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Characterization of CRISPR-Cas systems in the *Ralstonia solanacearum* species complex

André da Silva Xavier ... See all authors ›

Summary

Clustered regularly interspaced short palindromic repeats (CRISPRs) are composed of an array of short DNA repeat sequences separated by unique spacer sequences that are flanked by associated (*Cas*) genes. CRISPR-Cas systems are found in the genomes of several microbes and can act as an adaptive immune mechanism against invading foreign nucleic acids, such as phage genomes. Here, we studied the CRISPR-Cas systems in plant-pathogenic bacteria of the *Ralstonia solanacearum* species complex (RSSC). A CRISPR-Cas system was found in 31% of RSSC genomes present in public databases. Specifically, CRISPR-Cas types I-E and II-C were found, with I-E being the most common. The presence of the same CRISPR-Cas types in distinct *Ralstonia* phylotypes and species suggests the acquisition of the system by a common ancestor before *Ralstonia* species segregation. In addition, a Cas1 phylogeny (I-E type) showed a perfect geographical segregation of phylotypes, supporting an ancient acquisition. *Ralstoniasolanacearum* strains CFBP2957 and K60^T were challenged with a virulent phage, and the CRISPR arrays of bacteriophage-insensitive mutants (BIMs) were analysed. No new spacer acquisition was detected in the analysed BIMs. The functionality of the CRISPR-Cas interference step was also tested in *R. solanacearum* CFBP2957 using a spacer-protospacer adjacent motif (PAM) delivery system, and no resistance was observed against phage phiAP1. Our results show that the CRISPR-Cas system in *R. solanacearum* CFBP2957 is not its primary antiviral strategy.

Introduction

The Gram-negative plant-pathogenic bacteria *Ralstonia* spp. belong to a species complex, the *Ralstonia solanacearum* species complex (RSSC), which is recognized as a group of considerable genetic diversity encompassing phenotypically diverse strains that can be subdivided into four phylotypes (Allen *et al.*, 2005; Prior and Fegan, 2005). Phylotypes I, II and III contain strains predominantly from Asia, America and Africa and surrounding islands, respectively, whereas phylotype IV is comprised of strains from Indonesia and some strains from Japan, Australia and the Philippines. Phylotype IV is the most heterogeneous, containing strains assigned to *R. solanacearum*, *R. syzygii* and the Blood Disease Bacterium (BDB) (Prior and Fegan, 2005).

Safni *et al.* (2014) proposed a taxonomic restructuring of the RSSC into three soil-borne species, *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotypes I and III) and *R. syzygii* (phylotype IV, including *R. syzygii* and BDB strains). Recent genomic and proteomic approaches support this taxonomic and nomenclatural reclassification of RSSC (Prior *et al.*, 2016). These pathogens have a wide host range, infecting more than 200 botanical species belonging to more than 50 families, including economically important crops (Denny, 2006; Hayward, 1991). Despite the heterogeneity of the RSSC, all members colonize plant xylem vessels and induce wilting in host plants.

Bacteriophages belonging to four viral families (*Podoviridae*, *Myoviridae*, *Siphoviridae* and *Inoviridae*) have been described infecting RSSC strains, and some have shown promise for the control of bacterial wilt disease (Addy *et al.*, 2012; Elhalag *et al.*, 2018; Kawasaki *et al.*, 2009, 2016; Liao, 2018; Ozawa v, 2001; Su *et al.*, 2017; Toyoda *et al.*, 1991; Van Truong Thi *et al.*, 2016; Yamada, 2012) Nevertheless, more studies are needed for a future implementation of phage therapy in the fight against this destructive plant disease (Álvarez and Biosca, 2017).

Bacteria and their viruses go through continuous cycles of co-evolution, in which resistant hosts emerge and the genotypic composition of their populations change (Samson *et al.*, 2013). In this dynamic scenario, antiviral mechanisms play a key role in regulating bacterial populations (Koskella and Brockhurst, 2014). Bacteria use a wide range of strategies to evade viral infection (Labrie *et al.*, 2010), including the immune adaptive CRISPR-Cas [clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas)] system (Barrangou and Horvath, 2017). In the RSSC, natural resistance to viral infection has been observed, but the variability in resistance phenotypes suggests the involvement of more than one defence mechanism (Kawasaki *et al.*, 2016).

CRISPR-Cas systems provide sequence-specific protection against foreign nucleic acids, including viral genomes, plasmids and mobile genetic elements (Barrangou and Horvath, 2017). These systems are widely distributed in the genomes of archaea (85%) and bacteria (45%) (Grissa *et al.*, 2007), and their diversity allows them to be classified into two major classes consisting of six types and several subtypes according to their *Cas* gene composition (Makarova *et al.*, 2015; Mohanraju *et al.*, 2016).

A generic defence unit consists of a CRISPR array, comprising short palindromic repeats interspersed with hypervariable short DNA sequences, referred to as spacers, flanked by CRISPR-associated (*Cas*) genes. Three distinct steps control CRISPR-mediated immunity phenotype: adaptation, expression and interference (Magadán *et al.*, 2012). During the adaptation stage, fragments of foreign DNA, known as protospacers, are incorporated into the CRISPR array and constitute the memory of the microbial immune system. Then, spacers and repeats making up the CRISPR arrays are expressed as a long precursor RNA which is processed into small CRISPR RNAs. The latter guides the Cas proteins to target and cleave their cognate DNA or RNA (interference step) (Barrangou and Horvath, 2017; Makarova *et al.*, 2015; Silas *et al.*, 2016; Wright *et al.*, 2016).

Several plant-pathogenic bacteria harbour CRISPR-Cas systems, including *Xanthomonas oryzae*, *Pectobacterium atrosepticum*, *Erwinia amylovora* and *X. albilineans*. However, few comprehensive characterizations of these systems have been conducted in bacteria that cause plant diseases. In some cases, such as for *X. oryzae* and *E. amylovora*, the system has been explored as a tool for epidemiological studies (McGhee and Sundin, 2012; Midha *et al.*, 2017; Pieretti *et al.*, 2015; Richter and Fineran, 2013; Semenova *et al.*, 2009; Tancos and Cox, 2016).

The type I-E CRISPR-Cas system is found in a large number of bacteria, including *Escherichia coli*, *Salmonella* spp. and *Streptomyces* spp., and has been extensively studied (Fabre *et al.*, 2012; Guo *et al.*, 2011; Haft *et al.*, 2005; Kiro *et al.*, 2013; Shariat *et al.*, 2015). Its functional characterization has revealed a system that is often 'immunocompromised' in its native state as a result of the silencing of CRISPR-Cas promoters (Guo *et al.*, 2011; Kiro *et al.*, 2013; Medina-Aparicio *et al.*, 2011; Pul *et al.*, 2010; Westra *et al.*, 2010). Therefore, in such case, it does not actively participate in phage resistance. Other studies have suggested a different role for CRISPR-Cas systems, such as biofilm and pathogenicity regulation in some species (Westra *et al.*, 2014).

Here, we demonstrate the presence of CRISPR-Cas systems in RSSC strains and provide a comparative analysis of their diversity across strains. Furthermore, we show that the adaptation and interference activities of the CRISPR-Cas type I-E system do not provide

phage protection and that other defence system(s) are at play in *R. solanacearum* strain CFBP2957, an American isolate harbouring a CRISPR-Cas system.

