JULIANA CRISTINA FRALEON DE ALMEIDA

CARACTERIZAÇÃO MOLECULAR DE UM VÍRUS COM GENOMA DE DNA QUE INFECTA *Ralstonia* solanacearum E CARACTERIZAÇÃO DE PROTEÍNAS H-NS E SUA FUNÇÃO NA REGULAÇÃO DO SISTEMA CRISPR-CAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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APROVADA: 24 de fevereiro de 2017.

Francisco Murilo Zerbini Junior

Renan de Souza Cascardo
(Coorientador)

Poliane Alfenas Zerbini
(Orientador)

Dedico aos meus pais José Maria de Almeida e Ana Iracema Barbosa de Almeida, pois tudo que sou devo a eles.

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BIOGRAFIA

JULIANA CRISTINA FRALEON DE ALMEIDA é filha de José Maria de Almeida e Ana Iracema Barbosa de Almeida, brasileira, nascida em 1992 em Pará de Minas, Minas Gerais. Iniciei minha formação em 2010 quando ingressei no curso licenciatura em Ciências Biológicas, UFV- *Campus* Florestal e me formei em 2014. A oportunidade de estar em uma universidade descortinou novos horizontes, e o contato com a tríade ensino, pesquisa e extensão despertou em mim o desejo de aprender para ser útil. Motivada por este desejo, em julho de 2011 participei da seleção para o PET-Educação, sendo selecionada em segundo lugar. Permaneci no PET até a conclusão do curso, e não consigo mensurar em palavras o amadurecimento e crescimento acadêmico e pessoal que este programa me proporcionou. Sempre que possível participei também de Simpósios, Palestras, Seminários e Minicursos que eram realizados no *Campus* ao qual eu estava vinculada e também de outras instituições. Realizei uma pesquisa com alunos surdos de uma escola pública mineira, publicando um artigo com minhas análises, sendo premiada em apresentação oral no Simpósio de Integração acadêmica. Realizei também pesquisa sobre educação à distância, sendo também premiada por este trabalho. Em 2013, interessada em realizar pesquisa na área de Microbiologia, me tornei voluntária em um projeto de análise microbiológica da água no laboratório do *Campus* Florestal, onde aprendi um pouco mais sobre esta área e me encontrei como pesquisadora, aproveitando para aprender alguns procedimentos e técnicas desta área. A possibilidade de frequentar o laboratório de microbiologia e afinidade pelas disciplinas relacionadas a esta área me guiaram na decisão de me especializar em Microbiologia e esta decisão se fortaleceu quando participei do II Simpósio Internacional de Microbiologia na Universidade Federal de Viçosa conhecendo a extensão que as descobertas nesta área trazem para a sociedade. E em agosto de 2015 ingressei como discente de mestrado no Programa de Pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa com conclusão em fevereiro de 2015.

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RESUMO

ALMEIDA, Juliana Cristina Fraleon de, M.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Caracterização molecular de um vírus com genoma de dna que infecta *Ralstonia solanacearum* e caracterização de proteínas H-NS e sua função na regulação do sistema CRISPR-Cas.** Orientador: Poliane Alfenas Zerbini Coorientador: Renan de Souza Cascardo

Os vírus que infectam bactérias são os organismos mais abundantes do planeta, e desempenham importantes funções para a manutenção do ecossistema. Nos últimos anos, o uso de vírus como ferramenta de controle biológico tem ganhado bastante atenção, devido, principalmente, à dificuldade de se isolar novas drogas antimicrobianas e à seleção de bactérias resistentes às drogas já existentes. Devido a esse potencial como agentes de controle biológico, as descobertas sobre esses vírus tem aumentado o que amplia as perspectivas do manejo ecológico de doenças em plantas. Neste contexto, o presente trabalho teve como objetivos i) caracterizar um bacteriófago isolado de *Ralstonia solanacearum* proveniente do estado do Ceará, Brasil ii) caracterizar proteínas H-NS de *Ralstonia solanacearum* e estudar seu o efeito regulatório no sistema CRISPR-Cas. A partir de um isolado não patogênico de *Ralstonia solanacearum*, foi isolado um vírus filamentoso que possui um ciclo de multiplicação do tipo pseudo-lisogênico. O genoma viral foi completamente sequenciado, possui 6945 nucleotídeos e conteúdo de GC de 61,25%. A organização genômica é típica de vírus da família *Inoviridae*, gênero *Inovirus*. Comparação da sequência obtida com sequências de outros *Inovirus* sugere que o isolado viral é uma nova espécie do grupo φRSM, tentativamente denominada *Ralstonia solanacearum* Inovirus Brazil 1 (RSIBR1). Partículas de RSIBR1 foram transmitidas para *R. pseudosolanacearum*, que quando infectadas pelo RSIBR1 também perdeu a capacidade de causar doença em

