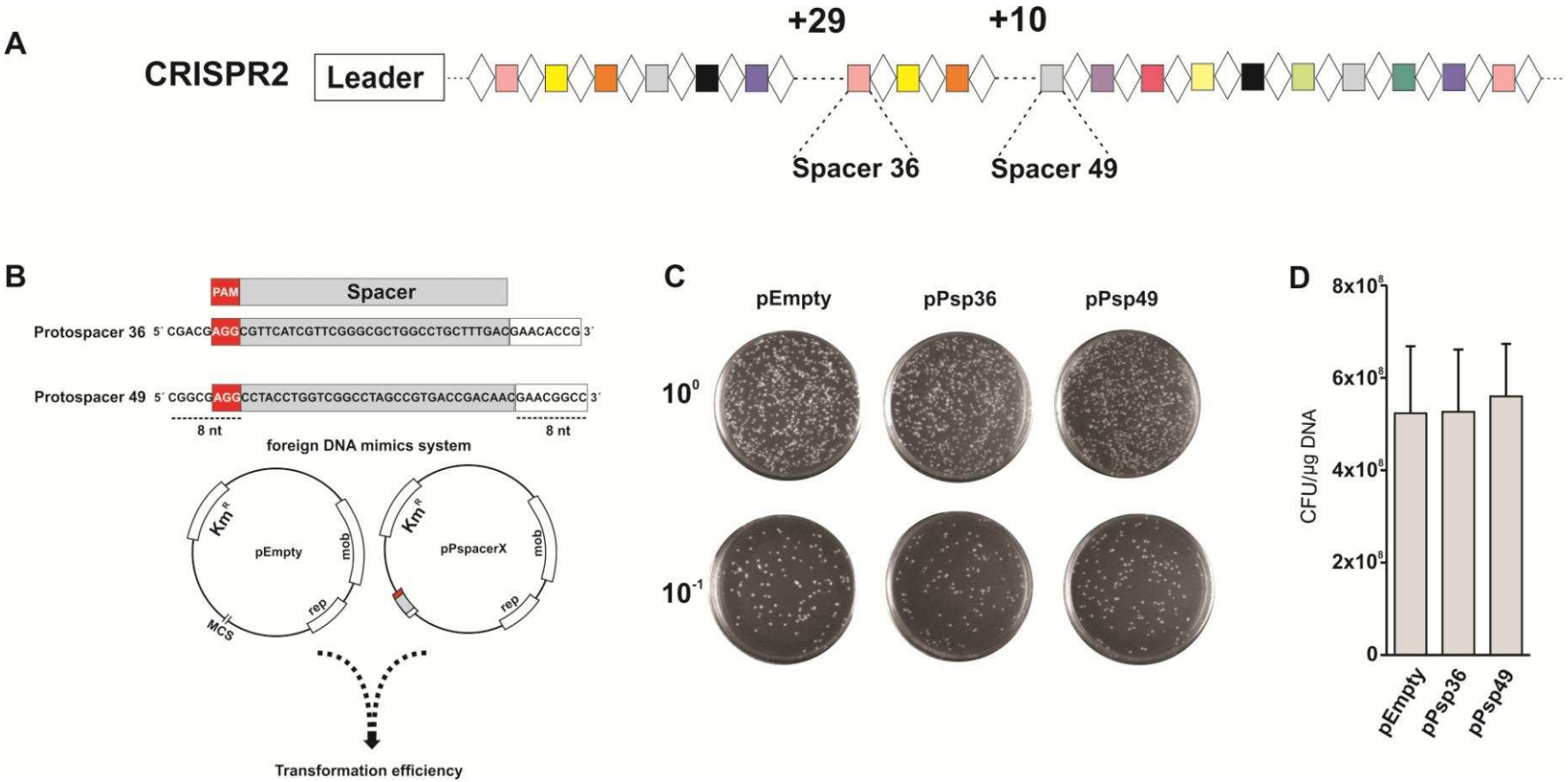
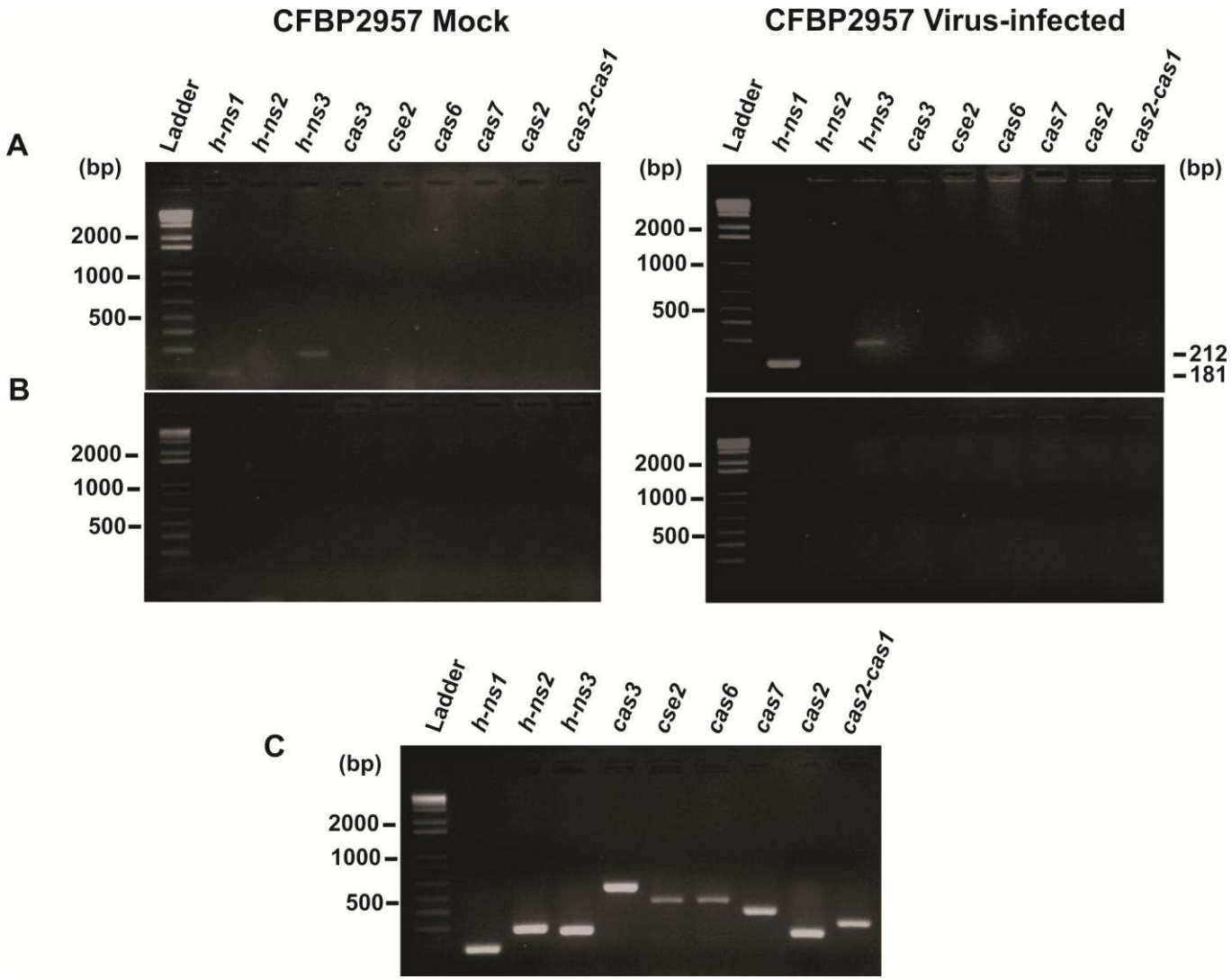
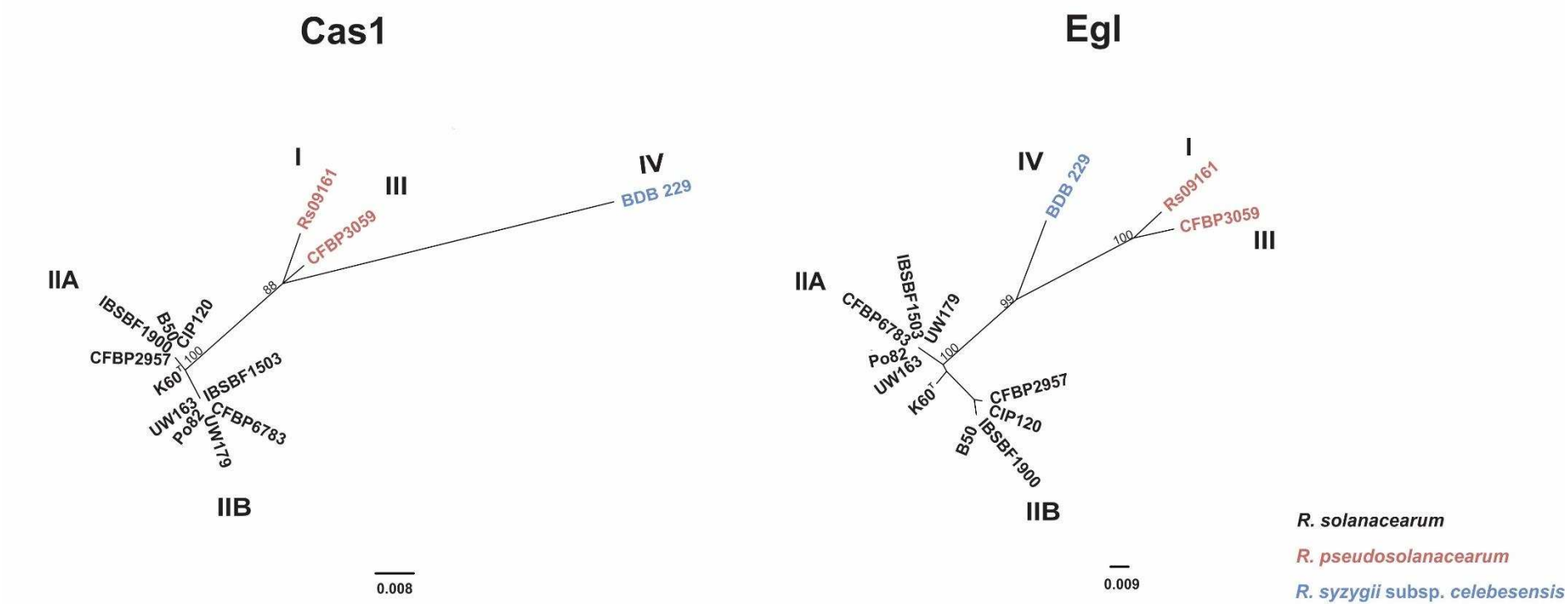

A
CRISPR2
Leader
+29
+10
Spacer 36
Spacer 49
B
PAM
Spacer
Protospacer 36 5´ CGACGAGGCGTTCATCGTTCGGGCGCTGGCCTGCTTTGACGAACACCG 3´
Protospacer 49 5´ CGGCGAGGCCTACCTGGTCGGCCTAGCCGTGACCGACAACGAACGGCC 3´
8 nt
8 nt
foreign DNA mimics system
Km^R
pEmpty
mob
rep
MCS
Km^R
pPspacerX
mob
rep
Transformation efficiency
C
pEmpty
pPsp36
pPsp49
10^0
10^-1
D
8x10^8
6x10^8
4x10^8
2x10^8
0
CFU/µg DNA
pEmpty
pPsp36
pPsp49

Figure 7


CFBP2957 Mock
CFBP2957 Virus-infected
A
B
C
Ladder
h-ns1
h-ns2
h-ns3
cas3
cse2
cas6
cas7
cas2
cas2-cas1
(bp)
2000
1000
500
212
181

Supplemental Figure S1



CHAPTER 3

MAPPING AND FUNCTIONAL ANALYSIS OF NATURAL MUTATIONS THAT PROTECTS *Ralstonia solanacearum* OF VIRAL INFECTION

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Mapping and functional analysis of natural mutations that protects *Ralstonia solanacearum* of viral infection

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Abstract

A wide range of mechanisms are responsible for antiviral resistance in microbial populations, providing adaptive advantages in the presence of the natural enemies with relevant role in the survival and evolution of the species. Besides CRISPR immunity, a variety of defense strategies has proven to be crucial in the combat between bacteria and viruses. Recently, the CRISPR-Cas system was detected an apparently intact state in some *Ralstonia* spp. isolates. In addition, the emergence of BIMs (Bacteriophages Insensitive Mutants) in cultures of the virus-sensitive CFBP2957 isolate post viral infection, raised suspicions about the involvement of the adaptive immunity. However, it was shown that the presence of CRISPRs loci in the CFBP2957 does not explain the resistance in the derived lines. Thus, this study aimed to characterize which immune strategies allow these resistant lines escape from viral infection. Two natural non-CRISPR BIMs (BIM4 and BIM30) derived from the virus-sensitive CFBP2957 Wild-Type (WT), were subjected against Ralstonia virus phiAP1 for resistance characterization. Both exhibit complete resistance and was observed that are permissive to viral adsorption. The survival rate of BIMs was high, which excluded the presence of abortive systems. Sequencing of the genome of BIMs and of the CFBP2957 WT revealed some specific mutations for BIMs (Single Nucleotide Polymorphism (SNPs) and small Insertions and Deletions (INDELs)) that may be responsible for the resistance. Most s SNPs were unique for each BIM, with exception of four of them that were shared. Additionally, few INDELs occurred (Two for BIM4 and one for BIM30), none was shared, but interestingly, two of the three INDELs (one in each BIM) beyond of the shared SNPs occurred in loci that encode membrane proteins including components of the secretion machinery and motility (Type II and Type IV), which is in agreement with a defect in twitching motility observed for the BIMs. These alterations can potentially being blocking the DNA

passage in the interface between adsorption and DNA ejection steps, but still is too early to assign specific mutations to the phenotype of BIMs. To this end, tests are being conducted to map with precision the determinants that control this complete antiviral resistance, as well as access the frequency of these mutations in the population of the host.

Introduction

In natural populations, the virus-mediated selection process in dynamic arms race between viruses and bacteria play a central role in maintaining bacterial diversity (Buckling and Rainey 2002a; Banfield and Young 2009), besides to drive the rapid molecular evolution, as effect of the natural cycles of adaptation and counter adaptation (Paterson et al, 2010; Dy et al, 2014; Van Valen, 1974). In this dynamic scenario, the prevalence of genotypes depends on the balance between adaptive advantages and biological sacrifice of some cognate functions, in a trade-off perspective (Meaden et al., 2015).

In addition to the adaptive immune CRISPR-Cas system (Marraffini, 2015), a large number of host innate mechanisms are relevant to the survival of the bacterial populations, including R-M systems (Tock and Dryden, 2005) abortive infection systems (Abi) (Chopin et al., 2005), the Bacteriophage Exclusion mechanism BREX (Goldfarb et al, 2015), viral adsorption and DNA injection blocking (Nordström and Forsgren, 1974; Lu et al., 1993), DNA interference drived by argonauts (Swarts et al., 2014). The viral resistance phenotype can be attributed to specific mutations that alter the critical host factors during the primary interactions with the pathogen (Leprohon et al., 2015). Prior to the arrival of viruses, resistant variants may exist in natural populations predominantly susceptible to viral infection. Once exposed to viral attack populations show oscillation in the mutants frequency in detriment to parental genotype (Luria and Delbrusk 1943; Abedon, 2012).

Plant pathogenic *Ralstonia* spp. are Gram-negative bacteria and belong to a heterogeneous group (Fegan and Prior, 2005; Safni et al., 2014) with global geographic distribution and unusually wide host range, infecting more than 200 species, including

economically important crops (Denny, 2006; Hayward, 1991; Hayward, 2000). These species have been targeted in some virotherapy approaches, often showing antiviral resistance in the population (Yamada, 2012, Fujiwara et al., 2011; Yamada et al., 2007), which apparently uses different strategies to escape of the infection (Kawasaki et al., 2016; Xavier et al., 2016 (Chapter 1)). Although some *Ralstonia* spp. isolates carry canonical CRISPR loci, it has been shown that for *R. solanacearum* this system is an immunocompromised unit in its native state, but despite this, BIMs (Bacteriophage Insensitive Mutants) arise with high frequency (Xavier et al., 2016 (Chapter 2)), which supports the existence of other evasion strategies. Although widely distributed and considered key factor in antiviral defense for various bacteria species, the CRISPR-Cas system in its native conditions seems to have lost its competence as an immune system in some hosts (Kiro et al., 2013; Medina-Aparicio et al., 2011; Westra et al., 2010; Pul et al., 2010; Guo et al., 2011) in some cases, interestingly acquired unrelated functions the host protection against DNA foreign (Westra et al, 2014; Zegans et al, 2009).

In order to determine the molecular basis of antiviral resistance in BIMs of *R. solanacearum*, we characterized of BIM4 and BIM30 that are virus-resistant derivative from parental virus-sensitive CFBP2957. In addition, we attempt to assess the costs for maintenance of this phenotype in variants of these phytopathogenic bacteria.

Results

In order to expand the knowledge about the natural strategies of defense against viruses in *R. solanacearum*, we investigated two BIMs (Bacteriophage Insensitive Mutants) (Supplemental Figure 1A), BIM4 and BIM30, which are extremely resistant to infection by Ralstonia virus phiAP1, because there were no detectable plaques when the high viral titer suspension was spotted onto the lawn of the BIM30 or BIM4 (Supplemental Figure 1B). In contrast, when the same high viral titer suspension was spotted onto the lawn of CFBP2957 WT resulted in the confluent lysis zone (Supplemental Figure 1B). These BIMs were obtained from previous research that indicated not be the CRISPR-Cas system the responsible for the resistance phenotype of these variants (Xavier et al., 2016, Chapter 2)).