Results

The analysis of 54 genomes of *Ralstonia* spp. strains, including 51 strains belonging to the RSSC and three non-plant pathogens, revealed the presence of canonical type I and II CRISPR-Cas loci, classified as I-E and II-C types (Makarova *et al.*, 2015) (Fig. 1). However, CRISPR-Cas systems appeared to be complete (CRISPR locus and Cas operon) in only 31% (16 of 52) of the genomic sequences analysed. Thirteen of the 16 strains had subtype I-E and only three had subtype II-C (Table 1). Ten strains of *R. solanacearum* (phylotype II), two of *R. pseudosolanacearum* (one in phylotype I and another in phylotype III) and one of *R. syzygii* ssp. *celebesensis* (BDB 229, formerly *Blood Disease Bacterium*, phylotype IV) had type I-E. The remaining three strains, one *R. syzygii* ssp. *syzygii* (phylotype IV) and two *R. solanacearum* (phylotype II), had type II-C (Table 1). Of note, CRISPR loci were not found in the three non-plant-pathogenic *Ralstonia* strains.
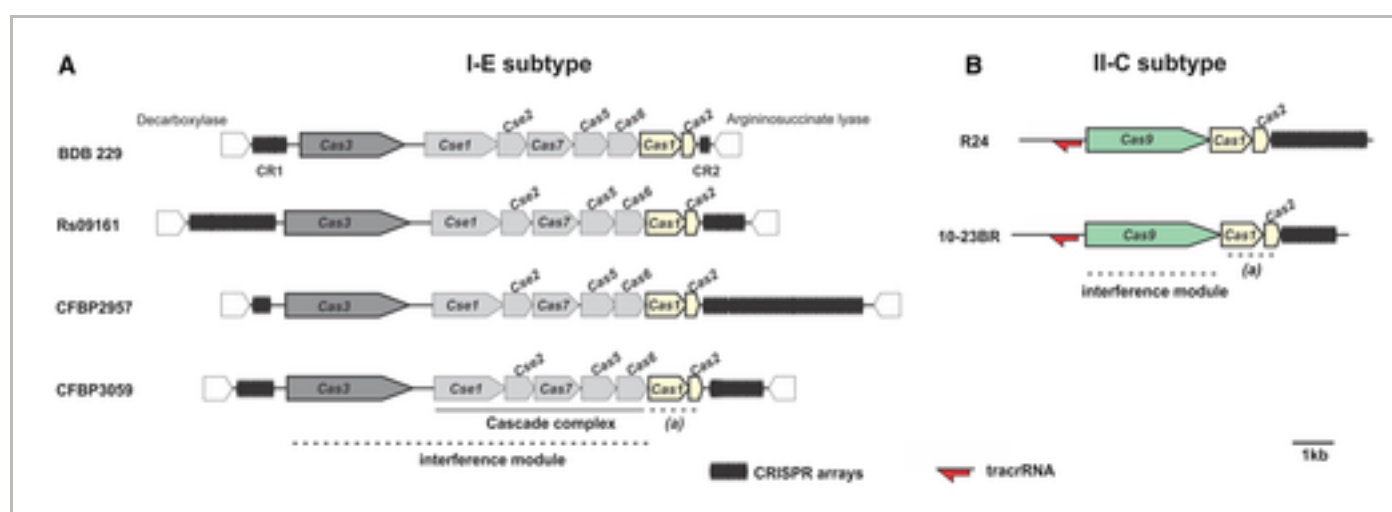

Figure 1

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Overview of the two types of CRISPR-Cas system found in *Ralstonia solanacearum* species complex (RSSC) strains. CRISPR-Cas loci in types I-E (A) and II-C (B). The open reading frames (ORFs) (arrows) and CRISPR arrays (black traces) are drawn to scale. Conserved ORFs in the CRISPR flank region are shown in white. Adaptation and interference modules are coloured yellow and gray, respectively (A) or yellow and green, respectively (B). The codes on the left are the identifiers of the RSSC strains. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Presence of CRISPR-Cas loci in the genomes of the *Ralstonia solanacearum* species complex (RSSC) and related *Ralstonia* strains.

Strains	CRISPR loci	Subtype	Phylotype	Host	Origin	Genome	Reference
Ralstonia solanacearum							
UY031	None	None	IIB	Potato	Uruguay	Complete genome	Guarischi-Sousa *et al.* (2016)
Po82	2	I-E	IIB	Potato	Mexico	Complete genome	Xu *et al.* (2011)
UW179	2	I-E	IIB	Plantain	Colombia	Draft	Ailloud *et al.* (2015)
Molk2	None	None	IIB	Banana	Philippines	Complete genome	Guidot *et al.* (2009)
IBSBF1503	2	I-E	IIB	Cucumber	Brazil	Complete genome	Ailloud *et al.* (2015)
K60T	2	I-E	IIA	Tomato	USA	Complete genome	Remenant *et al.* (2012)
IPO1609	None	None	IIB	Potato	Netherlands	Complete genome	Guidot *et al.* (2009)

Source of the strains: *R. solanacearum* CFBP2957 and K60[T] strains belong to the Culture Collection of Phytopathogenic Bacteria from the Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI, USA.

Despite the conserved architecture of CRISPR loci among the RSSC strains, they varied in size, ranging from four to 80 spacers per array (Fig. 1), as well as in their number, from one to three arrays per genome (Table 1). Type I-E systems were conserved in all 13 strains, in all cases flanked upstream by an operon containing genes coding for decarboxylases and downstream by a gene coding for an argininosuccinate lyase (Fig. 1A). These characteristics were not shared among strains containing type II-C (Fig. 1B). Pairwise identity analysis of the Cas proteins from type I-E (Cas3, Cse1, Cse2, Cas7, Cas5, Cas6, Cas2 and Cas1) of *R. solanacearum* CFBP2957 with the other strains showed a high identity (88%–100%) (Table 2). The Cas proteins of type II-C (Cas9, Cas1 and Cas2) were even more conserved, with 94%–100% identity (Table S1, see Supporting Information). As I-E was more frequent and found in

distinct lineages of the RSSC, further analyses were performed on this type.

Table 2. Cas protein comparisons among *Ralstonia solanacearum* species complex (RSSC) strains containing the type I-E CRISPR-Cas system.

Strains	Percentage identity of type 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 ssp. *celebesensis*								

[a] Phylotypes.

Phylogenetic trees based on the Cas1 protein of RSSC and other bacterial species containing CRISPR-Cas types I-A, I-B, I-C, I-E and I-F positioned all 13 RSSC strains in the I-E type clade (Fig. 2), confirming their classification. Phylogenetic analysis with only the RSSC Cas1 of type I-E showed a perfect congruence with the RSSC phylogenetic tree using the nucleotide sequence of the core gene *Egl* (Castillo and Greenberg, 2007). This suggests an ancient

acquisition of the CRISPR-Cas type I-E system, before the segregation of the *Ralstonia* species (Fig. S1, see Supporting Information).
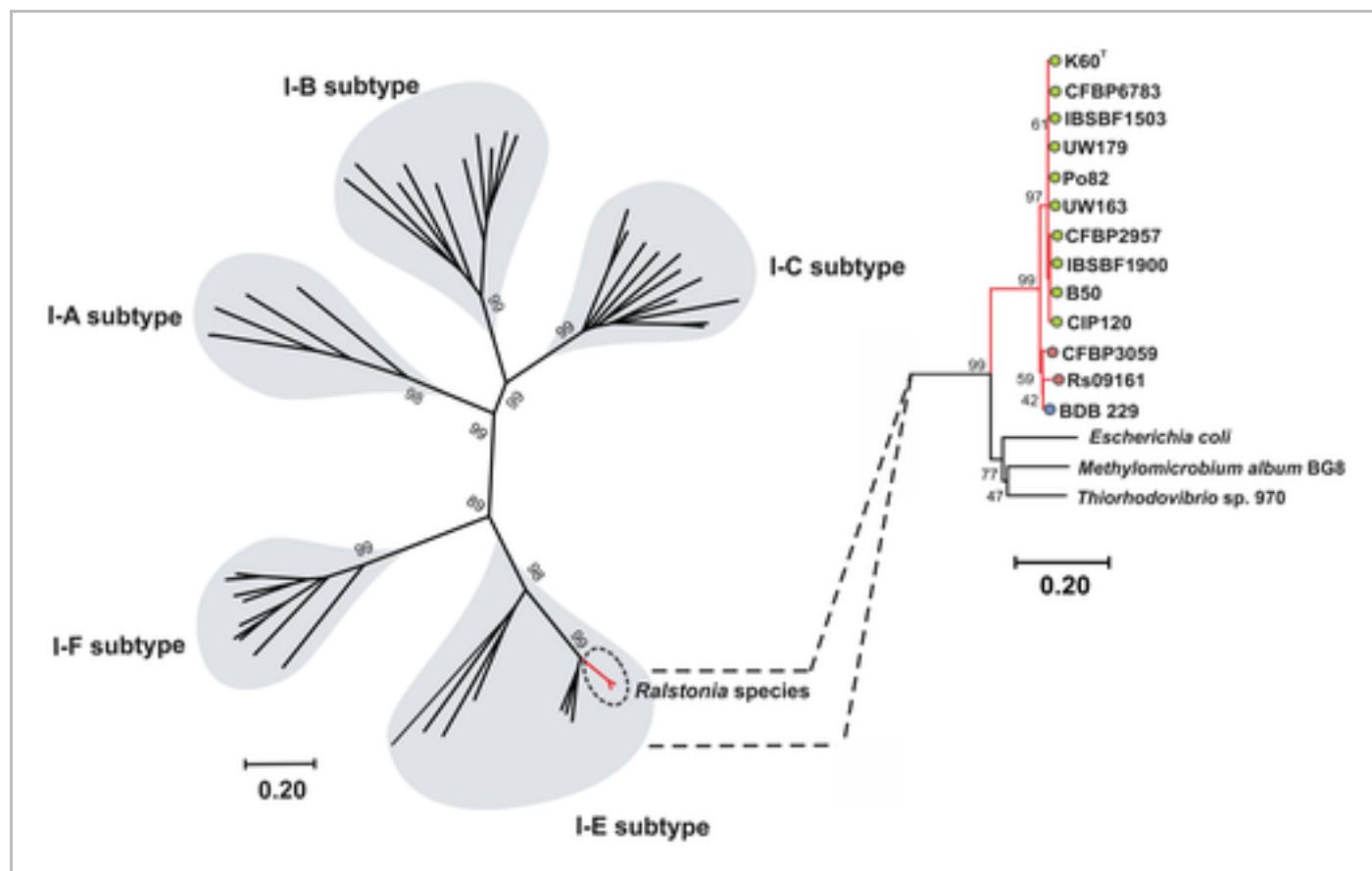