plantas de tomate, sugerindo que a infecção viral interfere com a patogenicidade de *Ralstonia* spp. Em estudos anteriores, foi demonstrado que o sistema CRISPR-Cas de *Ralstonia solanacearum* é inativo. Para avaliar se essa inatividade pode ser explicada pela presença de proteínas do tipo H-NS, foi realizada uma busca por genes que codificam H-NS no genoma de *Ralstonia solanacearum.* Três cópias de H-NS (H-NS 1, 2 e 3) foram localizadas no megaplasmídeo. Não foram identificadas cópias de H-NS codificadas no cromossomo bacteriano. As H-NS de *Ralstonia solanacearum* possuem os domínios de oligomerização e de ligação a DNA bem conservados. A análise filogenética de proteínas H-NS de diferentes bactérias agruparam de acordo com as famílias Enterobacteriaceae, Pseudomonadaceae e Ralstoniaceae. Além disso, foi observado que as proteínas H-NS da família Ralstoniaceae são altamente conservadas com H-NS codificadas por podovírus, sugerindo que as H-NS de Ralstoniaceae podem ser de origem viral. Foi realizada uma análise *in silico* nos promotores das proteínas CAS para verificar a presença de sítios de ligação de H-NS. Foram identificadas regiões de alto conteúdo AT, com curvatura típica potencial para a ligação de H-NS. A análise da expressão das H-NS mostrou que somente as H-NS 1 e 3 são expressas em *Ralstonia solanacearum*. Para confirmar que as proteínas H-NS possuem efeito regulatório na expressão dos genes Cas, foram construídos cassetes para a obtenção de mutantes para H-NS1, H-NS 2 e H-NS3. A obtenção dos mutantes e a análise da expressão dos genes Cas nos mutantes irão permitir elucidar o papel regulatório de H-NS na expressão do sistema CRISPR-Cas em *Ralstonia solanacearum*.

ABSTRACT

ALMEIDA, Juliana Cristina Fraleon de, M.Sc., Universidade Federal de Viçosa, february 2017. **Molecular charactetization of a ssDNA virus that infects *Ralstonia solanacearum* and characterization of H-NS proteins and their function in the regulation of the CRISPR-Cas system.** Orientador: Poliane Alfenas Zerbini Coorientador: Renan de Souza Cascardo

Viruses that infect bacteria are the most abundant organisms on the planet, and play important roles in maintaining the ecosystem. In recent years, the use of viruses as a biological control tool has gained a lot of attention, mainly due to the difficulty of isolating new antimicrobial drugs and the selection of bacteria resistant to existing drugs. Because of this potential as biological control agents, the findings on these viruses have increased what broadens the perspectives of the ecological management of diseases in plants. In this context, the present work had as objectives i) to characterize a bacteriophage isolated from *Ralstonia solanacearum* from the state of Ceará, Brazil ii) to characterize H-NS proteins of *Rastonia solanacearum* and to study its regulatory effect in the CRISPR-Cas system. From a non-pathogenic isolate of *Ralstonia solanacearum*, a filamentous virus was isolated which has a pseudo-lysogenic-like multiplication cycle. The viral genome was completely sequenced, containing 6945 nucleotides and a GC content of 61.25%. The genomic organization is typical of viruses of the family *Inoviridae*, genus *Inovirus*. Comparison of the sequences obtained with other inovirus sequences suggests that the viral isolate is a new species of the φRSS group, tentatively named *Ralstonia solanacearum* Inovirus Brazil 1 (RSIBR1). RSIBR1 particles were transmitted to R. pseudosolanacearum, which when infected by RSIBR1 also lost the ability to cause disease in tomato plants, suggesting that the viral infection interferes with the pathogenicity of *Ralstonia* spp. In previous studies,