Apparently both BIMs displayed normal morphology compared to parental CFBP2957 wild type (WT), including the abundant production of exopolysaccharide in rich media (Figure 1A). Despite the similarities with the WT phenotype, we found that both BIMs are defective in twitching motility (Figure 1B). BIMs were permissive to adsorption, showing only a slight reduction in the relative values compared to WT (Figure 1C) however the adsorption levels still remain substantially high, suggesting that additional mechanisms to adsorption blocked explain the resistance. In addition, the high percentage of survival (Figure 1D), excluded any altruistic behavior of Abi infection system (Dy et al., 2014b).

Through the phiAP1 DNA replication assay in both BIMs and CFBP2957 WT it was possible to monitor the viral DNA accumulation during infection. In CFBP2957 WT only at 45 minutes post inoculation it was possible to detect viral DNA (Figure 1E). Although with the method employed is not possible to trace the exact replication kinetics, it was useful, indicating

that different from the WT, the viral replication in BIMs does not occur or at least is defective. Thus, the resistance phenotype in both BIMs must be connected to a block in some step of the infection cycle beyond the viral adsorption step, possibly some interference in the DNA injection or the own viral replication, if the nucleic acid is injected competently.

To assist our investigations, the whole genome sequencing of BIMs and of the CFBP2957 WT was performed and identified 27 and 18 mutations in BIM4 and BIM30 respectively, including SNPs and INDELs, all absent in parental CFBP2957 WT (data not showed). For BIM 4, only two were INDELs, and the remaining were SNPs, with 4 being shared with BIM30 (Table 1) and the remaining 21 were exclusive to this lineage (Supplemental table S1). For the BIM 30, only one was INDEL and the remaining SNPs, with 4 being shared with BIM4 (Table 1) and the remaining 13 were exclusive (Supplemental Table S2). Every mutation (SNPs) shared between the BIMs occurred within coding sequences, in three genes encoding membrane proteins, although, only one of them was non-synonymous, which occurred in the gene encoding a putative hemagglutinin-related protein (L467S), a putative adhesin (Relman et al., 1989) (Table 1). The exclusive SNPs to each BIM have not yet been analyzed (Supplemental Table S1 and S2). Interestingly one of INDELs of BIM4 beyond the single INDEL of the BIM30 also occurred in membrane proteins, here associated with the machineries of secretion and motility GspL protein Type II secretion system (T2SS) and prepilin peptidade type IV pili (*pilD*), in both gene the effect of the mutation caused one frame shift at the beginning and in the central portion of the ORF, respectively (Table 1).

Discussion

In bacterial populations the resistance virus infection is gained through alteration or loss of host cellular factors, such as viral-binding sites located on the cell surface (Meaden et al, 2015; Labrie et al 2010; Koskella and Brockhurst, 2014) can result in dramatic changes in the host lifestyle when these alterations occur in components of metabolically important pathways, therefore leads to the prediction que resistance to viruses can be intrinsically costly (Meaden et al., 2015).

Here, the sequencing of the genome of BIMs (Bacteriophage Insensitive Mutants) of *R. solanacearum* revealed that alterations in genes that encoding cellular components of secretion machinery such as *GspL* Type II secretion system (T2SS) or *pilD* Type IV pili (Tfp) may be controlling the resistance in these variants (Table 1), consistent with the phenotype observed of the twitching-deficiency (Figure 1B). In bacteria the Tfp and T2SS systems facilitate the extracellular localization of proteins by Gram-negative bacteria and may be related since they share a number of protein homologs that are predicted, or have been demonstrated, to locate and function in a similar manner (Nunn, 1999; Sandkvist, 2001).

We observed that despite the complete resistance in the mutants, the viral adsorption occurred, indicating blocking any step between this initial interaction and cytoplasmic access, since that viral DNA replication was not detected in the BIMs (Figure 1E). Classical viral receptors found in Gram-negative bacteria for *Myoviridae* and *Podoviridae*, including *Ralstonia* viruses coincide with LPS fractions on the cell surface (Fujiwara et al, 2008; Yamada et al, 2010; Kawasaki et al., 2009, Rossmann et al., 2004; Wandersman and Schwartz, 1978; Filippov et al., 2011). Among the candidate mutations for the control of resistance in BIM4 and BIM30, we

were not able to find any that occurred in LPS biosynthesis components, supporting the observation of the competent adsorption (Figure 1C). In addition, to the primary contact, additional interactions should occur for a competent establishment of the virus, until it can inject the nucleic acid within the cell. Recently, a viral susceptibility factor that composes the Tfp machinery (*pilQ*), was characterized in *R. solanacearum* and supports this argument (Narulita et al., 2016). The authors showed the participation of pilQ in steps downstream of adsorption, which indicated it be a key factor in the infectivity of different *Ralstonia* viruses, including the *Autographivirinae* RSB1-types (Kawasaki et al, 2016)). The suggested mechanism for the involvement of channel pilQ is that it can mediate viral DNA transport, as a 'molecular syringae' across the outer membrane (OM) and periplasmic space, as occurs during the transforming DNA in *Neisseria meningitides*, where DNA is introduced into the cell through of the OM channel formed by PilQ complex in a non-specific manner (Assalkhou et al., 2007).

The positive adsorption without injection of viral DNA into the cells is a phenotype that can be found between variants of *Ralstonia* spp. resistant to viruses (Kawasaki et al., 2016). Thus, we hypothesize that the mutations that potentially altered the membrane proteins, including *GspL* and *PilD* committed the phiAP1 infectivity, through blocking of the translocation of nucleic acid, corroborating the previous hypotheses raised to explain similar phenotypes in *Ralstonia*-viruses interaction (Kawasaki et al., 2016; Hong et al., 2014; Narulita et al., 2016). Additional observations support our arguments, because mutations in the prepilin peptidase *pilD* or in *GspL* genes can be affect not only type IV piliation but also abolish the protein secretion process in many bacteria (Filloux, 2004).

Among its cognate functions, the T2SS in *Ralstonia* spp. secretes enzymes that degrade the plant cell through the wall, including pectinolytic (Tans-Kersten et al., 2001) and cellulolytic

enzymes (Liu et al., 2005). The Tfp in these species is associated with twitching motility, adherence to surfaces and natural DNA transformation, and variants that carry mutations in components of this complex are markedly less virulent and consequently defective in twitching motility (Kang et al 2002; Liu et al., 2001).

So far, we were not able to estimate the cost of resistance in the *R. solanacearum* BIMs, characterized here. However, considering the role of this membrane platform in the physiology of this pathogen, it is predictable that alterations, although minimal, lead to a scenario of trade-off evolution, common in systems containing variants resistant to viruses (Meaden et al., 2015). In this sense, Hendrick and Sequeira (1984) showed that the phenotype of resistance to virus exhibited by mutants of *Ralstonia* spp., which display various defects in LPS synthesis was accompanied by the loss of virulence, as in *Xanthomonas campestris* pv. *campestris*, where approximately 50% of virus-resistant mutants selected have become avirulent (Randhawa and Civerolo, 1986). Thus, we believe that the BIMs of *R. solanacearum* may be less competitive in plant environments considering a virus-free niche; this reinforces the important role of viruses as active modifiers agents of the evolutionary course of hosts.

Material and Methods

Bacterial isolates, virus and growth conditions

The CFBP2957 WT and its virus-resistant derivative lines (BIM4 and BIM30) were cultured in CPG containing casein (1g/L), peptone (10g/L) and glucose (5g/L) (Horita and Tsuchiya, 2002) at 28°C with shaking at 250 rpm. Ralstonia virus phiAP1, a phikmvvirus recently characterized (Xavier et al., 2016 (Chapter 1)) was propagated on GMI1000 strain as described elsewhere (Yamada et al., 2007) with some modifications (Xavier et al., 2016 (Chapter 1)). Boucher's minimal media (BMM) (Boucher et al., 1985) with glucose was used for motility twitching test.

DNA replication assay

In order to monitored phiAP1 DNA replication in BIMs and CFBP2957 WT we used the method of Hill et al. (1991). Total DNA was isolated from cultures infected at a m.o.i. of 5, digested with *Dra*I, an enzyme that cleaves the phiAP1 genome and allow to distinguish *Ralstonia* infected cultures of uninfected. Then, the fragments were electrophoretically separated on a 0.8% agarose gel stained with EZ-Vision Three (AMRESCO, Solon, OH), and visualized under UV light. The samples were collected every 15 minutes subjected to fast frozen -70°C, in a range of (0-120 minutes).

Adsorption assay

To verify if the BIMs were permissive to viral adsorption, phiAP1 suspension was added (multiplicity of infection, m.o.i = 1) to host cultures with OD_{600} = 0.7 and the mixtures incubated at 30°C for 15 minutes during adsorption. Then, tubes were removed and centrifuged (11,000 × g

for 3 minutes) to sediment the virus-adsorbed bacteria. The supernatants were assayed for unadsorbed phages (double-layer plate titration), and the counts were compared with the titre of the control without cells. The results were express as percentage of the initial phage counts (Marco et al., 2015).

Survival cells

To compare the survival percentage between CFBP2957 WT and the BIMs 4 and 30, the bacterial survival assay was performed according Sekulovic et al. (2015) with some modifications. The bacterial isolates were grown until exponential phase ($OD_{600nm} = 0.2$). Then, 0.9 ml of the culture was taken and mixed with phiAP1 in order to obtain a MOI of 5. Two controls were used, a without virus (mock) and another without bacteria. Both samples were mixed by inversion and incubated for 15 minutes at 30°C, after which aliquots were quickly diluted in triplicate. Then, 0.1 ml of these dilutions was plated on CPG agar and incubated for 48 hours to count the survivors colonies.

Twitching motility test

To compare the twitching motility between CFBP2957 WT and BIMs, bacterial cells ($1{,}5 \times 10^8$ CFU/ml) were spotted (initial spot diameter, 5 mm) on the surface of Boucher minimal medium (BMM) ((Boucher et al., 1985) and then, were air dried prior to the spotting. Petri dishes were then incubated at 28 °C and the diameter of the spots were measured daily for 3 days and the twitching activity was examined by microscopy.

BIMs Whole genome sequencing

Genomic DNA of the BIMs and of the respective parental virus-sensitive CFBP2957 WT was extracted using the protocol previously described (Garneau et al., 2010), with exception of the lysozyme step. Whole genome sequencing was performed using the Nextera XT kit (Illumina, San Diego, CA) as by the manufacturer and the sequencing (2×250 nt) performed on a MiSeq system (Illumina). The CFBP2957 WT genome was assembled with Ray Assembler version 2.3.1 using a k-mer size of 31 and compared to CFBP2957 reference sequence (PRJEA50685). The BIMS reads were aligned on the CFBP2957 reference sequence updated using BWA version 0.7.10. The SNPs and INDELs were found using a pipeline consisting of BAM files manipulation (merging, adding header) with Picard (version 1.123), indexing with Samtools (version 1.1) and SNPs/INDELs searching with GATK (version 3.3-0). The resulting vcf files were compared manually. Genome sequencing, assemblies and comparative analyses were performed at the Plateforme de Séquençage et de Génotypage des Génomes at the CHUL/CHUQ Research Center for sequencing.

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Table 1. List of mutations identified in the genomes of BIM4 and BIM30 shared (SNPs) or exclusives for each BIM (INDELs)

Isolates	Locus	Function	Mutation (nt)	Alteration (aa)
		SNPs		
BIM4 and BIM30	CAD14589.1	Hemagglutinin-related protein [*Ralstonia solanacearum*]	T→C*; C→T	L467S; L481L
	CUV56296 1	Putative transmembrane protein	T→C	L198L
	WP_039559476.1	Membrane protein [*Ralstonia solanacearum*]	T→G	L308L
		INDELs		
BIM4	WP 042548866 1	Histone	T→.	Frame shift
	WP 013204870 1	GspL protein Type II secretion system	GGCA→G	Frame shift
BIM30	WP 043892401 1	Prepilin peptidase Type IV pili (*pilD*)	GCGTT→G	Frame shift

* Nonsynonymous substitution

Supplemental Table S1. Exclusive mutations (SNPs) in BIM4

Locus	Position	Reference genome (CFBP2957 WT)	Mutation (BIM4)	Quality score
contig-2000004	17591	G	C	1466
contig-2000004	17585	C	T	1281
contig-2000004	17565	T	C	1184
contig-2000004	17558	C	G	1131
contig-2000004	17530	A	C	924
contig-5	5555	G	C	716
contig-2000015	7171	A	G	543
contig-4000018	1038	G	A	323
contig-5	5558	C	T	284
contig-2000015	7198	T	C	235
contig-4000018	928	A	G	214
contig-5	5687	T	C	202
contig-4000018	1014	G	A	172
contig-4000018	918	G	A	164
contig-4000018	915	C	T	148
contig-7000011	8466	T	C	146
contig-4000018	917	C	A	144
contig-5	6329	G	C	88
contig-4000018	1080	C	A	72
contig-1000028	31256	C	G	51
contig-8000017	33798	G	T	49

Supplemental Table S2. Exclusive mutations (SNPs) in BIM30.

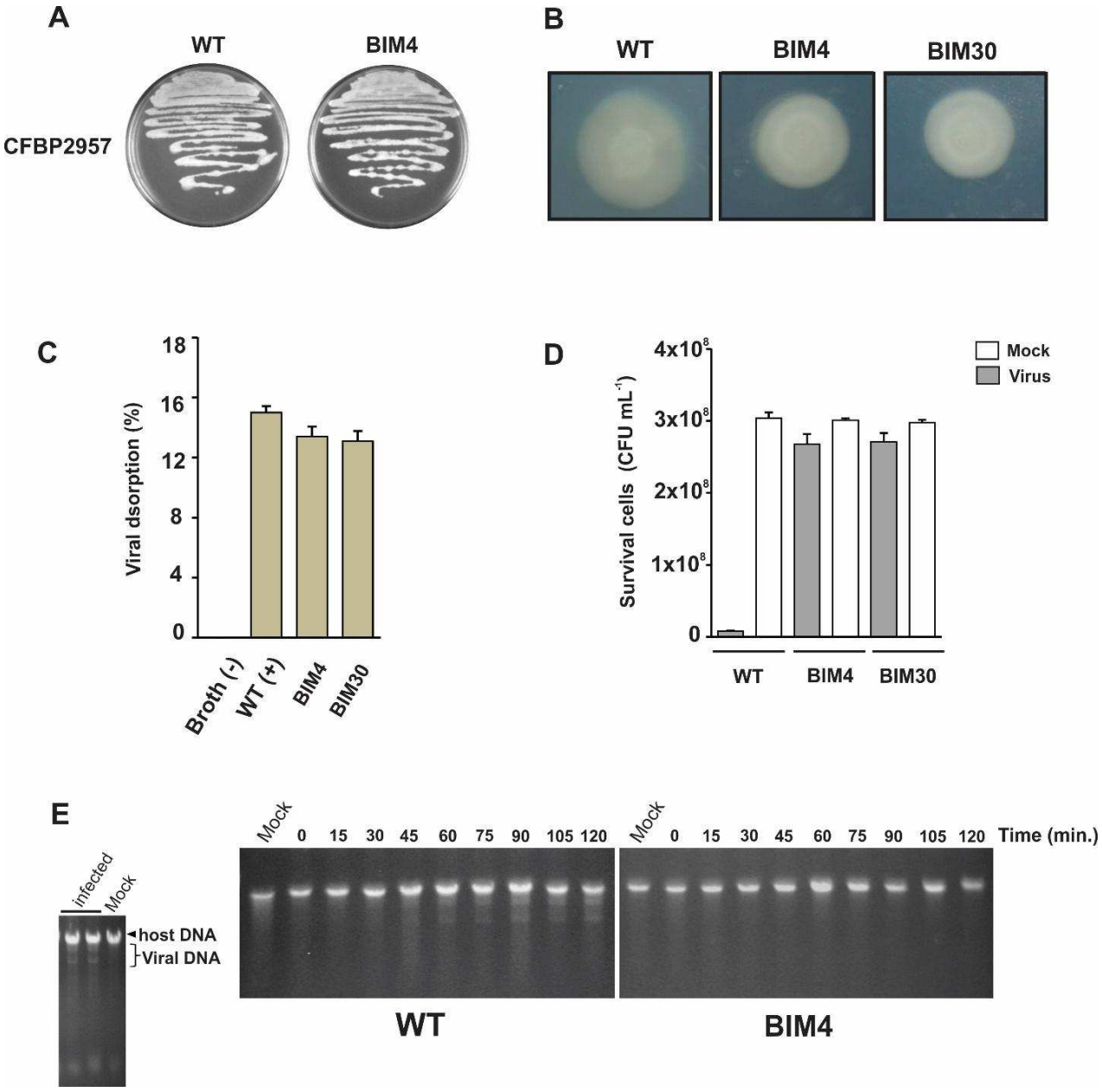
Locus	Position	Reference genome (CFBP2957 WT)	Mutation (BIM30)	Quality score
contig-3000028	54957	G	T	1623
contig-3000028	59342	G	T	1367
contig-1000025	49109	C	A	928
contig-7000011	8526	G	A	312
contig-4000008	143313	C	T	284
contig-2000015	6508	C	T	205
contig-7000011	8880	A	T	168
contig-7000011	8811	C	T	146
contig-5000000	20	T	G	131
contig-7000011	8910	G	C	92
contig-4000018	1755	A	G	85
contig-4000018	1571	A	G	68
contig-7000011	8510	G	T	65

Figure legends

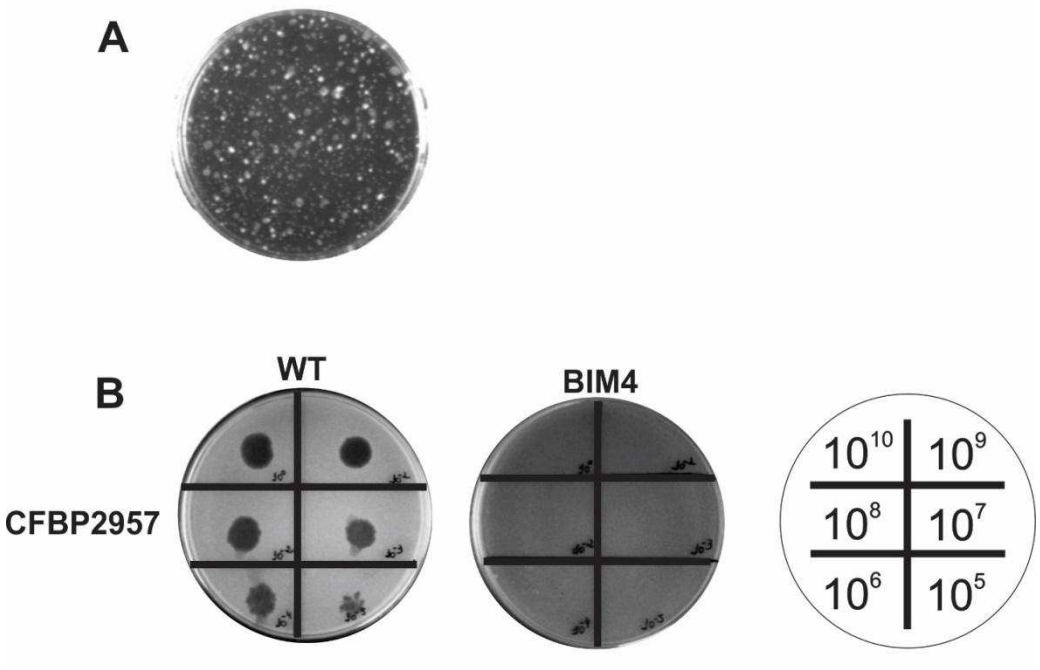
Figure 1. *Ralstonia solanacearum* BIMs are permissive to adsorption, but viral replication cannot be detected. **(A)** EPS production similar to CFBP2957 WT **(B)** Twitching motility in Boucher Minimal Medium. **(C)** Viral adsorption (Ralstonia virus phiAP1). **(D)** Survival cells. **(E)** DNA viral replication kinetics assay.