Figure 2

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Phylogeny of Cas1 proteins from different CRISPR-Cas types. The maximum likelihood tree was constructed using the MEGA 7.0 program and the Jones–Taylor–Thornton evolutionary model. Individual genes are labelled with taxon names and bootstrap values are indicated as percentage points. The Cas1E cluster that is coloured in red contains only *Ralstonia solanacearum* species complex (RSSC) strains, where each species is represented by the colours of the circles: *R. solanacearum* (light green), *R. pseudosolanacearum* (light red) and *R. syzygii* ssp. *celebesensis* (light blue). [Colour figure can be viewed at wileyonlinelibrary.com]

We also analysed the 32-bp spacers in the arrays of the 13 strains containing the type I-E system. Only 26% (200 of 734) of the spacers matched potential protospacers, 18% (136 spacers) of which corresponded to plasmids and 8% (64 spacers) to viral genomes (Fig. S2, see Supporting Information).

The CRISPR-Cas system does not confer resistance to phage phiAP1

To test the functionality of the *Ralstonia* type I-E system, we used *R. solanacearum* CFBP2957, a strain belonging to the diverse phylotype IIa subgroup, which is economically important and widespread in the Americas (Prior and Steva, 1990; Wicker *et al.*, 2012). In this strain, the

Cas genes are 9.5 kb in size and are flanked by two CRISPR arrays, a smaller one with seven repeat-spacer units (CRISPR1) and a larger one containing 59 repeat-spacer units (CRISPR2). Analysis of the leader sequences of both CRISPRs revealed typical A/T-rich sequences with conserved non-coding sequences (Jansen *et al.*, 2002). Based on its higher number of spacers, it is tempting to speculate that the CRISPR2 array of *R. solanacearum* CFBP2957 might be active (Horvath *et al.*, 2008).

We then tested whether the CRISPR-Cas system of *R. solanacearum* CFBP2957 was active against the virulent phage phiAP1, a member of the *Phikmvvirus* genus of the *Podoviridae* family isolated from a Brazilian soil sample (Xavier *et al.*, 2018). The *R. solanacearum* CFBP2957 was challenged with phage phiAP1. Bacteriophage-insensitive mutants (BIMs) were obtained on plates after a 72-h incubation period (Hynes *et al.*, 2017), and their CRISPR arrays were analysed (Fig. 3A,B). Simultaneously, *R. solanacearum* K60[T], a strain classified in phylotype subgroup IIa, as well as CFPB2957 (Prior and Steva, 1990; Wicker *et al.*, 2012), was subjected to the same phiAP1 challenge assay to check whether the immune response was strain dependent. For each wild-type (WT) parental strain, 30 BIMs were randomly selected and analysed to confirm the phage resistance phenotype by phage spot test. All BIMs were highly resistant to phiAP1, even when using high-titre phage lysates (Fig. 3C). Phage adsorption assays were performed and all BIMs allowed phage adsorption, indicating that the resistance phenotype was not caused by receptor mutation (Fig. S3A, see Supporting Information). Cell survival assay indicated that the phage-infected BIM cells could still be recovered, indicating that an abortive infection mechanism was not induced in BIMs (Fig. S3B). Viral DNA replication was not detected in BIMs (Fig. S3C).
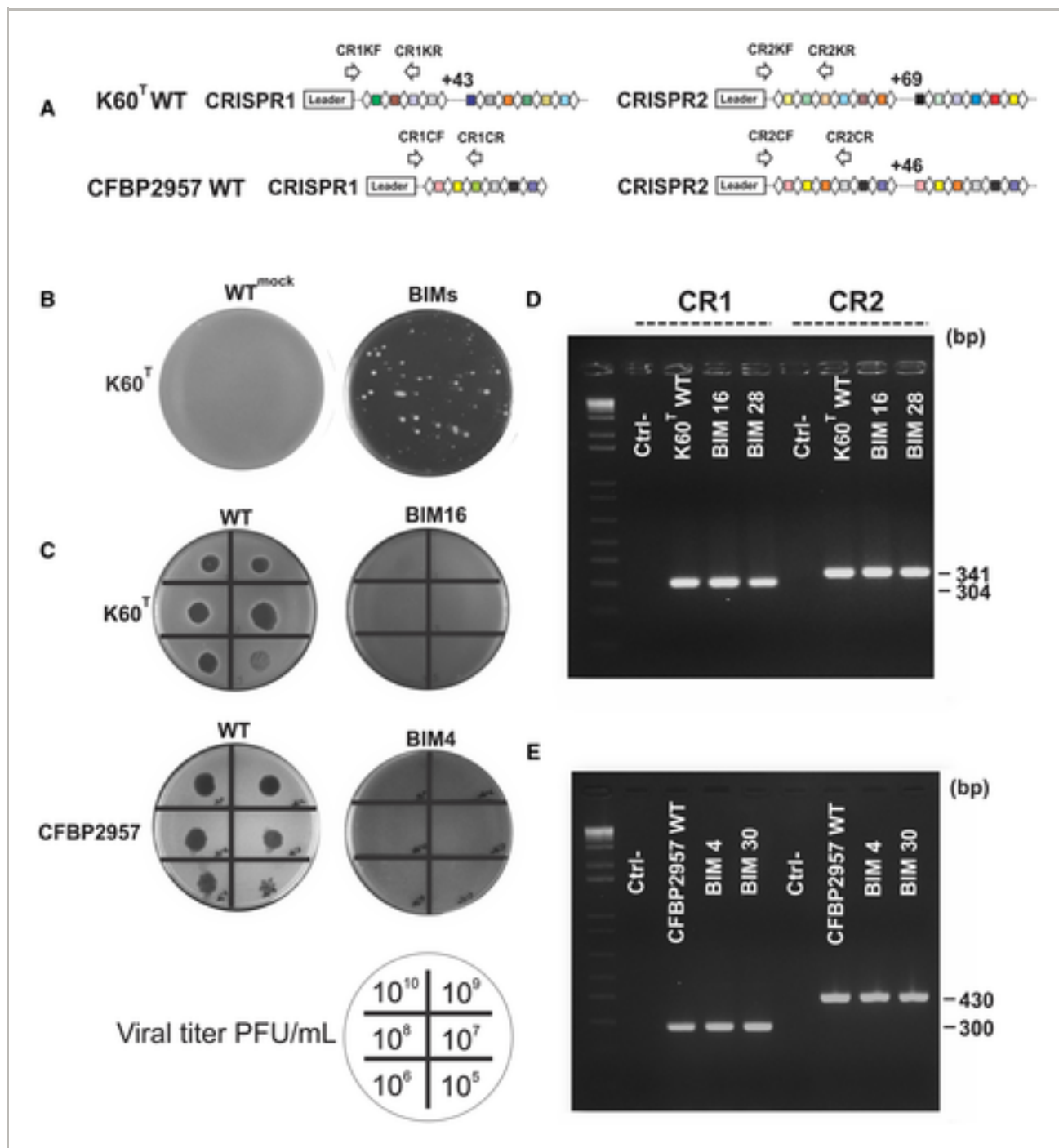
Figure 3

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Analysis of CRISPR sequences and bacteriophage-insensitive mutants (BIMs). (A) Polymerase chain reaction (PCR) screening of the spacer array in *Ralstonia solanacearum* K60^T and CFBP2957 strains. (B) Isolation of BIMs derived from the phage-sensitive strain K60^T after *Ralstonia* phage phiAP1 infection. (C) K60^T- and CFBP2957-derived BIMs exhibit complete phage resistance independent of viral titre. PCR screening to detect novel spacer acquisition in CRISPR1 (CR1) and CRISPR2 (CR2) for K60^T-derived BIMs (D) and CFBP2957-derived BIMs (E). Ctrl-, PCR-grade H$_2$O. [Colour figure can be viewed at wileyonlinelibrary.com]

We then performed polymerase chain reaction (PCR) screening to detect the integration of new spacers at the 5′-end of the CRISPR arrays. No spacer acquisition was detected in any of the BIMs derived from strains *R. solanacearum* K60^T (Fig. 3D) and *R. solanacearum* CFPB2957 (Fig. 3E). Sequencing of the PCR products also confirmed the absence of spacer acquisition.

Because ectopic spacer acquisition has been observed recently in some bacterial strains (Achigar *et al.*, 2017), we also analysed by PCR and sequencing the entire CRISPR arrays and still found no evidence of spacer acquisition in both sets of BIMs (Fig. 4). Together, our results showed that the adaptation stage was not active in *R. solanacearum* CFBP2957 and K60[T] under the laboratory conditions tested, indicating that another antiviral strategy mainly protects these *R. solanacearum* strains against phiAP1 infection. We also obtained BIMs of *R.pseudosolanacearum* GMI1000 (strain without CRISPR loci), which reinforces the idea that CRISPR is dispensable for phage resistance in RSSC (data not shown).
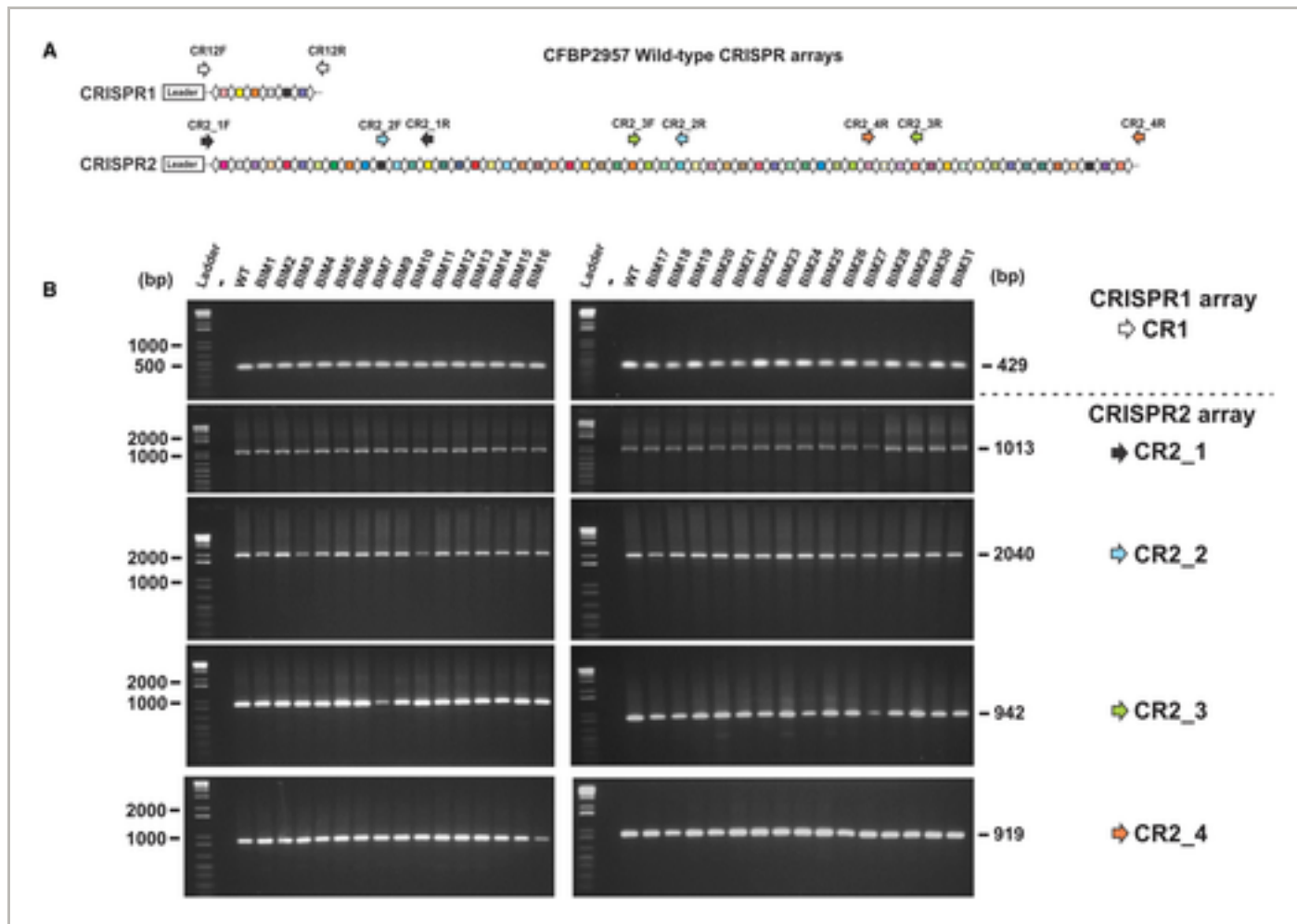

Figure 4

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Ralstonia solanacearum CFBP2957 CRISPR arrays. (A) Schemes for polymerase chain reaction (PCR) amplification of CRISPR1 array (CR1) and overlapping primers for full-length amplification of CRISPR2 array (CR2). (B) PCR detection of spacer acquisition in 30 CFBP2957-derived bacteriophage-insensitive mutants (BIMs) according to the strategies shown in (A). On the right side of the gels are shown the codes for each pair of primers employed in the amplification of the CRISPR arrays according to the nomenclature adopted in the scheme shown in (A). The only CR1 array fragment was amplified with the pair of primers represented by the white arrows, but, for full-length amplification of the CR2 array, the set of overlapping primers was necessary and they are represented by the black, blue, green and orange arrows, corresponding to four individually amplified fragments totalling the CR2 array.

Absence of DNA interference by the CRISPR-Cas system in *R. solanacearum* CFBP2957

To verify whether CRISPR-mediated interference is active in *R. solanacearum* CFBP2957, we attempted to introduce a plasmid targeted by one of the spacers found in CRISPR2. In *R. solanacearum* CFBP2957, spacers matching viral sequences were only detected in CRISPR2: spacers 36 [*Pseudomonas* phage JBD44, two single nucleotide polymorphisms (SNPs), 28/32 nucleotides, 90% identity] (Bondy-Denomy *et al.*, 2016) and 49 (*Ralstonia* phage phiRSA1, one SNP, 31/32 nucleotides, 97% identity) (Fujiwara *et al.*, 2008) (Fig. 5B). These two spacers (36 and 49) with sequences matching viral genomes were chosen and modified to become protospacers on an experimental model using a plasmid which is able to replicate in RSSC (Fig. 5A–D). Because the protospacer adjacent motif (PAM) had not been determined for the type I-E system in RSSC, we conducted a search for putative PAM sequences by aligning protospacers found in plasmid sequences and viral genomes. The prevalent nucleotide sequences upstream of the proptospacers were AGG and AAG, leading to the A*R*G consensus (Fig. 5C). Of note, the AGG sequence has been reported as a strong PAM in other systems (Leenay *et al.*, 2016). As reported previously, Type I systems can contain the tri-nucleotide PAM downstream of protospacers opposite to the 5′-handle crRNA (Gudbergsdottir *et al.*, 2011; Mojica *et al.*, 2009; Westra *et al.*, 2013).