Supplemental Figure S1. *R. solanacearum* BIMs are extremely resistant to phiAP1 infection. **(A)** BIMs derived of the parental virus-sensitive CFBP2957 WT post phiAP1 infection. **(B)** BIMs exhibits complete resistance in spot test, independent of viral titer.

Figure 1


A
WT
BIM4
CFBP2957
B
WT
BIM4
BIM30
C
Viral dsorption (%)
18
16
12
8
4
0
Broth (-)
WT (+)
BIM4
BIM30
D
Survival cells (CFU mL-1)
4x10^8
3x10^8
2x10^8
1x10^8
0
Mock
Virus
WT
BIM4
BIM30
E
infected
Mock
host DNA
Viral DNA
Mock
0 15 30 45 60 75 90 105 120
Mock
0 15 30 45 60 75 90 105 120
Time (min.)
WT
BIM4

Supplemental Figure S1



CHAPTER 4

BIOLOGICAL CHANGES DURING THE CONVERSION OF THE PHYTOPATHOGENIC *Ralstonia pseudosolanacearum* INTO A COMMENSAL BACTERIA BY AN INOVIRUS

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Biological changes during the conversion of the phytopathogenic *Ralstonia pseudosolanacearum* into a commensal bacteria by an inovirus.

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Abstract

Filamentous bacterial viruses contain a single-stranded DNA genome and compared to other bacterial viruses, have a peculiar lifestyle, because do not cause host cell lysis, but establish a persistent association with hostcells, often causing behavioral changes, with unpredictable effects on bacterial ecology. For years, a rapid loss of virulence has been observed in *Ralstonia* spp. isolates native of Brazil, as has been reported for Asian isolates infected by virus. In an attempt to elucidate which factors are associated with the phenomenon, we investigated one avirulent isolate of *R. solanacearum* (UB-2014), which was originally obtained from eggplant showing wilt symptoms. Pathogenicity tests in tomato and eggplant confirmed the loss of virulence of this strain. To verify if the presence of viruses was related to the phenotype, we performed viral purification and nucleic acids extraction and visualized a fragment with a length of ~ 7.0 Kb. The structural proteome revealed a characteristic profile present in members of the genus *Inovirus,* here associated to the isolate UB-2014 and tentatively named Ralstonia solanacearum Inovirus Brazil 1 (RsIBR1). When RsIBR1 particles were transmitted to GMI1000 VF (virus-free), a aggressive isolate of *R. pseudosolanacearum,* the infected isolate GMI1000 VI (virus- infected) showed abnormal characteristics, such as frequent aggregation, overproduction of a reddish-brown pigment in liquid culture, as well as loss of virulence, similar to those observed with the isolate UB-2014. In addition to these biological alterations, RsIBR1 infection affected the abilityof GMI1000 to inhibit other isolates of *Ralstonia* spp. and altered its susceptibility response during the secondary infection by Ralstonia virus phiAP1, a virus lytic. Here, we report the conversion of the phytopathogenic *R. pseudosolanacearum* into a commensal bacteria, that during its establishment causes only mild reversible phenotypic changes in tomato plants, after which they develop normally, similar to controls. The presence of UB-2014 and GMI1000 VI in

xylem vessels of plants without symptoms after 3 months, confirm that the infected isolates are able to colonize the plant, without causing disease, showing that the viral infection changed the lifestyle of these pathogens. Genomic analyses are underway to determine the full-length genome sequence of RsIBR1 that have significant role in modulating plant-bacteria interaction and microbial adaptation.

Introduction

Bacterial viruses have a significant role in the ecology of microbial communities and the vast majority are typical tailed virus with icosahedral heads (over 95%). The remaining 5% of viruses display a broad range of morphologies, e.g. filamentous, cubic or pleomorphic, with DNA or RNA genomes (Mai-Prochnow et al. 2015). The filamentous bacterial viruses belonging to the family *Inoviridae*, contain single-stranded DNA genomes and among their hosts are some Gram-positive bacteria and a wide range of Gram-negative bacteria, including plant pathogenic *Ralstonia* spp. (Chopin et al. 2002; Yamada, 2013; Mai-Prochnow et al. 2015). When compared to other bacterial viruses, the filamentous have a peculiar lifestyle, because the viral propagation in host is not accompanied by cell death, and mature viral particles are released in manner similar to "extrusion" (Yamada, 2013; Mai-Prochnow et al. 2015). Because inoviruses coexist with their host cells, they frequently can mediate conversion of bacterial phenotypes in various ways (Waldor and Mekalanos 1996; Chopinet al. 2002; Addy et al. 2012; Addy et al. 2012b).

Indirect effects on host physiology have been observed during infection by inoviruses in *Xanthomonas campestris* pv. *oryzae* and *X. campestris* pv. *campestris*, resulting in increased virulence in these two pathovars (Kamiunten and Wakimoto 1981; Tsenget al. 1990). In *Pseudomonas aeruginosa*, viral infection is associated with reduction of colonies size and interference in biofilm development (Webb et al. 2004; Rice et al. 2009). In *Ralstonia* spp.viral infection is associated with modulation of virulence, growth rate and EPS production (Addy et al. 2012; Addy et al. 2012b; Askora et al. 2014). More direct involvement of filamentous virus in host virulence is well characterized in *Vibrio cholerae*, where the pathogenicity of this bacterium depends of some key virulence factor, including the cholera toxin encoded by a gene on the filamentous virus CTXφ genome (Waldor and Mekalanos 1996). It is common to find these

filamentous viruses integrated into host chromosomes, as prophages (Delbrock 1946; Rakonjac 2012; Mai-Prochnow et al. 2015), in a state alternative to episomal propagation (pseudolysogeny).

Plant pathogenic *Ralstonia* spp. are Gram-negative bacteria and comprise a heterogeneous group with three soil-borne species, *R. solanacearum* (phylotypes IIA and IIB) *R. pseudosolanacearum* (phylotypes I and III) and *R. syzygii* subspecies (phylotype IV, including *R. syzygii* R24 and BDB; Blood Disease Bacterium) (Askora et al. 2009; Safni et al. 2014; Prioret al. 2016), that induce rapid and fatal wilting symptoms in host plants. These pathogens have an unusually wide host range, infecting more than 200 species belonging to more than 50 botanical families, including economically important plant species, they are habitants of soil, surviving even in the absence of a host (Hayward 1991; Hayward 2000; Álvarez et al. 2010).

Filamentous bacterial viruses (genus *Inovirus*) appear to be involved in interaction with *Ralstonia* spp., being found with frequency as pseudolysogens or as proviruses in genomes of various bacterial isolates (Yamada, 2013; Askora et al. 2014). Several of them have been well characterized in the Asian continent, including φRSS1, φRSM1, RSM3, RSM4 and PE226 (Yamada et al. 2007; Askora et al. 2009; Murugaiyan et al. 2011). The contrasting effects of these viruses on of these plant pathogens, increasing or reducing bacterial virulence, expose an interesting role for them in the epidemiology of the bacterial wilt in the field.

Here, we have isolated and biologically characterized RsIBR1, an inovirus that naturally infects a avirulent strain of *R. solanacearum* and that is also able to infect *R. pseudosolanacearum*, the two species that occur in America. Additionally, we describe

significant impacts of viral infection on the modulation of parasitic interaction plant-bacteria and microbial adaptation.

Results and Discussion

The identity of the *Ralstonia* spp. isolates was confirmed by PCR (Figure 1A). The isolate UB-2014 of *R. solanacearum* (phylotype II) is an avirulent isolate that shows abnormal characteristics of growth, including less turbidity and frequent aggregation in the liquid culture (Figure 1B). The avirulence phenotype of UB-2014 was confirmed by pathogenicity tests on tomato plants (Figure 1C). Similar phenotypic characteristic in some Asian isolates of *Ralstonia* spp. have been associated with the presence of filamentous viruses (*Inovirus*) (Addy et al. 2012; Addy et al. 2012b; Yamada, 2013). Thus, we hypothesized that a virus could be involved with the unusual characteristics of the isolate UB-2014 of *R. solanacearum*. In an attempt to detect the presence of any virus, Virus-Like Particles (VLPs) from UB-2014 were purified. Nucleic acid extraction of VLPs revealed a DNA band, with a length of ~ 7.0 kb, within expected size for a inovirus genome, as reported for *Ralstonia* spp. isolates (Askora et al. 2014). The VLPs were transmitted to *R. pseudosolanacearum* GMI1000 (Figure 2A). Liquid cultures of GMI1000 VI, as well as UB-2014, showed less turbidity and frequent aggregation (Figure 1B and Figure 2A). The analysis of structural proteins (Figure 2B) and of the nucleic acid present in the VLPs preparation confirmed the transmission of putative inovirus, here tentatively named Ralstonia solanacearum Inovirus Brazil 1 (RsIBR1). Interestingly the overproduction of the reddish-brown melanin-like is accompanied by viral infection because after infection GMI1000 exhibited this property as well as UB-2014, however much less in relation to production in the original host

(Figure 2B).The GMI1000 VI cells were not able to cause disease as observed for the natural host UB-2014 (Figure 2C). Interestingly, GMI1000 VI caused only mild symptoms (Figure 2D), which were reversible in older plants (Figure 2E). Additionally, the presence of UB-2014 and GMI1000 VI in xylem vessels of plants without symptoms after three months highlights the drastic change in the lifestyle of this pathogen. As observed for UB-2014 and GMII1000 VI, *Ralstonia* spp. cells infected by the species of inovirus phiRSS0, phiRSM1 and phiRSM3, also exhibited a considerable virulence decrease or even loss in some cases always accompanied by abnormal cell physiology (Addy et al. 2012).

It has been observed for some *Ralstonia* spp. isolates the overproduction of reddish-brown melanin-like in CPG rich media (González et al. 2007). In *Ralstonia* spp. occurs under very specific conditions in the stationary phase when grown in minimal medium containing tyrosine (Ahmad et al. 2016). The overproduction of reddish-brown melanin-like pigment detected in some isolates in the absence of abiotic stress may be a viral signature, indicating the presence of inoviruses (Yamada et al. 2007; Askora et al. 2014).

The microbial melanins are not essential for growth and survival, but they can provide some advantages to their producers, such as protection to UV radiation (Han, Iakovenko et al. 2015), tolerance to toxic radicals (Fogarty and Tobin 1996), oxidative stress (Rodríguez-Rojas et al. 2009), extreme cold and hot temperatures (Rosas and Casadevall 1997), in addition to protection of pathogenic microorganisms from the host immune response (Zughaieret al. 1999; Chai et al. 2010). Our hypothesis, as already raised by (Ahmad et al. 2016), is that the overproduction of these pigments induced by inovirus must protect UB-2014 and GMI1000 VI of the oxidative challenge from their hosts (Flores-Cruz and Allen, 2009).

Additionally, it was noted that the presence of RsIBR1 in GMI1000 altered the susceptibility response during a secondary infection by Ralstonia virus phiAP1, a lytic virus, making the bacterium more resistant to a second infection (Figure 3A). Cross-protection has been shown during secondary infections by phylogenetically related viruses in *Ralstonia* spp. (Yamada et al. 2007) and *Xanthomonas citri* pv. *citri* (Shieh et al. 1991). In *X. citri* pv. *citri* an important role in host immunity against viral secondary infection has been attributed to a viral repressor encoded by a inovirus (Cheng et al. 2009).

Thus, the presence of any repressor coded by RsIBR1 could explain the partial protection to phiAP1 infection, even though Ralstonia virus phiAP1 is a phikmvvirus. Alternatively, the physiological changes experienced by inovirus infected cells may be interfering indirectly in the interaction between the phikmvvirus phiAP1 and GMI1000 VI.

Recently was showed that the production of the bacteriocins by GMI1000 has a important role in the inhibition of the growth of other isolates of *Ralstonia* spp. and establishment in different habitats (Huerta et al. 2015). Here, the ability of GMI1000 to produce bacteriocins against isolates of two *Ralstonia* spp. was impaired by RsIBR1 infection (Figure 3B), indicatingthat in natural environment this infected isolate should be less competitive. .Thus, we accumulate more evidence about the roleof inoviruses in the modulation of the populations of *Ralstonia* spp. not only in Asia but also America.

Here, we report RsIBR1, an inovirus that have significant impact on the modulation of parasitic interaction plant-bacteria and microbial adaptation, involved in the conversion of the destructive phytopathogenic *R. pseudosolanacearum* in a commensal bacteria during its establishment and colonization in host plants.

Material and Methods

Bacterial isolates and growth conditions

R. pseudosolanacearum GMI1000 was obtained from the culture collection of the College of Agricultural and Life Sciences,University of Wisconsin-Madison, USA (kindly donated by Dr. Caitilyn Allen) and *Ralstonia* sp. UB-2014 strain from the culture collection of Embrapa-CNPH (kindly donated by Dr. Carlos Lopes). The other isolates used in this study belong to different culture collections and are listed in Table 1. The isolates were cultured in CPG medium containing Casamino acids, peptone and glucose (Horita and Tsuchiya, 2002) at 28°C with shaking at 200-300 rpm.

Ralstonia complex PCR and phylotyping

The UB-2014 strain was characterized by two PCR assays based on the hierarchical classification scheme (Opina et al. 1997; Fegan and Prior 2005). Multiplex PCR was performed with primers 759/760 as an internal marker providing the 280-bp reference PCR product which is amplified for plant pathogenic *Ralstonia* spp., besides a set of four primers for phylotyping (Opina et al. 1997).

Viral propagation and purification

To verify if the presence of viruses was related to an avirulence phenotype in the isolate UB-2014, the bacterium was cultivated to induce viral propagation, followed by purification as

proposed by Yamada et al. (2007), with some modifications. Two liters of a UB-2014 culture (optical density at 600 nm of 0.2) was incubated for four days at 28°C in static conditions. Intact bacterial cells were lysed with chloroform, shaked at 100 rpm during 30 minutes at 37ºC, and cell debris was removed by centrifugation at 8 000 *g* for 5 minutes 4ºC. The supernatant was passed through a 0.22 µm membrane followed by precipitation overnight with polyethylene glycol 6000 (5% wt/vol) and 0,5 M NaCl. The pellet was recovered by centrifugation at 15 000 *g* for 30 minutes at 4ºC and suspended in buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM MgSO4 and 0.01% gelatin). Particles were purified by CsCl-gradient ultracentrifugation and dialyzed three times for 30 min against 300 volumes of SM buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$ and 0.01% gelatin).

Nucleic acid isolation and partial characterization

The total nucleic acid was isolated from DNAse-treated purified particles using phenol-chloroform (Sambrook and Russell 2001). To confirm the nature of the genome, the nucleic acids were treated with RNAse A, DNAse I and S1 nuclease according to the manufacturer's recommendation. Genome size was estimated by 0,8% agarose gel electrophoresis

Analysis of virion-associated proteins

The profile of the putative virion-associated proteins was analyzed by SDS-PAGE. Purified particles were suspended in SDS-PAGE loading buffer (Moak and Molineux 2004) and boiled for 5 min before loading onto a 12% (w/v) polyacrylamide gel to characterize the

structural proteins. The profile bands were visualized by staining with Coomassie Brilliant Blue R250.

Viral inoculation assay

Approximately 100 µL of nuclease-treated viral purified was used to inoculate 900 µL of GMI1000 cells suspension, an aggressive isolate of *R. pseudosolanacearum*. The inoculated culture was incubated at 28°C in static conditions for four days. The infection was confirmed by SDS-PAGE profile and nucleic acid isolation from nuclease-treated purified particle.

Bacterial virulence assay

Eggplant and tomato plants were subjected to inoculations with UB-2014 to confirm the loss of the bacterial virulence. Additionally, to investigate the effect of the viral infection on the virulence of *R. pseudosolanacearum* GMI1000, we use the method of soil infestation with bacterial cell suspension (Fonseca et al. 2016) with minor modifications. The inoculum was prepared by suspending 24h-old bacterial cultures in distilled water and adjusting spectrophotometrically (A_{600}) to the concentration to 1.5×10^8 CFU/mL. For soil infestation were added 50 mL of the bacterial suspension were added to each pot (0.4 L) containing tomato plants (cv. Santa Clara, 4 weeks old). Immediately after infestation of the soil, random injuries were promoted with a sharp instrument and the plants were kept in growth chamber at 28°C with a photoperiod of 16h with light intensity of 40 µmole $s^{-1}m^{-2}$. Plants were evaluated during the first fifteen days for the presence or absence of symptoms. *R. solanacearum* and *R.*

pseudosolanacearum were tentatively reisolated from plants inoculated with UB-2014 and GMI1000 VI, respectively.