Figure 5
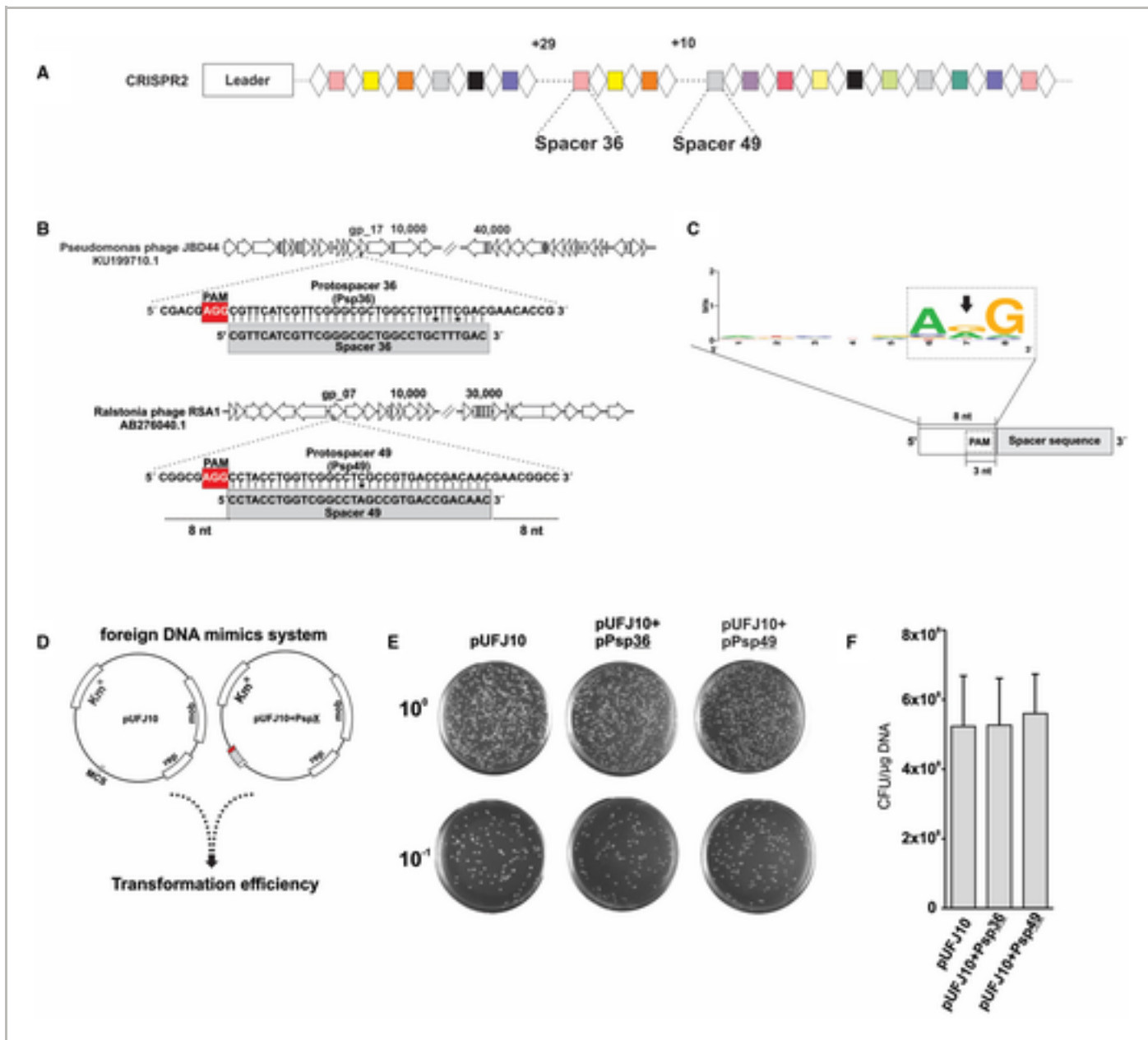
Lack of CRISPR interference in *Ralstonia solanacearum* CFBP2957. (A) Spacers 36 and 49 belong to the CRISPR2 array (CR2) and their sequences match with viral protospacers. (B) Putative phage genome targets of strain CFBP2957 CRISPR2, considered here as the possible sources of spacers 36 and 49. (C) Predominant putative protospacer adjacent motif (PAM) sequences found in the first three upstream nucleotides to the core of complementarity with the spacer sequences. To make sure that no additional unknown neighbour motifs of the PAM were excluded, the five nucleotides nearest upstream of the putative PAM sequences were also analysed. (D) Cloning of the protospacers to validate the cognate DNA delivery system. (E) Transformation of the strain CFBP2957 by electroporation using plasmids that contain protospacers. Samples were plated with or without dilution. (F) Comparison of transformation efficiency. pUFJ10 without protospacers (negative control); pUFJ10+Psp36 and pUFJ10+Psp49, pUFJ10 containing protospacer 36 or 49, respectively. Bars are presented as mean values from two independent experiments ± 1SD (standard deviation). [Colour figure can be viewed at wileyonlinelibrary.com]

Because the presence of the PAM flanking the protospacer makes it a preferred target for interference (Deveau *et al.*, 2008; Yosef *et al.*, 2013), we also cloned the 5′-AGG-3′ sequence next to the targeted plasmid protospacers (Fig. 5B–D). Interestingly, this PAM was also originally present in the respective protospacers targeted by spacers 36 and 49. The colony-forming unit (CFU)/µg of DNA values obtained indicated that transformation with plasmids containing spacer 36 (pPsp36) or 49 (pPsp49) was as efficient as that of the empty vector (pUFJ10), indicating that CRISPR interference is not functional in *R. solanacearum* CFBP2957 (Fig. 5E,F).

The Cas operon is not expressed in *R. solanacearum* CFBP2957

It has been shown in other bacteria that transcription of the *cas* operon can be repressed under normal growth conditions (Guo *et al.*, 2011; Medina-Aparicio *et al.*, 2011; Pul *et al.*, 2010; Westra *et al.*, 2014). To test whether the phenotype of CRISPR-Cas inactivity is caused by a similar repression in *R. solanacearum*, the expression of *Cas* genes was analysed using RNA isolated from phage-infected and non-infected *R. solanacearum* CFBP2957 cultures. Regardless of viral presence, *Cas* gene expression was not detected (Fig. 6), which probably explains the inactivity of the CRISPR-Cas system in this strain.
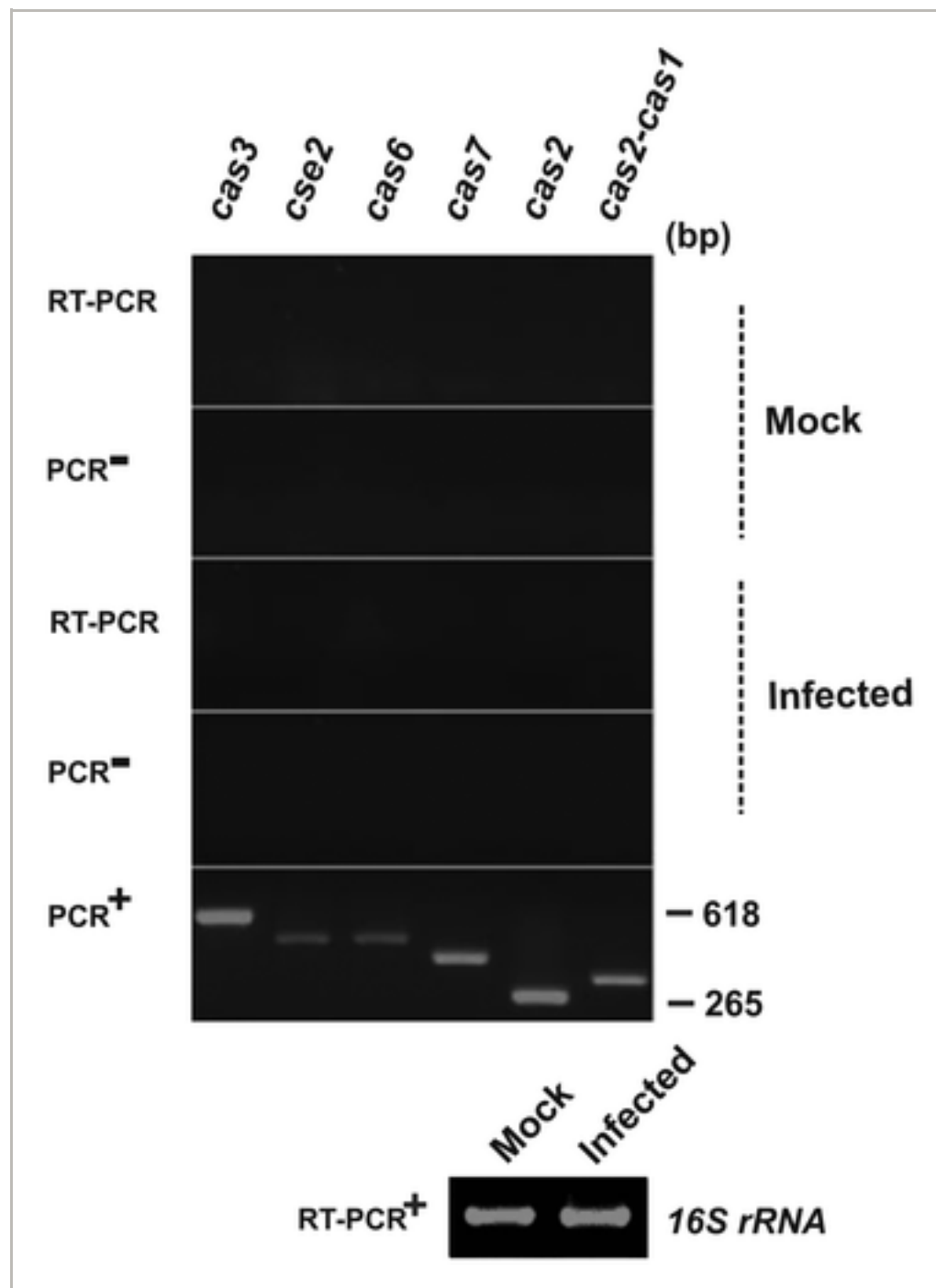
Figure 6

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Cas gene expression profile before (Mock) and after (Infected) phage challenge. Reverse transcription-polymerase chain reaction (RT-PCR) using RNA extracted from phage-infected *Ralstonia solanacearum* CFBP2957 or mock culture after DNAse I treatment. PCR⁻, negative control of PCR using RNA after DNAse I treatment. PCR⁺, positive control of PCR using genomic DNA from strain CFBP2957. RT-PCR⁺, positive control of RT-PCR using specific primers to 16S rRNA. The primers used in these reactions are listed in Table S4 (see Supporting Information).

Discussion

In RSSC strains, the type I-E CRISPR-Cas system was found at a higher frequency than the type II-C system (Table 1). This type I-E system is present in many proteobacteria (Haft *et al.*,

2005), including *E. coli* (Kunin *et al.*, 2007). The presence of type I-E systems in distinct strains and species, including *R. syzygii* ssp. *celebensis* strain BDB229 and *R. syzygii* ssp. *syzygii* strain R24 endemic to Indonesia (Remenant *et al.*, 2011), suggests that a common ancestor acquired this system. This probably occurred in the putative origin of *Ralstonia* spp. (Indonesia), before the fragmentation of Gondwana, according to the demographic history and probable migration of the last common ancestor of the RSSC (Wicker *et al.*, 2012). The phylogenetic tree of Cas1 (I-E type) produced a perfect geographical segregation of phylotypes, completely congruent with the phylogeny using the nucleotide sequence of the core gene *Egl* (Castillo and Greenberg, 2007). This evidence supports the hypothesis of the acquisition of the CRISPR-Cas locus being as old as the RSSC itself. However, the *Ralstonia* type II-C system is not located in a syntenic locus on the chromosome of the three strains found to carry it, suggesting that these modules were acquired via horizontal gene transfer. Reinforcing these findings, we noticed that the ORFs flanking the type II-C loci are mobile genetic elements (data not shown). It has been proposed that CRISPR-Cas systems can be readily transferred between bacteria, even beyond phylum boundaries (Bertelli *et al.*, 2016; Godde and Bickerton, 2006; Horvath *et al.*, 2009; Tyson and Banfield, 2008).

We did not detect CRISPR-Cas systems in the genomes of three non-plant-pathogenic *Ralstonia* spp. These findings are likely to be the result of a divergent evolutionary pathway between these two contrasting groups. Previous phylogenomic analyses based on 686 single-copy genes suggested that the last common ancestor of plant-pathogenic species was not shared with non-plant-pathogenic species (Zhang and Qiu, 2016).

Although the CRISPR-Cas system of *R. solanacearum* CFBP2957 contains the elements for immunity, we could not detect spacer acquisition and plasmid interference under our laboratory conditions. Seventy four per cent of the spacers did not match any sequence, including viral sequences, reinforcing the hypothesis that the CRISPR system in *R. solanaceraum* is not a main defence mechanism against viruses. However, we know only very little of the viral diversity present in the environment (Breitbart *et al.*, 2002; Paez-Espino *et al.*, 2016; Simmonds *et al.*, 2017), which could also explain the lack of a match with spacer sequences. It remains to be seen whether this system would be active under other environmental conditions, such as during bacterial growth into plant xylem vessels, when virulence genes are required. Our data suggest that the CRISPR-Cas system is not the dominant adaptation strategy used by *R. solanacearum* strain CFBP2957 to resist phage infection. The resistance mechanisms in the BIMs resistant to phiAP1 are currently under study.

The lack of *cas* gene expression could explain the absence of a protective phenotype against invasion by foreign DNA. In *Enterobacteriaceae*, the inactivity of type I-E CRISPR-Cas systems

has been associated with negative regulation by H-NS (Medina-Aparicio *et al.*, 2011). For example, it has been shown that transcription of the *cas* operon in *E. coli* K12 is repressed by H-NS (Pul *et al.*, 2010). H-NS proteins are general regulators of gene expression that act by compacting bacterial chromosomes with the help of AT-rich, curved DNA, characteristics often located in the close vicinity of promoters. The derepression of *cas* genes is sufficient to restore CRISPR-mediated immunity (Swarts *et al.*, 2012), showing that H-NS-mediated negative regulation is a reversible phenotype.