Microbial biological components

To test if RsIBR1 infection could affect GMI1000 susceptibility response during secondary infections by non-related virus, we inoculated virus-free and virus-infected GMI1000 with aliquots of 100 uL of purified particles of Ralstonia virus phiAP1, a lytic virus able to infect the isolate GMI1000 (Xavier et al., 2016 (Chapter 1)). Using the standard soft agar overlay method (Adams, 1959) the profile of plaques formation for each isolate was analyzed. In addition, to analyze if the presence of RsIBR1 disturbe the bacteriocins profile produced for GMI1000, we analized the inhibitory activity of the supernatant of infected or non-infected GMI1000 cultures against a collection of twenty-two isolates, including *R. solanacearum* and *R. pseudosolanacearum* isolates (Table 1) as described (Moore et al. 2013; Huerta et al. 2015) with some modifications Approximately 20 µL of the supernatant of each isolate (GMI1000 VF or GMI1000 VI) were deposited in cavities (5 mm diameter) obtained after to puch the CPG medium containing separately each one of twenty-two isolates of *Ralstonia* spp. (Table 1). After 48 hours, the inhibition zone radius was measured.

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Figure legends

Figure 1. UB-2014 is an avirulent isolate of *R. solanacearum* (phylotype II). **(A)** *Ralstonia* spp. molecular identification and Phylotype multiplex PCR. **(B)** UB-2014 exhibits abnormal behavior, such as frequent aggregation and supernatant brown coloration *in vitro*. **(C)** UB-2014 is unable to cause disease in a susceptible host (*Solanum lycopersicum* cv. Santa Clara, three weeks post-inoculation). GMI1000 is a isolate of *R. pseudosolanacearum* (phylotype I); CFBP2957 is a isolate of *R. solanacearum* (phylotype II).

Figure 2. RsIBR1 is an inovirus associated with the phenotype exhibited by UB-2014 and converts the phytopathogenic *R. pseudosolanacearum* GMI1000, into a commensal bacteria. **(A)** GMI1000 VI exhibits the same abnormal behavior as UB-2014, such as frequent aggregation and overproduction of reddish-brown pigment melanin. **(B)** Profile of the RsIBR1 virion-associated proteins. **(C)** RsIBR1 infection makes GMI1000 unable to cause disease in a susceptible host (*Solanum lycopersicum* cv. Santa Clara, three weeks post-inoculation). **(D)** Inoculation of GMI1000 VI in tomato plants causes only a mild wilt **(E)** which later reverse and the plants develop similarly to mock-inoculated plants. GMI1000 VF, virus-free; GMI1000 VI, virus-infected.

Figure 3. RsIBR1 infection affects the competitive ability of *R. pseudosolanacearum*. (A) GMI1000 VI is more resistant to a secondary infection by phikmvvirus Ralstonia virus phiAP1,

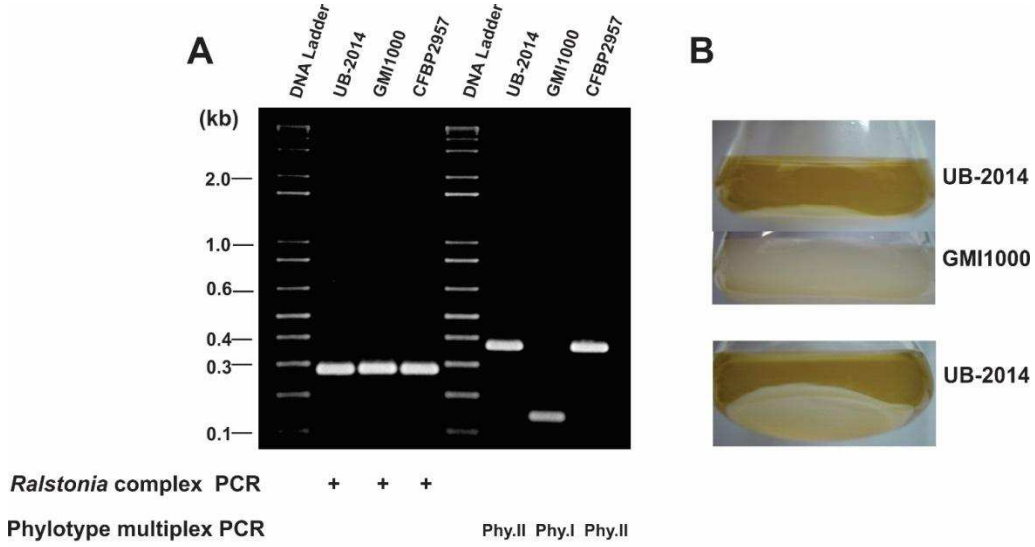
but shows (B) decreased intraspecific and interspecific antibiosis. RSB1 and V45 are isolates of *R. solanacearum* (phylotype II) and V4 is isolate of *R. pseudosolanacearum* (phylotype I).

Table 1. Interference of RsIBR1 infection on intra and interspecific antagonism of *R. pseudosolanacearum* GMI1000

					Inhibition zone (cm)[d]	
Isolates	**Source[a]**	**Host**	**Origin[b]**	**Phyl-seq[c]**	**GMI1000 VF**	**GMI1000 VI**
***Ralstonia pseudosolanacearum* native isolates**						
RS476	Embrapa-CNPH	Tomato	MA/Northeast	I/ND[e]	-	-
RS470	Embrapa-CNPH	Tomato	MA/Northeast	I/ND	-	-
CRMRS42	UFRPE	Bell pepper	PE/Northeast	I/18	-	-
***Ralstonia solanacearum* native isolates**						
RS333	Embrapa-CNPH	Tomato	TO/North	IIA/6	-	-
RS263	Embrapa-CNPH	Potato	SP/Southeast	IIA/ND	-	-
RS278	Embrapa-CNPH	Tomato	RS/South	IIB/NS[f]	-	-
RS99	Embrapa-CNPH	Potato	PR/South	IIB/NS	-	-
RS503	Embrapa-CNPH	Eggplant	CE/Northeast	IIA/ND	-	-
RS300	Embrapa-CNPH	Tomato	MG/Southeast	IIB/NS	-	-
RS480	Embrapa-CNPH	Geranium	SP/Southeast	IIB/NS	-	-
RSCOI	In this study	Tomato	MG/Southeast	II/ND	-	-
RSB1	In this study	Eucalypt	BA/Northeast	II/ND	1,84[g]	0,75
RSB2	In this study	Eucalypt	BA/Northeast	II/ND	-	-
B11	UFRPE	Banana	AM/North	IIA/24	-	-
AMC22	UFV[1]	Eucalypt	AP/North	IIA/NS	-	-
***Ralstonia* sp. native isolates (Plant pathogenic)**						
RS17738	AGR	Tobacco	RS/South	ND	-	-
RS5191	AGR	Tobacco	RS/South	ND	-	-
V4	INPA	Tomato	AM/North	I/ND	2,01	1,09
V43	INPA	Scarlet eggplant	AM/North	ND	-	-
V45	INPA	Cucumber	AM/North	II/ND	2,25	0,97
V55	INPA	Tomato	AM/North	ND	-	-
***R. solanacearum* type-isolate**						
IBSBF292	IBSBF	Tomato	USA	IIA/7	-	-

[a]Source of the isolates: EMBRAPA-CNPH, Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisas em Hortaliças, Distrito Federal, Brazil. UFRPE, Universidade Federal Rural de Pernambuco. Recife, Brazil. UFV[1], Laboratório de Patologia Florestal Molecular, Universidade Federal de Viçosa**,** Viçosa, Brazil. AGR, Agronômica-Laboratório de Diagnóstico Fitossanitário, Porto Alegre, Brazil. INPA, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil. IBSBF, Instituto Biológico, São Paulo, Brazil. [b]Origin of the isolates: AM=Amazonas State; AP=Amapá State; BA=Bahia State; CE=Ceara State; MA= Maranhao State; MG=Minas Gerais State; PE=Pernambuco State; PR=Paraná State, RS=Rio Grande do Sul State; SP=São Paulo State; TO=Tocantins State. [c]Phylotype-sequevar. [e]ND=Non determined. [f]NS=novel sequevar. [g]Average of inhibition zone, 48 hours after addition of the GMI1000 VI and GMI1000 VF supernatants on each one of the twenty-two *Ralstonia* spp. isolates.

Figure 1


A
DNA Ladder
UB-2014
GMI1000
CFBP2957
DNA Ladder
UB-2014
GMI1000
CFBP2957
(kb)
2.0
1.0
0.6
0.4
0.3
0.1
B
UB-2014
GMI1000
UB-2014
Ralstonia complex PCR + + +
Phylotype multiplex PCR
Phy.II Phy.I Phy.II

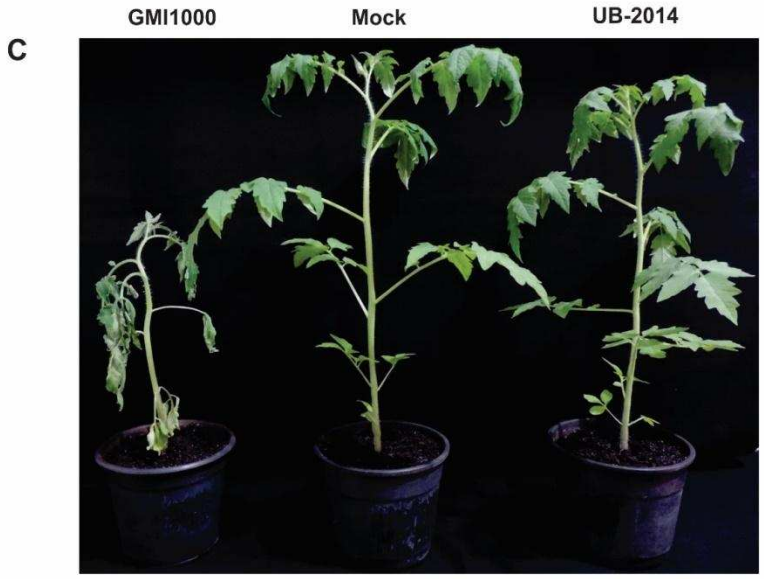

C
GMI1000
Mock
UB-2014

Figure 2

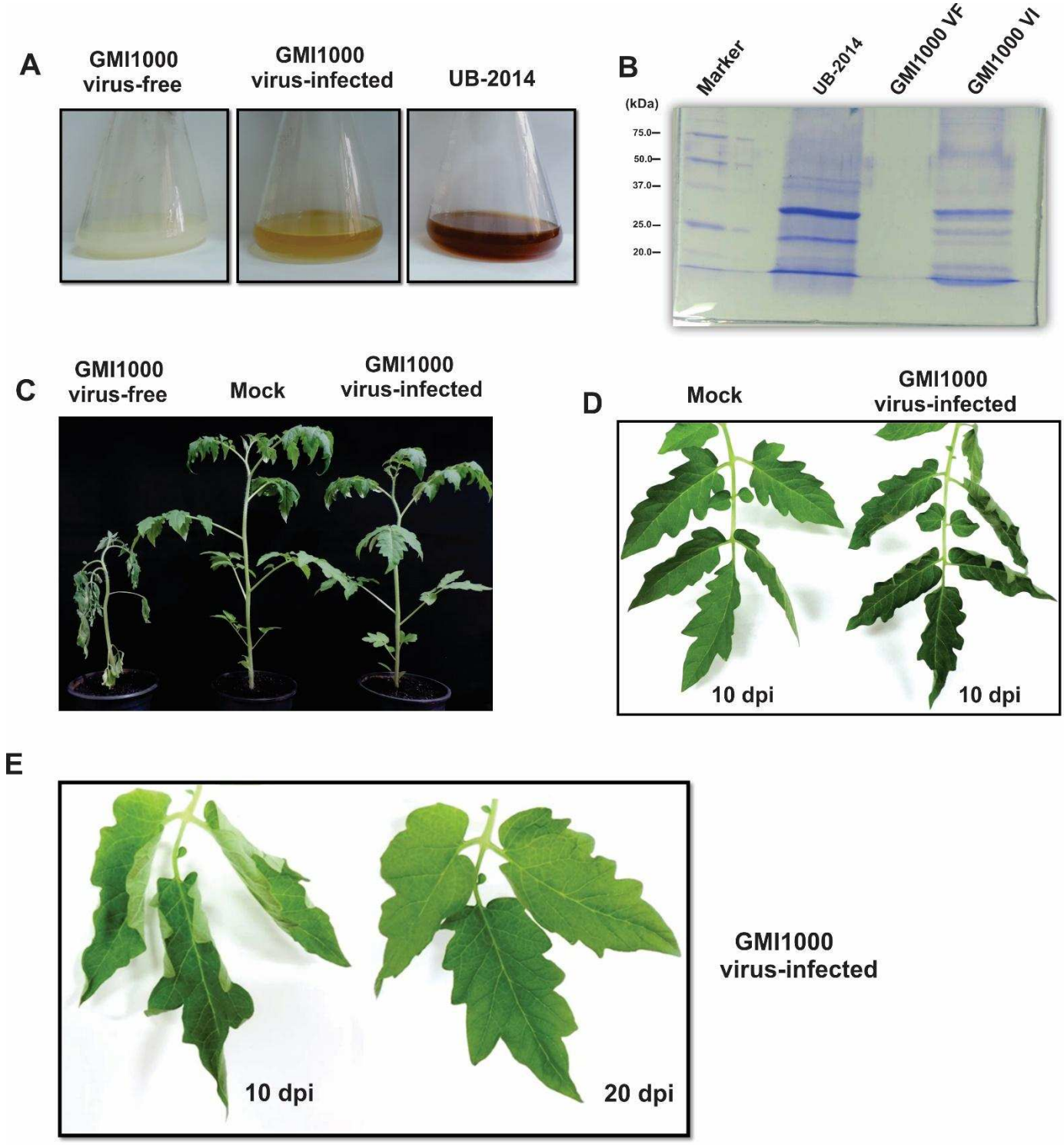
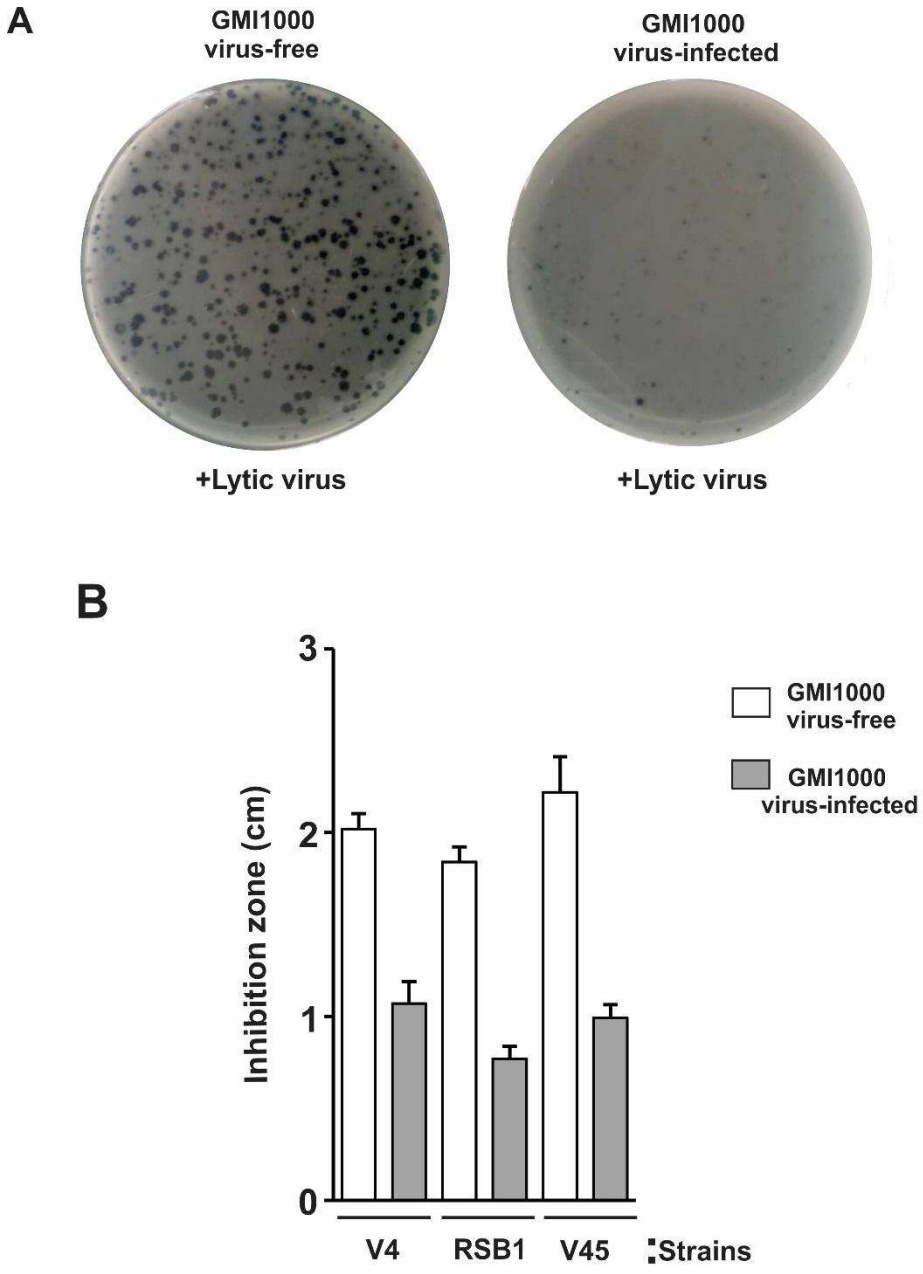

A
GMI1000 virus-free
GMI1000 virus-infected
UB-2014
B
Marker
UB-2014
GMI1000 VF
GMI1000 VI
(kDa)
75.0
50.0
37.0
25.0
20.0
C
GMI1000 virus-free
Mock
GMI1000 virus-infected
D
Mock
GMI1000 virus-infected
10 dpi
10 dpi
E
10 dpi
20 dpi
GMI1000 virus-infected

Figure 3


A
GMI1000
virus-free
GMI1000
virus-infected
+Lytic virus
+Lytic virus
B
Inhibition zone (cm)
3
2
1
0
GMI1000
virus-free
GMI1000
virus-infected
V4
RSB1
V45
Strains

CHAPTER 5

A NOVEL *MYCOVIRUS* ASSOCIATED TO *Alternaria alternata* COMPRISES A DISTINCT LINEAGE IN *Partitiviridae*

Xavier, A.S., Barros, A.P.O., Godinho, M.T., Souza, F.O., and Alfenas-Zerbini, P. A novel mycovirus associated to *Alternaria alternata* comprises a distinct lineage in *Partitiviridae.* ***Archives of Virology***, will be submitted.