Many RSSC strains carry multiple genes coding for H-NS proteins (Stoebel *et al.*, 2008). In *R. solanacearum* CFBP2957, three *h-ns* genes are present on the megaplasmid (Fig. 7A), and the deduced H-NS proteins, although smaller than those from other bacteria, contain the two conserved functional domains (Fig. 7B). The phylogeny of the H-NS amino acid sequences from different species was congruent with the taxonomic grouping, allowing bacterial families to be separated, such as *Enterobacteriaceae*, *Pseudomonadaceae* and *Burkholderiaceae* (Fig. 7C). Interestingly, two of the *Ralstoniah-ns* genes (*h-ns1* and *h-ns3*) are related to a viral H-NS from the EBPR podovirus 1 (Skennerton *et al.*, 2011). It is tempting to speculate that viruses may also be using H-NS-dependent mechanisms to escape from the CRISPR-Cas system (Skennerton *et al.*, 2011), or even that H-NS from RSSC strains has been acquired from viral donors.
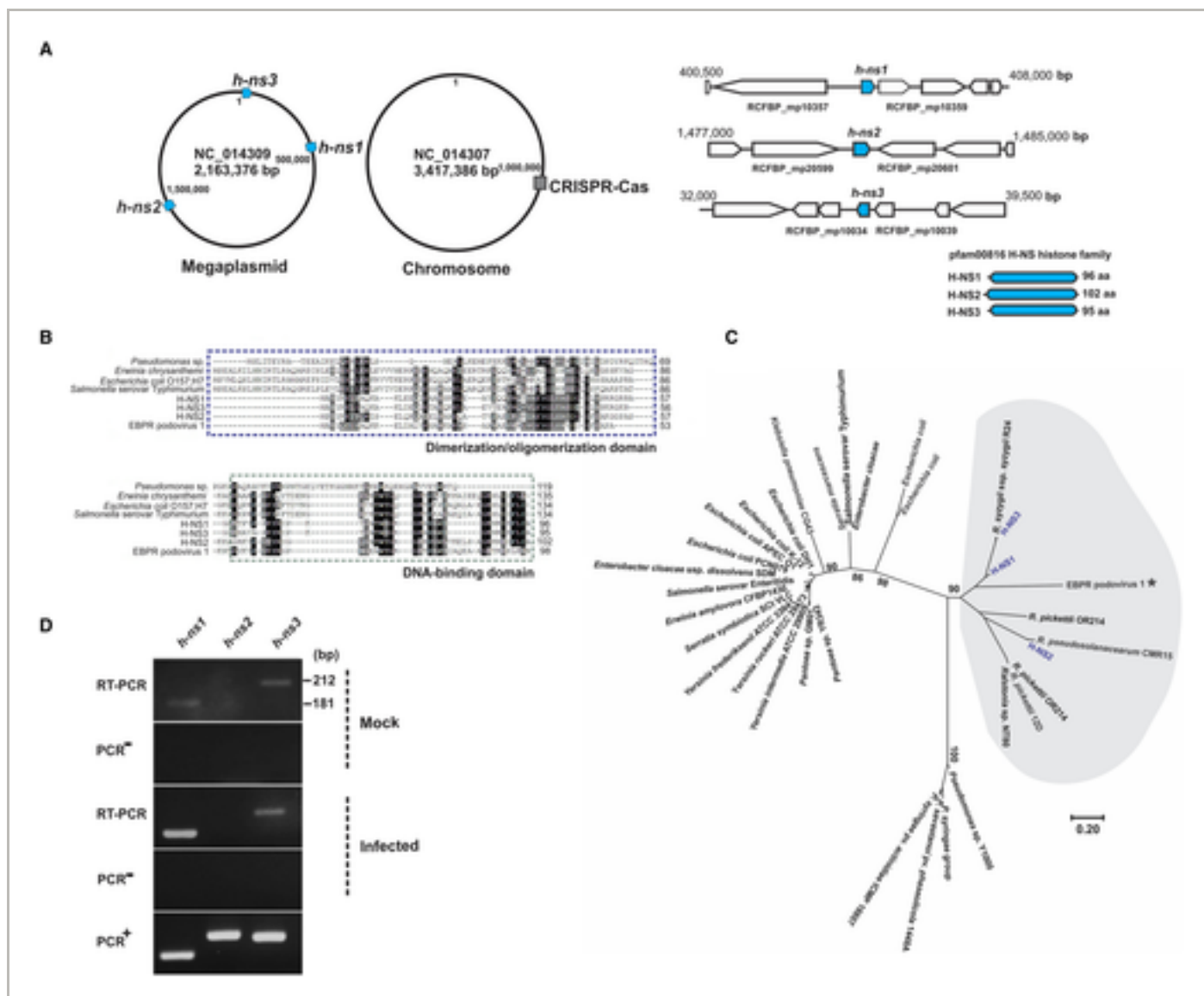
Figure 7

Characterization of H-NS proteins of *Ralstonia solanacearum*. (A) Genomic loci of the three *h-ns* genes found in the *R. solanacearum* CFBP2957 megaplasmid (blue arrows) and the CRISPR-Cas system found in the *R. solanacearum* CFBP2957 chromosome (grey square) are indicated. Details of genomic coordinates, neighbour flank regions and conserved domains are shown. (B) CLUSTAL alignment of the H-NS proteins found in CFBP2957, together with other canonical and/or related H-NS, indicating the functional domains of these proteins. (C) Phylogenetic tree of the bacterial and viral H-NS proteins, including the three H-NS proteins found in CFBP2957 indicated in light blue. The grey cluster contains only RSSC strains, except for the EBPR podovirus 1. (D) The *h-ns* expression profile investigated by reverse transcription-polymerase chain reaction (RT-PCR) using RNA extracted from phage-infected CFBP2957 or mock culture after DNAse I treatment. PCR⁻, negative control of PCR using RNA after DNAse I treatment. PCR⁺, positive control of PCR using genomic DNA from strain CFBP2957. The primers used are given in Table S4 (see Supporting Information). [Colour figure can be viewed at wileyonlinelibrary.com]

It should be noted that environmental signals or stresses can also interfere with *Cas* expression (Koskenniemi *et al.*, 2011; Laakso *et al.*, 2011; Melnikow *et al.*, 2008; Rodriguez *et al.*, 2011). It has been shown that the expression of some CRISPR elements can be conditioned by environmental stimuli, as in *Salmonella* and *Campylobacter* (Jerome *et al.*, 2011; Sheikh *et al.*, 2011). Therefore, the expression profile of the *cas* genes from RSSC strains may be different in the natural environment. Previous transcriptomic studies have shown that gene expression profiles of RSSC strains drastically change in plants when compared with growth in a synthetic rich medium (Ailloud *et al.*, 2015; Jacobs *et al.*, 2012; Puigvert *et al.*, 2017).

The presence of a CRISPR-Cas system in over 30% of the *Ralstonia* genomes investigated suggests a meaningful role of this system in the biology of this Gram-negative bacterium. The absence of acquired CRISPR-based immunity during phage infection, as well as the lack of plasmid interference, showed that, in the laboratory conditions used, which were successful for other bacteria (Hynes *et al.*, 2017), the CRISPR-Cas system is not functional in *R. solanceraum* and other phage resistance mechanisms are necessary in this host. It remains to be seen whether the CRISPR-Cas systems of *Ralstonia* play other roles or whether their activities can be detected under different experimental conditions.

Experimental Procedures

Bacterial strains, bacteriophage and growth conditions

Ralstonia solanacearum strains were cultured in CPG medium containing casamino acids (1 g/L), peptone (10 g/L) and glucose (5 g/L) (Horita and Tsuchiya, 2001) at 28 °C with shaking at 250 rpm. Ralstonia phage phiAP1, a recently characterized *Phikmvvirus* (Xavier *et al.*, 2018), was propagated on *R. solanacearum* CFBP2957 and K60^T (Table 1). NEB® 5-α competent *E. coli* (Ipswich, MA, USA) cells were grown at 37 °C using Luria–Bertani (LB) broth (Hofnung, 1993).

CRISPR bioinformatics analyses

Fifty-four genomes of RSSC strains and three genomes of the non-plant-pathogenic species *R. mannitolilytica*, *R. eutropha* and *R. pickettii*, including full-length or draft versions, were analysed. The complete genome sequences or contigs (for the drafts) were downloaded from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/genome/browse) and are listed in Table 1. To find CRISPR arrays and *cas* genes, we used the CRISPR database (https://crispr.i2bc.paris-saclay.fr) (Grissa *et al.*, 2007), CRISPI (https://crispi.genouest.org) (Rousseau *et al.*, 2009), CRISPRfinder software tools (https://crispr.u-psud.fr/Server) (Grissa *et al.*, 2007), CRISPR Recognition Tool CRT (Bland *et al.*, 2007) and manual inspection in Geneious R8.1 (Biomatters Ltd., Auckland, New Zealand). Putative PAM sequences were identified through the alignment of putative protospacers found in plasmid sequences and viral genomes, and visualized using WebLogo (https://weblogo.berkeley.edu/logo.cgi) (Crooks *et al.*, 2004). The spacer content was analysed and potential protospacers were classified into 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 ≥27/32 nucleotides matching were selected). Pairwise comparisons of the amino acid sequences of the Cas proteins were performed with Geneious R8.1, and alignments were performed using the MAFFT algorithm (Edgar, 2004).

Phylogeny

The phylogeny of Cas1 of RSSC strains was analysed with Cas1 from bacterial species that contained different CRISPR-Cas types, including I-A, I-B, I-C, I-E and I-F (Tables S2 and S3, see Supporting Information). In addition, the phylogeny of only Cas1 of RSSC strains and a phylogeny of the nucleotide sequence of *egl* (Castillo and Greenberg, 2007) were constructed. The sequences were aligned with ClustalX2 and a maximum likelihood tree was constructed in the MEGA 7.0 program using the Jones–Taylor–Thornton (JTT) evolutionary

model (Kumar *et al.*, [2016](#)). Phylogenetic trees were visualized using FigTree (https://tree.bio.ed.ac.uk/software/figtree/).

Isolation of BIMs and spot assay

BIMs were obtained by challenging the phage-sensitive *R. solanacearum* strains CFBP2957 and K60^T with phage phiAP1 (Deveau *et al.*, [2008](#); Hynes *et al.*, [2017](#)). Briefly, *R. solanacearum* strains were grown in CPG broth to an optical density at 600 nm (OD$_{600}$) of 0.2 at 28 °C. A 0.25-mL aliquot was mixed with 100 μL of purified phiAP1 [10^{10} phage-forming units (PFU) /mL]. After 15 min of incubation, the mixture was added to 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 h. Thirty resistant colonies derived from each phage-sensitive parental strain were selected. Resistant colonies were picked and, after three successive replications on CPG agar, single colonies were preserved and confirmed for the resistance phenotype with a spot test (Pantůček *et al.*, [2008](#)) using 10 μL of viral suspension (10^{10}, 10^9, 10^8, 10^7, 10^6 and 10^5 PFU/mL).