A novel mycovirus associated to *Alternaria alternata* comprises a distinct lineage in *Partitiviridae*

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Supplementary Tables: 3

Abstract

Here, we report a novel mycovirus that infects *Alternaria alternata*. The mycovirus has isometric particles of approximately 30 nm and the genome consists of two molecules of dsRNA, dsRNA1 with 1833 bp, encoding a putative RNA-dependent RNA polymerase (RdRp) and dsRNA2, with 1680 bp in length, encoding the putative capsid protein (CP). RdRp analysis revealed low aminoacid identity with RdRps with species in the genus *Gammapartitivirus*, and the alignment of the RdRp revealed all the six conserved motifs present in members of *Partitiviridae*. The putative coat protein (CP) analysis revealed no similarity with any typical mycoviral protein, except with the putative CP of Botryosphaeria dothidea partitivirus 1 (BdPV1), a divergent partitivirus. We propose that Alternaria partitivirus 1 (AtPV1) is a novel species and comprises a distinct lineage related to genus *Gammapartitivirus* in the family *Partitiviridae*, apparently on the threshold of radiation of a new genus, together with BdPV1. Vertical transmission tests showed that AtPV1 was transmitted to 100% conidial progeny and standard curing was unable to eliminate it from the host, characterizing it as a persistent virus. The absence of a virus-free isogenic lineage prevented us from accessing the details of the interaction between AtPV1 and *Alternaria alternata.* In this way, it remains unclear whether the morphological plasticity observed or the inability of the *Alternaria alternata* isolate Avi1 to cause disease in plants is associated with AtPV1 infection.

Introduction

Viruses that infect fungi (mycoviruses) are widely distributed in the major taxonomic groups of filamentous fungi and yeasts (Pearson et al., 2009; Xiao et al., 2014; Xie and Jiang, 2014). RNA genomes predominate among mycoviruses with only one example of a mycovirus with a single-strand DNA (ssDNA) genome (*Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1* (SsHADV-1) (Yu et al., 2010). Mycoviruses are classified into 15 families (*Alphaflexiviridae, Barnaviridae, Chrysoviridae, Endornaviridae, Gammaflexiviridae, Hypoviridae, Megabirnaviridae*, *Metaviridae*, *Mycomononegaviridae, Narnaviridae*, *Partitiviridae, Pseudoviridae, Quadriviridae, Reoviridae* and *Totiviridae*), and are mostly packaged into icosahedral particles, although some have naked genomes associated with host vesicles (Ghabrial et al., 2015; Ghabrial and Suzuki, 2009; Lin et al., 2015; Lin et al., 2012).

Most infections caused by mycoviruses are cryptic, because they do not lead to any phenotypic changes in the host. However, some mycoviruses are associated with serious disorders in the natural physiology of the host such as debilitation, hypovirulence, toxin production and morphological changes (Nuss, 2010). The success in biological control mediated by hypovirulent strains of *Cryphonectria parasitica,* as well as constant reports of new mycoviruses causing hypovirulence, suggest that mycovirus-based control of fungal diseases can be expanded to other pathosystems (Ghabrial and Suzuki, 2009; Pearson et al., 2009; Xie and Jiang, 2014). Investigation of other biological phenomena associated with mycoviral infection, apart from hypovirulence, remains incipient, possibly underestimating the true viral diversity existing in fungi, and the ecological implications of the interaction of persistent viruses with their hosts (Roossinck, 2010).

Partitiviruses have been mainly associated with cryptic infections in both plants and fungi (Roossinck, 2013). The widespread persistent and the diversity of these viruses suggest

that they can behave as cytoplasmic epigenetic elements, that could lead to a rapid adaptation of the host that replicates these viruses (Roossinck, 2013). There are some examples of partitiviruses exhibiting deleterious or positive effects on the host´s physiology (Boccardo and Candresse, 2005; Nakatsukasa-Akune et al., 2005; Zheng et al., 2014).

Alternaria alternata is a weak plant pathogen causing opportunistic infections on a number of crops, but can also be a saprophytic fungus isolated from various dead plant materials (Rotem, 1994; Thomma, 2003; Woudenberg et al., 2013). Pathogenic strains of *A. alternata* are known to produce host-selective toxins that are necessary for pathogenicity (Tsuge et al., 2013). The presence of viral dsRNA was reported in six isolates of *A. alternata,* displaying no apparent phenotypic changes, obtained from cotton seeds. The dsRNA profile of the isolates was variable, ranging in number from one to four dsRNA molecules, and in size from 1.0 to 5.1 kbp (Shepherd, 1988). In *A. alternata* pathotype Japanese pear segments of dsRNAs ranging of 1.0–8.0 kbp in length were detected in isolates able or not to produce toxin, not being detected any apparent phenotypic changes associated tothe presence of the dsRNAs (Hayashi et al., 1988). The unclassified quadripartite mycovirus, Alternaria alternata virus-1 (AaV-1), described affecting the growth of *A. alternata* pathogenic strains which cause black spot disease on Japanese pear, comprised the first report of a mycovirus in *A. alternata* clearly affecting the development of the host (Aoki et al., 2009).

Here, we analyze an isolate of *A. alternata* (isolate Avi1) which displays phenotypic plasticity and carries two dsRNA segments. Phylogenetic analysis suggests that the dsRNAs comprises a novel virus, tentatively named Alternaria partitivirus 1 (AtPV1), that corresponds to a distinct lineage related to viruses in the genus *Gammapartitivirus*, apparently on the threshold of radiation of a new genus in the family *Partitiviridae*.

Results

Alternaria alternata AVi1 isolate carrying dsRNAs exhibits phenotypic plasticity in vitro

During the propagation of *A. alternata* AVi1 isolate, a remarkable phenotypic plasticity was observed in different Petri dish replicates incubated under the same conditions (Figure1A). Three experiments were conducted (*n*= 100) at different times to validate the phenomenon observed in this strain. Equimolar amounts of potato dextrose agar media were measured in each Petri dish, the mycelia discs of Avi1 strain were always removed from the periphery of donor culture and during the incubation at 25°C the Petri dishes were repositioned daily in order to reduce sources of environmental variation. To investigate whether the presence of mycoviruses might be associated with such physiological response, the mycelium of this isolate was subjected to extraction of total nucleic acids for screening for DNA and RNA viruses. After treatment with DNase I and S1 nuclease, two dsRNA elements with approximately 1,900 and 1,500 nucleotides were detected, indicating viral infection (Figure 1B). Viral particles with size around 25-30 nm were isolated from the mycelium of the Avi1 isolate (Figure1C) and extraction of dsRNA from the purified viral particles confirmed bipartite genomic profile previously detected in the initial screening (Figure1D).

dsRNAs are the genomic components of a new partitivirus

The raw sequencing data consisted of 133,177 reads, with an average read length of 433 nt. The contigs were obtained by *de novo* assembly, and those ranging between 700 and 2,000 nucleotides were selected and submitted to BLAST analysis. BLAST*n* and BLAST*x* analyses

indicated highest identity (65-80%) with Botryosphaeria dothidea partitivirus 1 (BdPV1). To check if the complete genome was assembled, we performed another assembly using the BdPV1 genome as a reference, and comparative mapping allowed us to obtain the complete sequences of the two dsRNAs segments. The genomic dsRNA1 has 1833 nt and contains a single ORF (ORF1) that encodes a putative RNA-dependent RNA polymerase (RdRp) (Figure1D). The RdRp revealed 75% amino acid identity with the RdRp of BdPV1, and low sequence identity (22-30%) with RdRps of members of the *Gammapartitivirus* in the family *Partitiviridae* and polyproteins of *Potyviridae* and *Caliciviridae* (Table S1). The dsRNA2 has 1680 nt and also contains a single ORF (ORF2), that encodes a putative capsid protein (CP) (Figure1D). BLASTp analysis of the putative CP revealed no detectable sequence similarity with any mycovirus protein, except with the putative CP of BdPV1 (56% identity) and a hypothetical protein of *Exophiala dermatitidis,* a black yeast (Suppl. Table S1). The taxonomic criteria for demarcation of a new species in the genus *Gammapartitivirus* are percent identity ≤80% for the CP and ≤90% for the RdRp (Nibert et al., 2014).. The 5'UTRs of dsRNA1 and dsRNA2 were 34 and 71 nt in length, respectively and share 90% identity. The 3'UTRs of the dsRNA 1 and 2 were 83 and 103 nt length, respectively, and shared 78% identity. The comparison between the untranslated regions of AtPv1 and BdPV1 revealed the presence of the conserved sequences CGAAAAUKAGUCACAAYAUYAYA in the 5'UTR and UCUCAUUAG in the 3'UTR in all genomic segments (Figure 2A). The 5' and 3'UTR of the dsRNA1 and dsRNA2 of AtPv1, are predicted to fold into secondary structures (Figure 2B) as in other partitiviruses. Togther, these analyses suggest that the two dsRNA segments represent the genome of a new virus in the family *Partitiviridae,* for wich we propose the name Alternaria partitivirus 1 (AtPV1).

According to the taxonomic criteria for genus attribution in the *Partitiviridae*, ambiguous results were obtained for attribution of a genus to AtPV1 and BdPV1, where the values of pairwise identity of the RdRp are on the threshold for radiation of a new *Gammapartitivirus*-related genus.

AtPV1 and BdPV1 comprise a divergent lineage with a Gammapartitivirus-related RdRp

Phylogenetic analysis of the amino acid sequence of the RdRp encoded by dsRNA1 of AtPV1 and RdRps of representative members of the five genera of the family *Partitiviridae* shows a separation of five clusters that correspond to the current genera, and a divergent branch containing AtPV1 and BdPV1, most closely related to gammapartitiviruses (Figure 3). Multiple protein alignment confirmed the predicted domains of viral RdRp including the six conserved motifs (III to VIII) similar to the RdRp sequences of members of family *Partitiviridae*. However, a considerable degeneration of these motifs are found in AtPV1 and BdPV1. Interestingly, most of the alterations detected between the divergent viruses (AtPV1 and BdPV1) and other members of *Partitiviridae* are conserved between AtPV1 and BdPV1, showing a high conservation in these motifs in these two viruses (Figure 4).

Genus-specific elements can be found in the 5 'UTR of viruses in the four genera of the family *Partitiviridae* (*Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus* and *Deltapartitivirus*) (Hacker et al., 2006; Lesker et al., 2013; Nibert et al., 2014). In BdPV1 and AtPV1 and in the other viruses classified in the genus *Gammapartitivirus* the specific element GCHAA can be found (Figure 5).

Similarly to phylogeny (Figure 3), the pairwise identity matrix generated with the amino acid sequences of RdRp of members of the five genera of the family *Partitiviridae* clearly

positioned AtPV1 together with BdPV1, in a subgroup or a new lineage closer to viruses in the genus *Gammapartitivirus* (Figure 6). Comparative analysis using the length of proteins, or the length of the dsRNA segments proposed by Nibert et al. (2014) corroborate the phylogeny in the current *Partitiviridae* taxonomy, but the inclusion of the AtPV1 and BdPV1 sequences again caused ambiguity in the taxonomic position. For both of them the length of the genomic segments and proteins are unusual for *Gammapartitivirus* (Table S3) and group them with *Alphapartitivirus* (Figure 7).

AtPV1 exhibits a persistent lifestyle

To analyse the horizontal and vertical transmission of AtPV1, co-cultivation assay was conducted using the parental mycovirus-infected (mycovirus +) Avi1 isolate, virus free *Alternaria alternata* and other species of *Alternaria*. A death zone (incompatibility) was checked for all combinations (Avi1/*A. brassicicola*, Avi1/*A. tomathophila*, Avi1/*A. alternata*, Avi1/*A. dauci* and Avi1/*A. grandis*) (Figure 8A). AtPV1 was not passed to other species of virus-free *Alternaria* during co-cultivation with the Avi1 strain (Figure 8B). As expected for *Partitiviridae*, the transmission to the conidial and spheroplasts progenies was extremely efficient: out of one hundred conidia or spheroplasts analyzed, all were infected (Figure 8C and D). To obtain the virus-free Avi1 isogenic lineage, we attempted to cure the virus-infected Avi1 using chemotherapy or a mixed treatment with thermotherapy and chemotherapy. Both dsRNA curing strategies failed and AtPV1 can still be detected in cured-tentative lines, displaying a persistent lifestyle (Figure 8E).

Discussion

A growing number of partitiviruses has been reported mostly in several groups of fungi, which indicates a wide distribution of these entities (Jiang et al., 2014; Vainio et al., 2013; Wang et al., 2014; Xiao et al., 2014; Zheng et al., 2014). The present study revealed the genomic and morphological properties of Alternaria partitivirus 1 (AtPV1), a novel dsRNA mycovirus that infects *Alternaria alternata.* The dsRNA segment profile of AtPV1 confirmed their origin and indicates that despite of the divergent CP, AtPV1 is able to form particles, as has been observed for other partitiviruses such as PSV-F, BdPV1, UvPV1, UvPV2 and CaRV1 (Kim et al., 2005; Wang et al., 2014; Zhong et al., 2014a; Zhong et al., 2014b). To date, the set of available sequences do not provide footprints to trace the origin of these different CPs. Unlike gammapartitiviruses wich have orphan CPs, the AtPV1 and BdPV1 CPs share a considerable degree of identity (56%). AtPV1 could not be indicated to any genus in the family *Partitiviridade*, although it is closely related to gammapartitiviruses (Figure 3, Table S2). As BdPV1, it possesses unique characteristics that according to the current criteria of *Partitiviridae* taxonomy the classification of these two viruses can not be cleary defined. The length of the RdRp and CP proteins as well dsRNAs segments is not consistent with the average length observed in other gammapartitiviruses. The RdRp identity is below the threshold value when compared with members of *Gammapartitivirus* and the phylogenetic analysis indicates a formation of a divergent cluster. The six typical RdRp motifs present in *Partitiviridae* are degenerate, showing a close relationship only between BdPV1 and AtPV1.We have chosen to call this cluster a divergent lineage until new taxonomic parameters are suggested so that they can support the inclusion of these taxa in the genus *Gammapartitivirus*, or allow the proposal of a new genus.

In horizontal transmission assays, AtPV1 could not move to the other *Alternaria* spp. isolates, similar what has been obtained with BdPV1in transmission assays (Wang et al., 2014). In our system, although this negative result may be due to vegetative incompatibility between the different species (Wu et al., 2012), for some mycovirus this barrier is not decisive to the transmission between hosts, because the particles of some mycovirus can be released overcoming the absence of anastomosis (Charlton and Cubeta, 2007; Xiao et al., 2014). AtPV1 was efficiently transmitted to the asexual progeny, as expected for partitivirus and was maintained in the Avi1 strain even after several tentatives to cure the dsRNA, showing a relationship of persistence with its host. In both plants and fungi, partitiviruses are transmitted at high rates to the progeny, are maintained in low titers, but in the case of plants they are not horizontally transmitted (Roossinck, 2010). Cryptic infections provide clues to a long history of coexistence between the virus and its host, particularly demonstrated by Chiba et al. (2013) with RnPV1, a partitivirus that causes severe symptoms in *Cryphonectria parasitica* but not in its natural host, *Rosellinia necatrix*. Transfection assays using purified viral particles of BdPV1 showed that the infection of this partitivirus, closely related to AtPV1, does not have any apparent measurable effect on the physiology of the host fungus, displaying a cryptic lifestyle (Wang et al., 2014).

It is probable that the AtPV1 infection in the Avi1 isolate may be responsible for triggering its morphological instability, and also for the inability of the Av1 isolate to cause disease in plants. Phenotypic plasticity is defined as the ability of one genotype to produce multiple phenotypes depending on environmental conditions (West-Eberhard and Jane, 2003). Abiotic or biotic factors can induce plasticity, and results in changes that can vary from pathogenicity to highly adaptive alternative phenotypes (Whitman and Agrawal, 2009). At this

point we can not say that the phenotipic plasticity, or that the lack of ability to cause disease in plants are associated with partitivirus infection, because in Avi 1 isolate, AtPV1 behaves as a persistent virus, preventing the isolation of an isogenic lineage virus-free.

Material and Methods

Fungal isolates and culture conditions

The *A. alternata* isolate AVi1 was isolated from cabbage leaves (*Brassica oleracea*) with typical symptoms of alternariosis in crop fields in the city of Viçosa, Minas Gerais, Brazil. Additional isolates of *Alternaria* (*A. alternata*, *A. brassicicola*, *A. dauci*, *A. grandis* and *A. tomathophila*) used in this study belong to the Culture Collection of the Universidade Federal de Viçosa and were provided by Dr. Robert Barreto. The isolates were grown on potato dextrose agar (PDA) medium at 25ºC and stored on PDA at 4ºC. Mycelium for particle purification and viral dsRNA isolation was grown for 10 days on an orbital shaker (125 rpm) (Lin et al., 2015), at 25ºC in liquid V8 medium (Simmons and Roberts, 1993).

dsRNA isolation

The dsRNA was extracted from 30g of frozen mycelia by selective absorption to columns of cellulose fibrous powder (Sigma-Aldrich) using the method described by Morris and Dodds (1979) with the following modifications: 200 mL of extraction buffer pH 7.5 (100 mM Tris, 2 mM EDTA, 200 mM NaCl, 1.6% βeta-mercaptoethanol, 0.4g bentonite) were added to

30g of mycelial powder and homogenized. After homogenization, an equal volume of phenol-chloroform-isoamilic alcohol (25:24:1) was added. After extraction, the dsRNA was treated with DNase I (Promega) and S1 nuclease (Promega) to confirm the nature of the nucleic acid.

Purification and electron microscopy of virus particles

Viral particles were purified according to Zheng et al. (2014). The fractions containing virus particles were stained with uranyl acetate 2% and observed using a transmission electron microscope (TEM) (Zeiss Libra 120). The nucleic acid from virus particles was extracted with phenol-chloroform (Sambrook et al., 1989).

Sequencing, assembly and annotation

The dsRNA purified from virus particles was sequenced using the 454 GS-FLX platform. (Macrogen Inc). The reads were trimmed and quality filtered and the virus genome was assembled using *de novo* assembly in CLC Genomics Workbench 5.5.1 (CLC Bio), Geneious 8.0 and DNASTAR. The assembled contigs were compared with sequences in GenBank using BLAST algorithms (Altschul et al., 1990; Altschul et al., 1997). The assembled contigs were manually inspected to verify the expected genomic size and the sequences on its extremities were mapped according to the reference genome of the mycovirus BdPV1 (KF688741; KF688741), the sequence that showed higher nucleotide identity. Open reading frames (ORFs) in each genomic component (dsRNA 1 and dsRNA 2) were determined using ORF Finder program (http:// www.ncbi.nlm. gov/gorf/gorf.html) and deduced amino acid sequences were subject to a BLASTp search. The search for conserved motifs in aminoacid

sequences was performed in CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd) and PROSITE databases (http://www.expasy.ch/). The sequences reported in this paper have been deposited in the GenBank database under the accession numbers _____.

Phylogeny and sequence analysis

Multiple sequence alingments were made for the full-length genome and for the CP and RdRp coding sequences using MUSCLE (Edgar, 2004) and pairwise comparisons were performed using SDT v. 1.0 (Muhire et al., 2013). The dataset included sequences with greatest similarity with the contigs assembled based on the BLAST*n* analysis. Pairwise identity scores from global alignments using the RdRp amino acid sequences of AtPV1, BdPV and members of the five genera of the *Partitiviridae* family were calculated. The scores were generated using EMBOSS 6.3.1: with default settings (Blosum62 matrix) plus gap opening extension penalties of 10 and 0.5, as recommended taxonomic criteria in the family *Partitiviridae* (Nibert et al., 2014).

Phylogenetic trees were constructed using Bayesian inference with MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003), with the model selected by MrModeltest v. 2.2 (Nylander, 2004) in the Akaike Information Criterion (AIC). The analyses were carried out running 20,000,000 generations and excluding the first 2,000,000 generations as burn-in. Trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/)

To verify if there was correspondence among the length of CP and RpRp and the phylogeny clades the sequences were analysed using Qtiplot v. 0.9.8.9 as recommended by Nibert et al. (2014).

Vertical and horizontal transmission of AtPV1

The contribuition of vertical transmission to the dispersion of AtPV1 was assessed by chek the presence of the two dsRNAs segments in the asexual progeny of a single conidium obtained from the dsRNA-containing AVi1 strain. For sporulation, the strain AVi1 was incubated at 25ºC on PDA medium in 9-cm Petri dishes for 10 days under a 12h/12h photoperiod. Single conidia cultures were obtained as described by Wu et al. (2012).

The horizontal transmission experiment was carried on PDA in Petri dishes using the pairing culture technique with isolates without dsRNA (Wu et al., 2010). The dsRNA-containing isolate AVi1 served as donor, whereas other isolates of *A. alternata* and other plant pathogenic *Alternaria* species (*A. brassicicola, A. dauci, A. grandis* and *A. tomathophila*) without dsRNA served as recipients. After incubation of the cultures at 25 ° C for 10 days, mycelia plugs located in the anastomotic areas were excised and grown on PDA to verify the presence of the dsRNA segments.

Viral dsRNA cure assay

A combined strategy involving thermotherapy and chemoterapy was used in an attempt to obtain a virus-free strain of *A. alternata*. Mycelial plugs of AtPV1-infected strain AVi1 were inoculated on PDA containing 5µg/mL of cycloheximide (Sigma-Aldrich) and incubated at 28ºC for 5 days. Hyphal tips were transplanted into PDA in successive increasing concentrations of the antibiotic (10, 20, 30, 40, 50 and 70µg/mL) and incubated at 34°C. PDA plugs of 4 mm in diameter were individually inoculated into liquid V8 medium and cultured for two weeks, and

the presence of dsRNA was checked. Spheroplast regeneration of AtPV1-infected *A. alternata* was done as proposed by Zhou and Boland (1997).

Molecular detection of AtPV1

Total RNA was extracted from mycelium of *A. alternata* regenerated from spheroplasts using the RNAid Plus Kit (MP Biomedical, Inc.) according to the manufacturer's instruction. Total RNA (1µg) was treated with DNAse I according to standard techniques (Sambrook et al., 1989) and used for cDNA synthesis. cDNA synthesis from the viral RNA was done using the specific primers designed based on the genome sequence obtained in this work. For dsRNA1 we used the primer RN1-R (5´ TAA TGG AAT GCA GTA ACG CAT CTC 3´) and for the dsRNA2 the primer RN2-R (5´ GTC GTA CAG AGT ACA ACT AAG TAG 3´). The AtPV1 RNAs were detected by multiplex PCR using the specific primers RN1-F (5´ ATC TTG CGA GAC AGG AAT ATG C 3´) and RN1-R for RNA1 and RN2-F (5´ CTC CTC AGC TAT GGT TTG AGT C 3´) and RN2-R to amplify RNA2. As an internal control, the mRNA that codes GAPDH was amplified using oligo d(T) for cDNA synthesis and the primers described by Woudenberg et al. (2013).

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Figure legends

Figure 1. Presence of dsRNAs in *A. alternata* AVi1 isolate. **(A)** Phenotypic plasticity exhibited by *A. alternata* AVi1 . AVi1 isolate cultured on PDA plates, grown for 10 days on 25°C. **(B)** dsRNA profiles extracted from mycelium of *A. alternata* AVi1 isolate, 1. Total RNA; 2. Total RNA treated with DNAse and S1 nuclease **(C)** Electron micrograph of particles of AtPv1 negatively strained with 2% uranyl acetate. The scale bar indicates 50 nm. **(D)** dsRNA profiles extracted from purified virus particles of AtPV1 after digestion with DNase I and S1 nuclease. **(E)** Genomic organization of AtPV1, a bipartite *A. alternata*-associated mycovirus. RN1F/RN1R and RN2F/RN2R are primers and their respectives annealing positions for partial amplification of *RdRp* and *CP* genes.

Figure 2. Conserved motifs and secondary structure on the 5´ and 3´ UTRs of AtPV1. **(A)** Multiple-sequence alignments for the terminal regions of the dsRNAs of AtPV1 and BdPV1. Black, gray, and light gray backgrounds, nucleotide identities with no less than 100%, 80%, and 60%, respectively**. (B)** Secondary structures proposed for terminal region of dsRNAs 1 and 2 of AtPV1.

Figure 3. AtPV1 is a member of the *Gammapartitivirus* genera and together with BdPV1 comprises a divergent lineage**.** Phylogenetic tree based on RdRp aa sequences of *Partitiviridae* family members calculated by Bayesian MCMC coalescent analysis. The posterior probability values (PP) calculated using the best trees found by MrBayes are shown beside each node as filled circles (> 90%), open circles (60-79%) or semi-filled circles (80-89%). The outgroup taxa are the picobirnaviruses oPBV and hPBV. The host of each partitiviruses is indicated in the

colors of the circles: fungus (light blue), plant (green), protozoa (orange) or fungus-plant (light blue/green). The divergent cluster of AtPV1 and BdPV1 in *Gammapartitivirus* is shown in red. See Table S1 for the abbreviation code for partitiviruses names and their GenBank accession numbers.

Figure 4. Alignment of amino acid sequences of the RdRp motifs of AtPV1, BdPV1 and other *Gammapartitivirus* support the existence of a distinct cluster. Conserved amino acids, motifs III–VIII, are showed in horizontal lines above the amino acid areas. Filled stars indicate identical amino acid residues (shaded), unfilled stars and colons indicate similar residues only among partitiviruses species recognized by the ICTV or shared between all taxa including BdPV1 and AtPV1, respectively. Numbers in brackets correspond to the number of amino acid residues separating the motifs. The asterisks indicate the different amino acid between BdPV1 and AtPV1. See Table S1 for the abbreviation code for partitiviruses names and their GenBank accession numbers.

Figure 5. Conserved sequence at 5'UTR of gammapartitiviruses genomes. Aprox. 20 nt of 5' UTR of each genomic component of gammapartitiviruses are shown. Gray shading was used to highlight conserved nucleotides, and in black the Genus-specific element of *Gammapartitivirus.*

Figure 6. Pairwise sequence identity matrix**.** The pairwise of amino acids sequence of the RdRp of members of five genera of *Partitiviridae.* In red AtPV1 and BdPV1.

Figure 7. RdRp-CP scatter plots for AtPV1, BdPV1 and other partitiviruses**.** The lengths of RdRp (X-axis) vs. CP (Y-axis) are plotted for sixty partitiviruses for which full-length

sequences of both genome segments are available in GenBank (Table S1). The plotteds values for viruses from each of the five *Partitiviridae* genera are surrounded by boxes.

Figure 8. Multiplex RT-PCR for AtPV1 detection**. (A)** AtPV1 horizontal transmission design experiment; (B-E). **(B)** AtPV1 detection in single conidial lines in recipient *Alternaria* species; **(C)** AtPV1 detection in single conidial lines; **(D)** AtPV1 detection in regenerated spheroplast lines; **(E)** AtPV1 detection in tentatively cured lines. A.br, *A. brassicicola*; A.to, *A. tomatophila*; A.al, *A. alternata*; A.dau, *A. dauci*; A.gr, *A. grandis*; P, parental; R, recipie

Supplementary Table S1. Summary of the results of a BLASTP search with dsRNA 1 ORF1-encoded RdRp and dsRNA 2 ORF2-encoded putative capsid protein

Description	ORF1 Size (aa)	Total Score	Query cover	E-value	Identity (%)	Acession
putative RNA-dependent RNA polymerase [Botryosphaeria dothidea partitivirus 1]	572	914	100%	0.0	75%	AGZ84316.1
RNA-dependent RNA polymerase [Ustilaginoidea virens partitivirus]	539	146	83%	2e-34	29%	AGO04402.1
RNA dependent RNA polymerase [Grapevine partitivirus]	457	145	71%	2e-34	30%	AFX73023.1
RNA-dependent RNA polymerase [Mycovirus FusoV]	519	143	76%	2e-33	27%	NP_624350.1
putative RNA dependent RNA polymerase [Aspergillus ochraceous virus]	539	142	78%	7e-33	29%	ABV30675.1
RNA-dependent RNA polymerase [Verticillium dahliae partitivirus 1]	539	138	81%	8e-32	30%	AGI52210.1
RNA-dependent RNA polymerase [Ophiostoma partitivirus 1]	539	137	82%	3e-31	28%	CAJ31886.1
dsRNA-dependent RNA polymerase [Aspergillus ochraceous virus]	539	136	82%	4e-31	28%	ABC86749.1
RNA-dependent RNA polymerase [Verticillium dahliae partitivirus 1]	539	135	82%	1e-30	29%	YP_009164038.1
RNA-dependent RNA polymerase [Aspergillus fumigatus partitivirus-1]	542	134	83%	4e-30	27%	CAY25801.2
putative replicase [Gremmeniella abietina RNA virus MS1]	539	133	77%	4e-30	28%	NP_659027.1
RNA-dependent RNA polymerase [Gremmeniella abietina RNA virus MS1]	539	132	77%	1e-29	29%	AII16004.1
RNA directed RNA polymerase [Ustilaginoidea virens partitivirus]	347	129	57%	1e-29	33%	AHH35116.1
RNA-dependent RNA polymerase [Gremmeniella abietina RNA virus MS2]	539	131	77%	3e-29	29%	YP_138540.1
RNA-dependent RNA polymerase [Verticillium albo-atrum partitivirus-1]	539	130	77%	7e-29	28%	AIE47664.1
RNA-dependent RNA polymerase [Colletotrichum truncatum partitivirus 1]	539	129	82%	2e-28	28%	ALF46547.1
RNA-dependent RNA polymerase [Botryotinia fuckeliana partitivirus 1]	540	128	82%	3e-28	27%	YP_001686789.1
RNA-dependent RNA polymerase [Penicillium stoloniferum virus S]	539	125	71%	4e-27	28%	YP_052856.2
RNA-dependent RNA polymerase [Discula destructiva virus 1]	539	125	84%	5e-27	28%	NP_116716.1
RNA-dependent RNA polymerase [Penicillium stoloniferum virus S]	539	124	71%	5e-27	28%	CAJ01909.1
RNA-dependent RNA polymerase [Discula destructiva virus 2]	539	123	84%	2e-26	27%	NP_620301.1
RNA-dependent RNA polymerase [Cytospora sacchari partitivirus MP-2014]	405	112	62%	3e-23	29%	AHV83754.1
RNA-dependent RNA polymerase-like protein [uncultured virus]	473	112	56%	4e-23	29%	AGW51759.1
putative dsRNA-dependent RNA polymerase [Penicillium stoloniferum virus F]	538	109	60%	9e-22	27%	YP_271922.1
putative RNA-dependent RNA polymerase [Pinus sylvestris partitivirus NL-2005]	358	107	49%	1e-21	28%	AAY51483.1
RNA-dependent RNA polymerase [Colletotrichum acutatum RNA virus 1]	540	105	65%	1e-20	28%	AGL42312.1
RNA-dependent RNA polymerase [Botryosphaeria dothidea virus 1]	544	104	65%	3e-20	26%	AIE47694.1
RNA-dependent RNA polymerase [Valsa cypri partitivirus]	444	99.0	58%	1e-18	27%	AIS37548.1
RNA-directed RNA polymerase [Toxocara canis]	452	97.8	46%	3e-18	29%	KHN74396.1
RDRP [Ustilaginoidea virens partitivirus 3]	522	97.4	64%	6e-18	27%	AGJ03719.1
PREDICTED: uncharacterized protein LOC106655011 [Trichogramma pretiosum]	397	90.1	41%	5e-16	29%	XP_014230682.1
RNA-dependent RNA polymerase [Ustilaginoidea virens partitivirus 2]	529	87.4	80%	1e-14	26%	YP_008327312.1
RNA-dependent RNA polymerase [Pyrus pyrifolia]	477	83.6	42%	1e-13	28%	BAA34783.1
RNA dependent RNA polymerase [Persimmon cryptic virus]	477	81.3	55%	7e-13	25%	YP_006390091.1
RNA-dependent RNA polymerase [Beet cryptic virus 2]	475	80.9	42%	1e-12	26%	AGQ49482.1
RNA-dependent RNA polymerase [Beet cryptic virus 2]	475	79.3	42%	3e-12	26%	ADP24757.1
polyprotein [Falcovirus A1]	2420	74.7	68%	2e-10	25%	YP_009133208.1
RNA-dependent RNA polymerase [Gremmeniella abietina RNA virus MS1]	171	63.9	28%	3e-08	32%	AII15952.1
polyprotein [Sugarcane streak mosaic virus]	3130	47.8	24%	0.044	30%	AEQ35297.1
Description	**ORF2 Size (aa)**	**Total Score**	**Query cover**	**E-value**	**Identity (%)**	**Acession**
putative coat protein [Botryosphaeria dothidea partitivirus 1]	494	523	98%	1e-178	56%	AGZ84317.1
hypothetical protein HMPREF1120_06590 [Exophiala dermatitidis NIH/UT8656]	452	326	90%	3e-102	43%	XP_009159042.1

Supplementary Table S2. Pairwise identity scores from global alignments using the RdRps amino acid sequences of AtPV1 and BdPV1 as comparative reference against members of the five genera of the family *Partitiviridae*

Genus	AtPV1 RdRp (% identity)	BdPV1 RdRp (% identity)
Gammapartitivirus		
DdV1	**24.8**[a]	**24.7**
VdPV1	**25.3**	22.6
UvPV2	23.3[b]	**25.1**
UvPV1	23.2	23.1
CaRV1	22.7	19.9
BfPV1	**25.5**	22.4
AfuPV1	**24.6**	23.9
PsV-S	22.6	22.7
PsV-F	19	20.1
OPV1	23.1	22.7
GaRV_MS1	**26.1**	**24.6**
FsV1	23.9	24
DdV2	24.4	24.4
AoV	**26.1**	**25.4**
Divergent lineage		
AtPV1	**100**	**75**
BdPV1	**75**	**100**
Cryspovirus		
CSpV1	19.6	19.7
Deltapartitivirus		
RoCV1	18	19.9
RsCV3	20.3	19.1
RsCV2	20.5	19.7
PerCV	18.3	17.8
FCCV	17.8	18.6
PepCV1	20.4	17.8
Betapartitivirus		
SsPV1	17.3	17.1
HetPV7	16.8	17
RnPV1	16.9	15.5
RCCV2	16.8	16.7
RhSV717	14.8	14.7
Alphapartitivirus		
RhsdRV2	18.7	18.8
RCCV1	17.4	15.7
RSCV1	18	15.6
RnPV2	18.4	18.3
HetPV1	19.1	16.8

[a]Values that satisfy the taxonomic criterion for members within *Gammapartitivirus* genus, threshold value 24.6% RdRp identity**.** [b]Values that support the radiation of a new genus *Gammapartitivirus*-related.