DNA isolation

The genomic DNAs from BIMs and their parental strains were extracted as described previously (Garneau *et al.*, [2010](#)), except that the lysozyme step was not performed. The purity and concentration of the DNA were estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and samples were diluted in PCR-grade water to a final concentration of 20 ng/μL.

CRISPR array amplification and sequencing

To investigate whether the complete resistance phenotype on BIMs was linked to spacer acquisition in *R. solanacearum* CFBP2957 or K60^T CRISPR arrays, primers were designed to amplify by PCR the CRISPR1 and CRISPR2 arrays. Primers for PCRs were designed based on the sequence of CRISPR arrays found on the genomes of *R. solanacearum* CFBP2957 [PRJEA50685] and K60^T [PRJEB8309] (Table [S4](#), see Supporting Information). PCR was performed according to standard protocols using 20 ng/μL of genomic DNA. The PCR products were analysed in a 2.5% agarose gel stained with EZ-Vision Three (Amresco, Solon, OH, USA) and visualized under UV light. PCR products were sequenced (Plateforme de Séquençage et de Génotypage des Génomes at CHUL/CHUQ) and the sequences were analysed using Geneious R.8.

Protospacer cloning

Protospacers 36 and 49 (matching viral sequences) of the *R. solanacearum* CFBP2957 CRISPR locus were cloned into vector pUFJ10 (Gabriel *et al.*, 2006). We included eight nucleotides upstream (containing the PAM) and eight nucleotides downstream (containing probable enhancer motifs) of the protospacer present in each target genome. The frequency of the probable PAM motifs contained on the investigated protospacers was checked in putative target DNA (plasmids and phages) of CRISPR loci from other RSSC strains. Primers were designed with restriction sites for *Eco*RI and *Xba*I (Table S4), compatible with the multiple cloning sites of pUFJ10. Plasmid DNA was isolated with a Qiagen (Crawley, UK) Maxi-Prep kit as recommended by the manufacturer. Plasmid DNA and inserts were double digested with *Eco*RI and *Xba*I enzymes, and ligated using T4 DNA ligase (Invitrogen) at 16 ºC as in standard techniques (Sambrook *et al.*, 2001) and in the manufacturer's recommendations. Heat shock transformations using NEB® 5-α competent *E. coli* cells (high efficiency) were performed according to the manufacturers' protocols, and the putative clones were confirmed by PCR. Two clones were confirmed after sequencing and named as pPsp36 (protospacer 36 cloned into pUFJ10) and pPsp49 (protospacer 49 cloned into pUFJ10).

DNA interference assay

To verify whether the CRISPR interference step was active in *R. solanacearum*, we transformed the strain CFBP2957 with 1 µg of plasmid DNA via electroporation (Allen *et al.*, 1991) using pPsp36, pPsp49 and pUFJ10. The transformation experiments were performed in triplicate for each treatment and repeated twice.

Expression of *cas* genes

Total RNA from *R. solanacearum* CFBP2957 was isolated from samples collected from cultures grown to OD_{600} = 0.2 using TRIzol Reagent (Invitrogen). Two groups of samples were analysed: uninoculated cultures (mock) and cultures grown for 60 min after inoculation with phage phiAP1 (infected). Pellets obtained from 25 mL of culture were resuspended in 1 mL of TRIzol reagent and transferred into a 2-mL tube containing 250 mg of glass beads (106 µm, Sigma, St. Louis, MO, USA). The mixture was homogenized with a Mini-Beadbeater-8 cell (BioSpec Products), four times for 2 min. The samples were treated with 20 U of DNAse I (Invitrogen) for 60 min at 37 °C in the presence of 80 U RNaseOUT (Invitrogen). The cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The PCRs were performed with Feldan's Taq DNA polymerase according to the manufacturer's instructions and using specific primers for *cas* genes (Table S4). Each reaction consisted of 5 µL of 10 × Taq buffer, 1 µL of dNTP (10 mM), 1 µL of each primer (50 µM), 5 µL of 5 × Band sharpener solution, 0.25 µL of Taq DNA polymerase (5 U/µL) and 0.25 µL of bacterial DNA in a final volume of 50 µL. The

amplification consisted of a denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C/45 s, 60 °C/45 s and 72 °C for 1 min/kb, and a final extension of 72 °C for 5 min. Before the cDNA synthesis, the absence of genomic DNA in the DNAse-treated RNA samples was confirmed by PCR with the primers listed in Table S4.

Characterization of H-NS proteins *in silico*

To investigate the presence of H-NS proteins in *R. solanacearum* CFBP2957, we performed a search for these genes in its genome (megaplasmid NC_014309 and chromosome NC_014307) via remote Blast with the software Blast2go (Conesa and Götz, 2008; available at https://www.blast2go.com). The putative H-NS proteins were selected for additional characterization. Details of the conserved domains were accessed in the Conserved Domains Database CDD (available at https://www.ncbi.nlm.nih.gov/Structure/cdd) and PROSITE databases (available at https://www.expasy.ch/). In addition, when *h-ns* loci were found, we characterized the flanking regions by manual inspection in Genetic R8.1. Alignments of H-NS proteins of *R. solanacearum* CFBP2957, together with other canonical and/or related H-NS proteins, were performed using CLUSTALX2 and edited using Color Align Conservation (Stothard, 2000) (https://www.bioinformatics.org/sms2/color_align_cons.html). The maximum likelihood tree containing 33 H-NS proteins, including the H-NS proteins found in *R. solanacearum* CFBP2957 and EBPR podovirus 1, was constructed in the MEGA 7.0 program using the JTT evolutionary model (Kumar *et al.*, 2016).

Author Contributions

P.A-Z. and S.M. directed the project. A.S.X., S.M. and P.A-Z. designed the experiments. A.S.X. performed the analysis of CRISPR diversity across *Ralstonia*. A.S.X., G.M.R., A.G.M. and D.M.T. worked on the functional analysis of the adaptation and interference activity of the *R. solanacearum* CRISPR-Cas system. A.S.X., J.C.F.A. worked on the characterization of the H-NS

proteins. A.S.X., J.C.F.A. and P.A-Z. wrote the manuscript and S.M. commented on the manuscript.

Filename	Description
mpp12750-sup-0001-FigS1.docx Word document, 618.3 KB	**Fig. S1** Topological comparison between phylogenetic trees of Cas1 (flexible genome) and Egl (core genome) proteins. For the phylogeny, the amino acid sequences of Cas1 and Egl proteins were used from the 13 *Ralstonia solanacearum* species complex (RSSC) strains listed in Table S3 (see Supporting Information). The maximum likelihood trees were constructed using the MEGA 7.0 program and the Jones–Taylor–Thornton (JTT) evolutionary model. Bootstrap values are indicated as percentage points. Each taxon name is indicated in distinct colours corresponding to the three *Ralstonia* species of the RSSC: *R. solanacearum* (light green), *R. pseudosolanacearum* (light red) and *R. syzygii* ssp. *celebesensis* (light blue). The distinct *Ralstonia* phylotypes, as well as the geographical origin, are indicated near to each segregate cluster. I, phylotype I; II, phylotype II; III, phylotype III; IV, phylotype IV.
mpp12750-sup-0002-FigS2.tif TIFF image, 926.5 KB	**Fig. S2** Potential targets of the *Ralstonia solanacearum* species complex (RSSC) strains of CRISPR-Cas system type I-E. Distribution of putative protospacers into three categories using the following parameters: spacers with potential protospacer matches to fewer than six single nucleotide polymorphisms (SNPs) (≥27/32 nucleotides matching).
mpp12750-sup-0003-FigS3.docx	**Fig. S3** *Ralstonia solanacearum* bacteriophage-insensitive mutants (BIMs) are permissive to adsorption, but viral replication cannot be detected. (A) Viral adsorption (*Ralstonia* virus phiAP1). (B) Survival cells. (C) DNA viral replication kinetics assay.

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