Supplementary Table S3. Ranges of dsRNAs genomic components and protein lengths codified within each genera of the family *Partitiviridae*

Genus	dsRNA1 (nt)	dsRNA2 (nt)	RdRp (aa)	CP (aa)
Betapartitivirus	2180-2444	2135-2354	663-746	636-686
Alphapartitivirus	1776-2027	1708-1866	538-621	463-521
Gammapartitivirus	1645-1793	1445-1611	519-540	413-443
Deltapartitivirus	1563-1734	1415-1581	472-481	337-430
Cryspovirus	1786	1374	524	319
BdPV1 and AtPV1	1826-1833	1623-1680	571-572	494-501

Figure 1

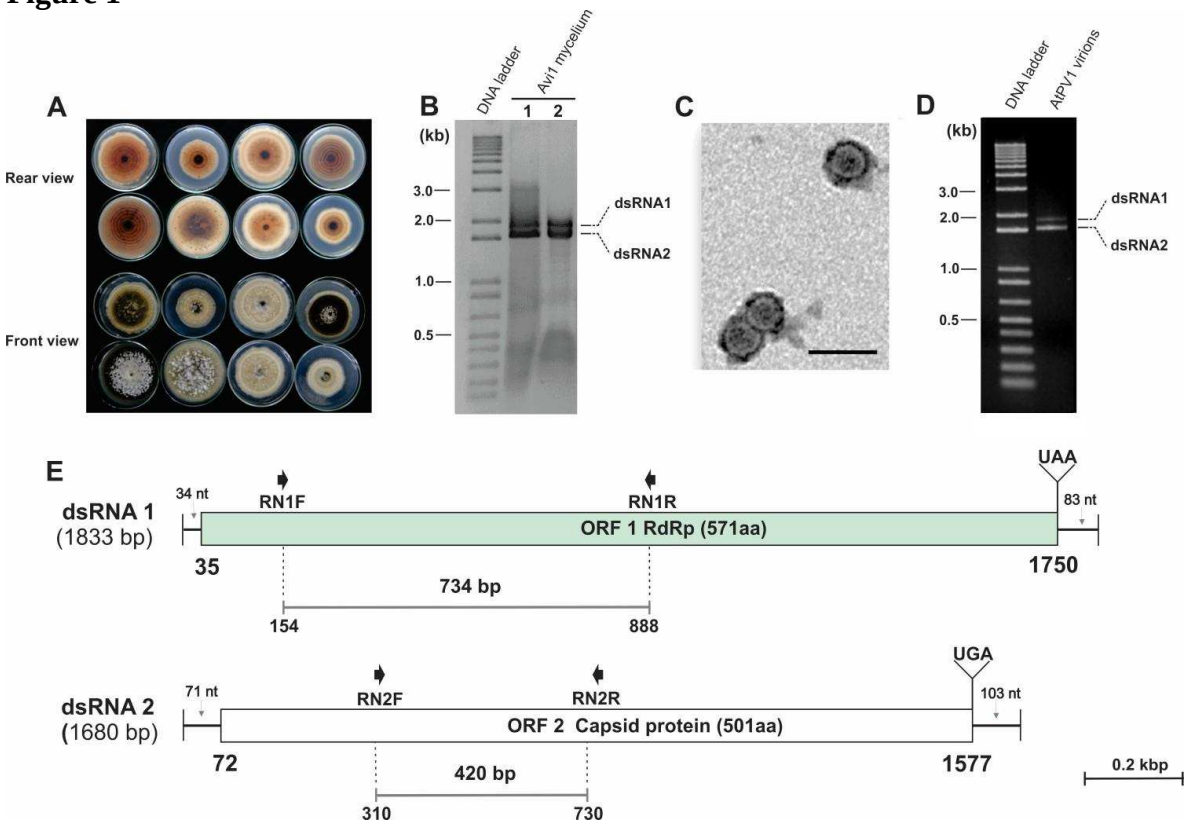

A
Rear view
Front view
B
DNA ladder
AvV1 mycelium
1 2
(kb)
3.0
2.0
1.0
0.5
dsRNA1
dsRNA2
C
D
DNA ladder
AfPV1 virions
(kb)
3.0
2.0
1.0
0.5
dsRNA1
dsRNA2
E
dsRNA 1
(1833 bp)
34 nt
RN1F
RN1R
UAA
83 nt
ORF 1 RdRp (571aa)
35
1750
734 bp
154
888
dsRNA 2
(1680 bp)
71 nt
RN2F
RN2R
UGA
103 nt
ORF 2 Capsid protein (501aa)
72
1577
420 bp
310
730
0.2 kbp

Figure 2

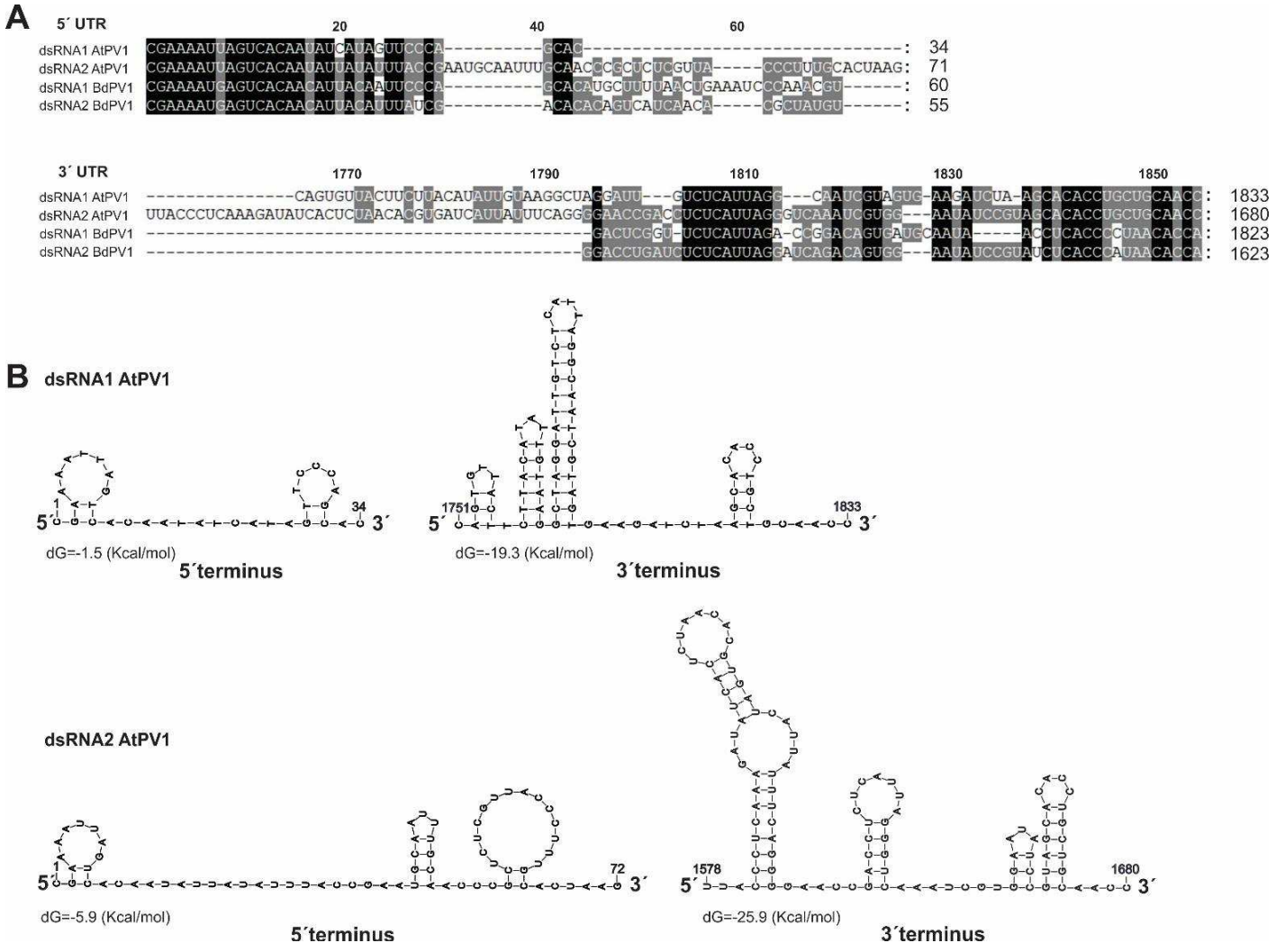

A
5´ UTR
dsRNA1 AtPV1
dsRNA2 AtPV1
dsRNA1 BdPV1
dsRNA2 BdPV1
3´ UTR
B
dsRNA1 AtPV1
dG=-1.5 (Kcal/mol)
5´terminus
dG=-19.3 (Kcal/mol)
3´terminus
dsRNA2 AtPV1
dG=-5.9 (Kcal/mol)
5´terminus
dG=-25.9 (Kcal/mol)
3´terminus

Figure 3

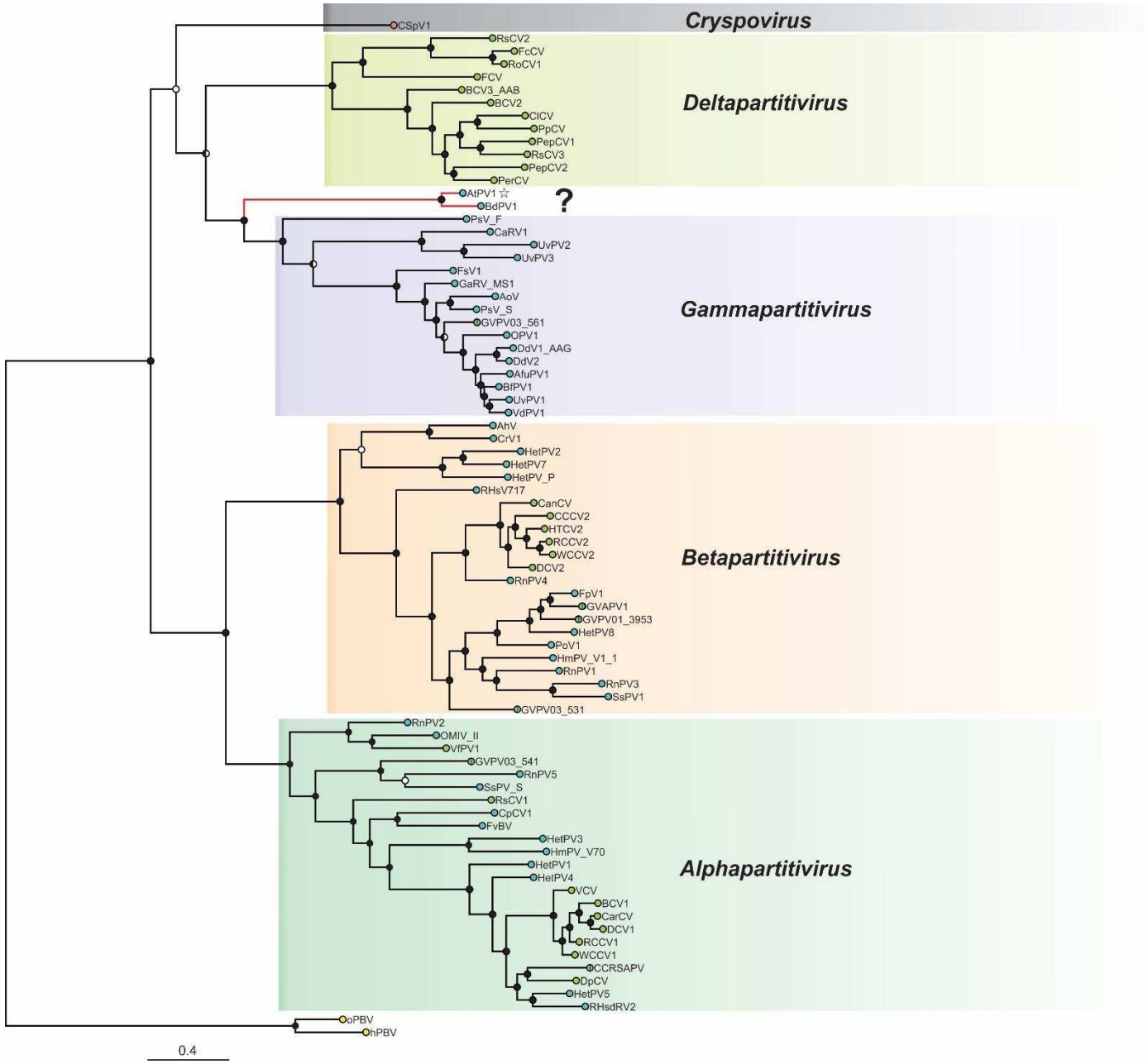

Cryspovirus
CSpV1
Deltapartitivirus
RsCV2
FcCV
RoCV1
FCV
BCV3_AAB
BCV2
ClCV
PpCV
PepCV1
RsCV3
PepCV2
PerCV
?
AtPV1 ☆
BdPV1
Gammapartitivirus
PsV_F
CaRV1
UvPV2
UvPV3
FsV1
GaRV_MS1
AoV
PsV_S
GVPV03_561
OPV1
DdV1_AAG
DdV2
AfuPV1
BfPV1
UvPV1
VdPV1
Betapartitivirus
AhV
CrV1
HetPV2
HetPV7
HetPV_P
RHsV717
CanCV
CCCV2
HTCV2
RCCV2
WCCV2
DCV2
RnPV4
FpV1
GVAPV1
GVPV01_3953
HetPV8
PoV1
HmPV_V1_1
RnPV1
RnPV3
SsPV1
GVPV03_531
Alphapartitivirus
RnPV2
OMIV_II
VfPV1
GVPV03_541
RnPV5
SsPV_S
RsCV1
CpCV1
FvBV
HetPV3
HmPV_V70
HetPV1
HetPV4
VCV
BCV1
CarCV
DCV1
RCCV1
WCCV1
CCRSAPV
DpCV
HetPV5
RHsdRV2
oPBV
hPBV
0.4

Figure 4

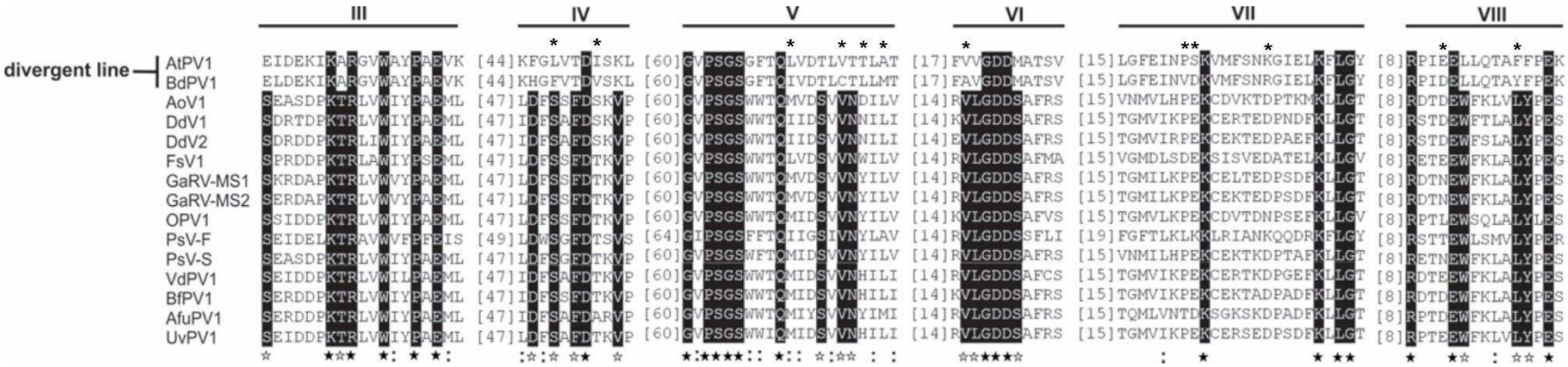

III
IV
V
VI
VII
VIII
divergent line
AtPV1
BdPV1
AoV1
DdV1
DdV2
FsV1
GaRV-MS1
GaRV-MS2
OPV1
PsV-F
PsV-S
VdPV1
BfPV1
AfuPV1
UvPV1

Figure 5

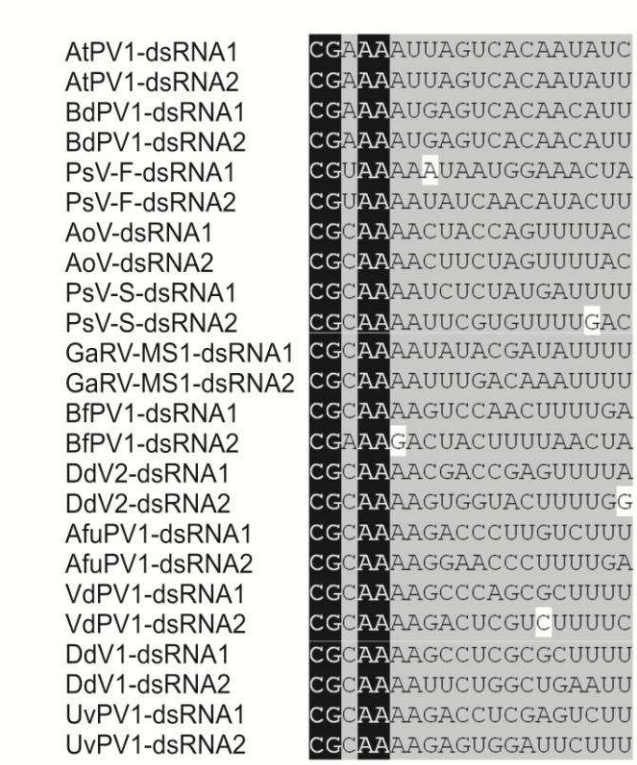

AtPV1-dsRNA1 CGAAAAUUAGUCACAAUAUC
AtPV1-dsRNA2 CGAAAAUUAGUCACAAUAUU
BdPV1-dsRNA1 CGAAAAUGAGUCACAACAUU
BdPV1-dsRNA2 CGAAAAUGAGUCACAACAUU
PsV-F-dsRNA1 CGUAAAAAUAAUGGAAACUA
PsV-F-dsRNA2 CGUAAAAUAUCAACAUACUU
AoV-dsRNA1 CGCAAAACUACCAGUUUUAC
AoV-dsRNA2 CGCAAAACUUCUAGUUUUAC
PsV-S-dsRNA1 CGCAAAAUCUCUAUGAUUUU
PsV-S-dsRNA2 CGCAAAAUUCGUGUUUUGAC
GaRV-MS1-dsRNA1 CGCAAAAUAUACGAUAUUUU
GaRV-MS1-dsRNA2 CGCAAAAUUUGACAAAUUUU
BfPV1-dsRNA1 CGCAAAAGUCCAACUUUUGA
BfPV1-dsRNA2 CGAAAGACUACUUUUAACUA
DdV2-dsRNA1 CGCAAAACGACCGAGUUUUA
DdV2-dsRNA2 CGCAAAAGUGGUACUUUUGG
AfuPV1-dsRNA1 CGCAAAAGACCCUUGUCUUU
AfuPV1-dsRNA2 CGCAAAAGGAACCCUUUUGA
VdPV1-dsRNA1 CGCAAAAGCCCAGCGCUUUU
VdPV1-dsRNA2 CGCAAAAGACUCGUCUUUUC
DdV1-dsRNA1 CGCAAAAGCCUCGCGCUUUU
DdV1-dsRNA2 CGCAAAAUUCUGGCUGAAUU
UvPV1-dsRNA1 CGCAAAAGACCUCGAGUCUU
UvPV1-dsRNA2 CGCAAAAGAGUGGAUUCUUU

Figure 6

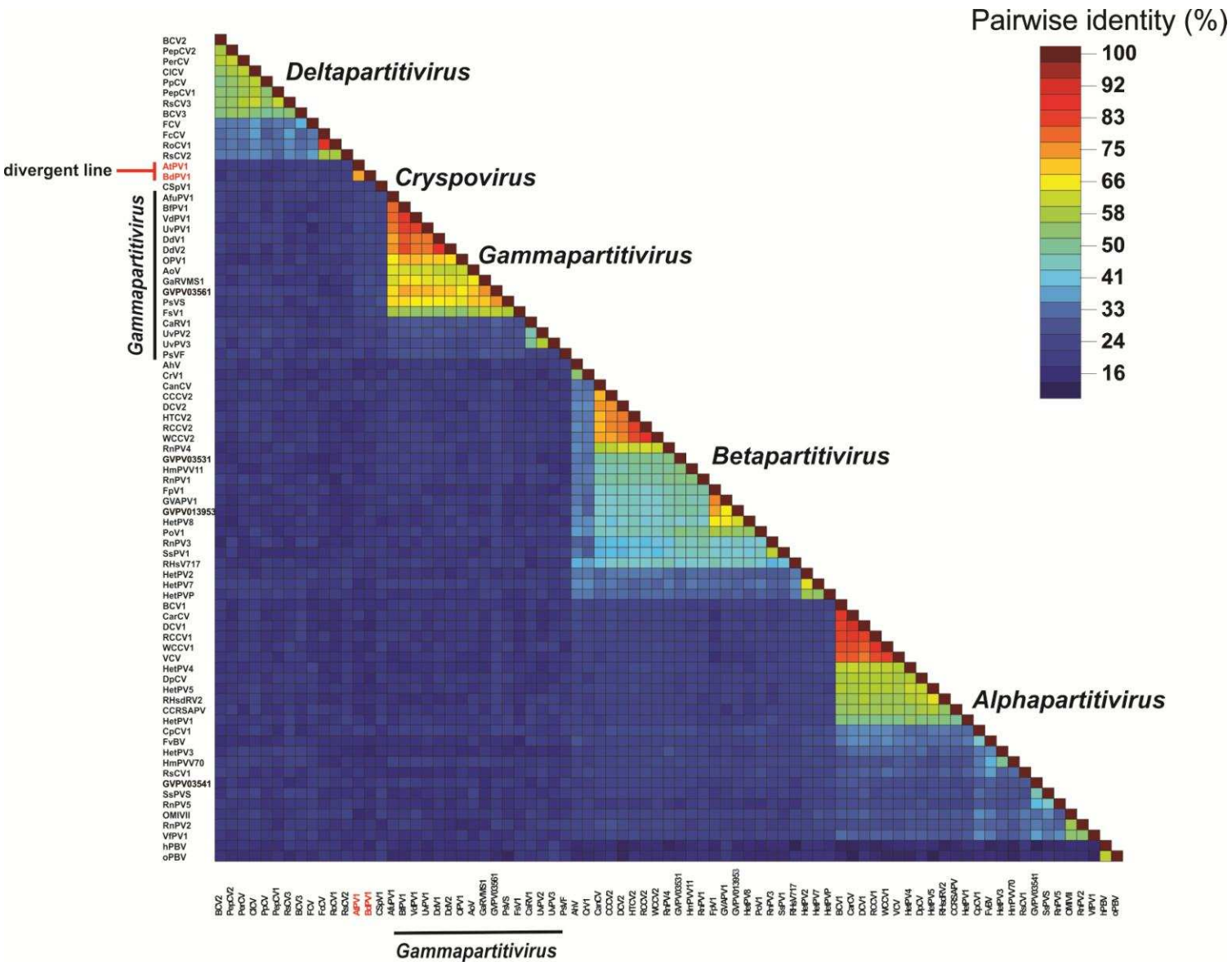

Pairwise identity (%)
100
92
83
75
66
58
50
41
33
24
16
Deltapartitivirus
Cryspovirus
Gammapartitivirus
Betapartitivirus
Alphapartitivirus
divergent line
Gammapartitivirus
BCV2
PepCV2
PerCV
ClCV
PpCV
PepCV1
RsCV3
BCV3
FCV
FcCV
RsCV1
RsCV2
AtPV1
BdPV1
CSpV1
AfuPV1
BfPV1
VdPV1
UvPV1
DdV1
DdV2
OPV1
AoV
GaRVMS1
GVPV03561
PsVS
FsV1
CaRV1
UvPV2
UvPV3
PsVF
AhV
CrV1
CanCV
CCCV2
DCV2
HTCV2
RCCV2
WCCV2
RnPV4
GVPV03531
HmPVV11
RnPV1
FpV1
GVAPV1
GVPV013953
HetPV8
PoV1
RnPV3
SsPV1
RHsV717
HetPV2
HetPV7
HetPVP
BCV1
CarCV
DCV1
RCCV1
WCCV1
VCV
HetPV4
DpCV
HetPV5
RHsdRV2
CCRSAPV
HetPV1
CpCV1
FvBV
HetPV3
HmPVV70
RsCV1
GVPV03541
SsPVS
RnPV5
OMIVII
RnPV2
VfPV1
hPBV
oPBV

Figure 7

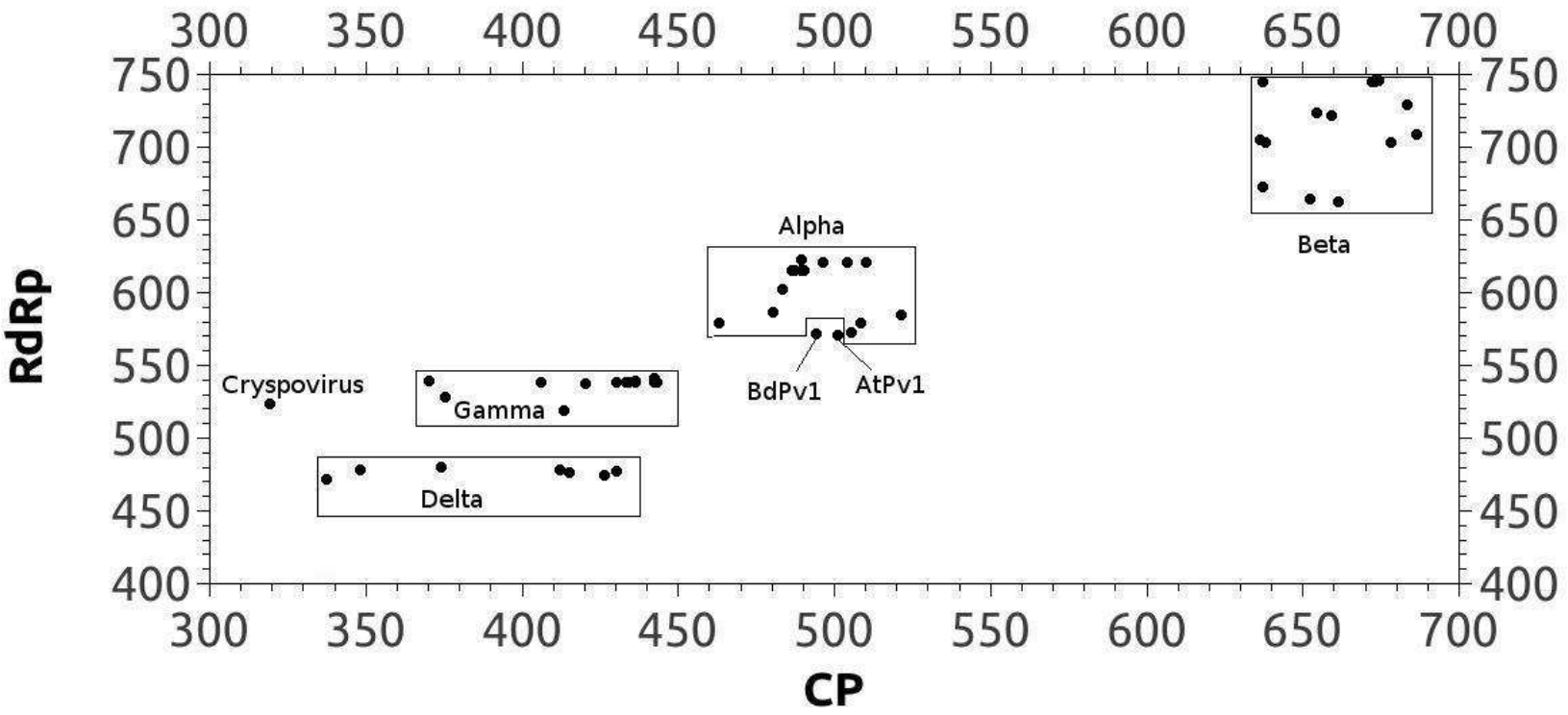
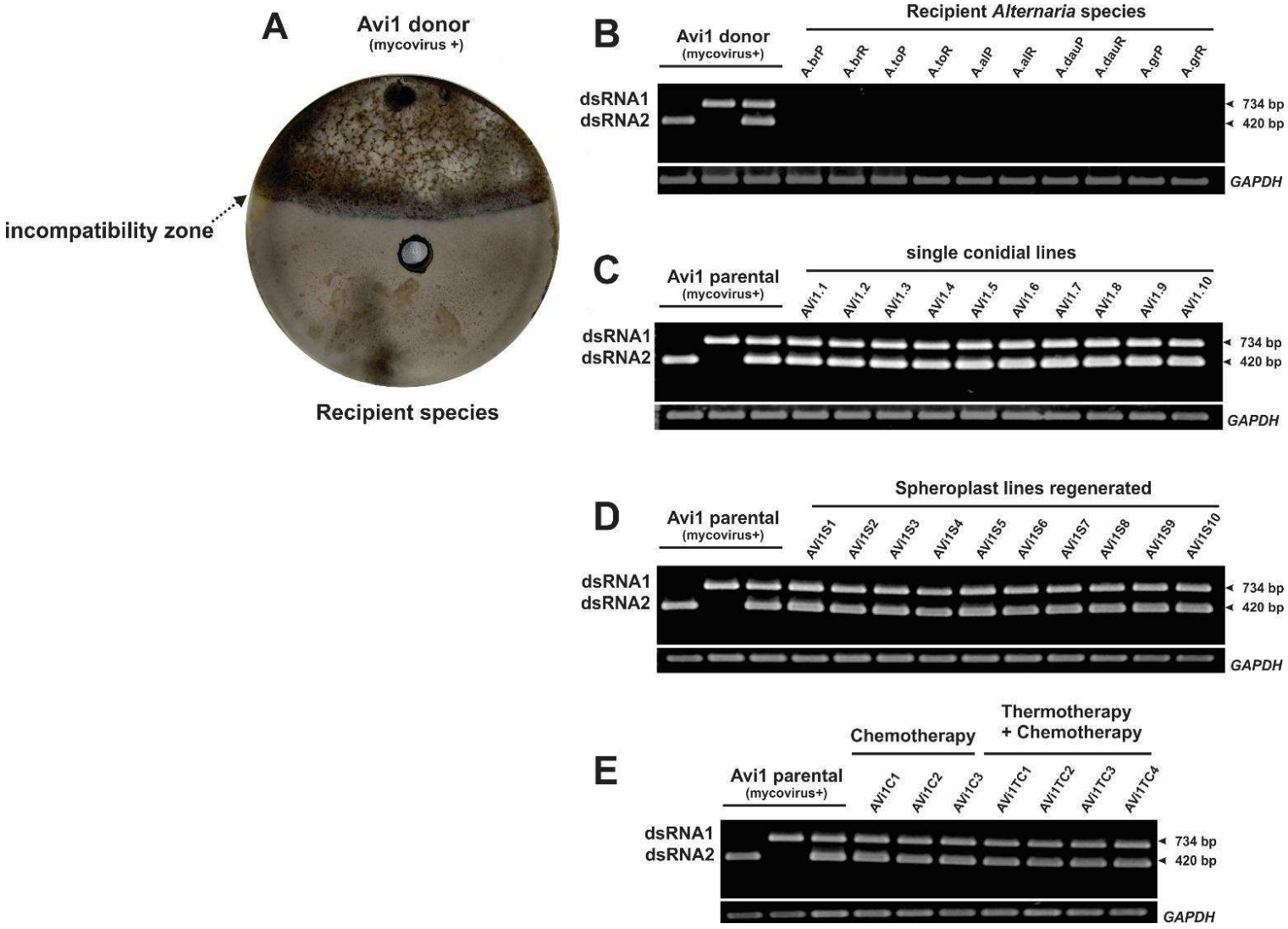

RdRp
CP
300
350
400
450
500
550
600
650
700
750
Alpha
Beta
Cryspovirus
Gamma
Delta
BdPv1
AtPv1

Figure 8


A
Avi1 donor
(mycovirus +)
incompatibility zone
Recipient species
B
Avi1 donor
(mycovirus+)
Recipient Alternaria species
A.brP
A.brR
A.toP
A.toR
A.alP
A.alR
A.dauP
A.dauR
A.grP
A.grR
dsRNA1
dsRNA2
734 bp
420 bp
GAPDH
C
Avi1 parental
(mycovirus+)
single conidial lines
Avi1.1
Avi1.2
Avi1.3
Avi1.4
Avi1.5
Avi1.6
Avi1.7
Avi1.8
Avi1.9
Avi1.10
dsRNA1
dsRNA2
734 bp
420 bp
GAPDH
D
Avi1 parental
(mycovirus+)
Spheroplast lines regenerated
Avi1S1
Avi1S2
Avi1S3
Avi1S4
Avi1S5
Avi1S6
Avi1S7
Avi1S8
Avi1S9
Avi1S10
dsRNA1
dsRNA2
734 bp
420 bp
GAPDH
E
Chemotherapy
Thermotherapy
+ Chemotherapy
Avi1 parental
(mycovirus+)
Avi1C1
Avi1C2
Avi1C3
Avi1TC1
Avi1TC2
Avi1TC3
Avi1TC4
dsRNA1
dsRNA2
734 bp
420 bp
GAPDH

GENERAL CONCLUSIONS

- ✓ The robust mechanism of pathogenesis of Ralstonia virus phiAP1, including the fast lysis and EPS-degrading activity highlights the potential this virus for the management of bacterial wilt, but the host resistance should be managed;

- ✓ The transcription of the *h-ns* genes and the CRISPR inactivity suggests that this system in *R. solanacearum* can be under transcriptional repression;

- ✓ The alterations in genes that coding cellular components of secretion pathway such as *GspL* (Type II secretion system (T2SS) and *pilD* (Type IV pili (Tfp) may be controlling the resistance in the each BIM, but this can have a high cost to *R. solanacearum*, that use these systems for the basal metabolism and pathogenesis;

- ✓ During the conversion of the phytopathogenic *R. pseudosolanacearum* in a commensal bacteria by RsIBR1 infection, its possible that the overproduction of melanin can protect this bacterium of the oxidative challenge into vessels host in the new lifestyle. Together, the biological alterations linked to RsIBR1 infection showed a significant impact on the modulation of parasitic interaction plant-bacteria and microbial adaptation;

- ✓ According to the peculiar characteristics, Alternaria partitivirus 1 (AtPV1), is tentatively a novel species proposed that compose a distinct lineage related to genus *Gammapartitivirus* in the family *Partitiviridae*, apparently on the threshold of radiation

of the a new genus, together with BdPV1. The absence of a virus-free isogenic lineage prevented us from accessing the details of the interaction between AtPV1 and *Alternaria alternata.* In this way, its unclear whether the morphological plasticity observed or the lack of ability to cause disease in plants of the Avi1 isolate is associated with AtPV1 infection;

- ✓ Lastly, the discovery and characterization of new viruses that infect phytophatogens in Brazil, in addition to expanding the informations about the microbe viral diversity, is little known in America, expose the biotechnological potential of these natural enemies of plant pathogens and reveal the interesting role their in modulation and evolutionary course of hosts.