it has been shown that the CRISP-Cas system of *Ralstonia solanacearum* is inactive. To assess whether this inactivity can be explained by the presence of H-NS type proteins, we searched for genes encoding H-NS in the *Ralstonia solanacearum* genome. Three H-NS copies (H-NS 1, 2 and 3) were located in megaplasmid. No copies of H-NS encoded on the bacterial chromosome were identified. *Ralstonia solanacearum* H-NS have the well-conserved oligomerization and DNA binding domains. Phylogenetic analysis of H-NS proteins from different bacteria grouped according to the families Enterobacteriaceae, Pseudomonadaceae and Ralstoniaceae. In addition, it was observed that the H-NS proteins of the Ralstoniaceae family are highly conserved with H-NS encoded by podovirus, suggesting that the H-NS of Ralstoniaceae may be of viral origin. An *in silico* analysis was performed on the promoters of the CAS proteins to verify the presence of H-NS binding sites. Regions with high AT content were identified, with a typical potential curvature for H-NS binding. Analysis of H-NS expression showed that only H-NS 1 and 3 are expressed in *Ralstonia solanacearum*. To confirm that H-NS proteins have a regulatory effect on the expression of Cas genes, cassettes were constructed to obtain mutants for H-NS1, H-NS2 and H-NS3. Obtaining the mutants and analyzing the expression of the Cas genes in the mutants will enable elucidating the regulatory role of H-NS in the expression of the CRISPR-Cas system in *Ralstonia solanacearum*.

INTRODUÇÃO GERAL

Os vírus que infectam bactérias são os organismos mais abundantes da terra, ubíquos e parasitas intracelulares obrigatórios. O seu material genético pode ser tanto de RNA ou DNA, pode estar linear ou circular e ainda pode ser fita dupla ou fita simples. É recoberto pelo capsídeo, que pode, ou não, estar ligado a uma cauda de fibras necessárias para reconhecer e se ligar aos receptores das células bacterianas. Apesar da maioria desses vírus serem poliédricos, com cabeça e cauda, cerca de 5% possuem formato filamentoso ou sem forma definida (Haq *et al.*, 2012; Patel *et al.*, 2015; Salmond & Fineran, 2015).

De acordo com o mecanismo de multiplicação, a maioria dos vírus bacterianos podem ser classificados em dois grupos: virulentos e temperados, que propagam utilizando o ciclo lítico e ciclo lisogênico respectivamente. Durante o ciclo lítico, após a multiplicação viral na célula, novas partículas são montadas e liberadas por lise da célula hospedeira. No ciclo lisogênico, o genoma do vírus se integra no cromossomo do hospedeiro, sendo chamado de profago e replica junto com o genoma da célula hospedeira, sendo transmitido para as células filhas. Em condições de estresse do hospedeiro, os profagos podem se excisar do genoma e entrar em ciclo lítico (Elbreki *et al.*, 2014; Patel *et al.*, 2015; Negash & Ejo, 2016). No entanto, alguns vírus são exceções a esses ciclos de multiplicação. Um exemplo são os vírus filamentosos pertencentes à família *Inoviridae*. Os vírus desta família se multiplicam no citoplasma da célula hospedeira, e as partículas virais são liberadas por extrusão, sem levar à lise da célula. Alternativamente, o genoma desses vírus também podem se integrar no cromossomo do hospedeiro, ou ainda, pode replicar como um elemento

298 extracromossomal, sem a formação de novas partículas. Esse tipo de replicação é
299 denominada “infecção estável” (Rakonjac *et al.*, 2011; Mai-Prochnow *et al.*, 2015)

300 Os vírus que infectam bacterias historicamente contribuíram muito para o
301 desenvolvimento da biologia molecular e biotecnologia, sendo ferramentas muito
302 importantes na resolução de vários desafios nestes campos do conhecimento (Haq *et*
303 *al.*, 2012). Atualmente, devido à resistência bacteriana aos antibióticos, a aplicação
304 desses organismos no biocontrole tem ganhado bastante atenção (Inal, 2003; Haq *et*
305 *al.*, 2012; Patel *et al.*, 2015).

306 Diversos vírus capazes de infectar bactérias fitopatogênicas de importância
307 econômica são conhecidos: *Xanthomonas campestris* (Lin *et al.* 1999), *Ralstonia*
308 *solanacearum* (Yamada *et al.*, 2007), *Xillella fastidiosa* (Ahern *et al.*, 2014),
309 *Xanthomonas axonopodis* pv. *citri* (Ahmad *et al.* 2014b, Ahmad *et al.* 2014a) e
310 *Xanthomonas campestris* pv. *campestris* (Hung *et al.* 2002). A infecção viral em
311 bactérias, além de lise celular pode causar alterações fisiológicas importantes no
312 hospedeiro, como por exemplo a redução da virulência da bactéria e
313 consequentemente o estabelecimento da doença. Por exemplo, a infecção lisogênica
314 do fago filamentoso XacF1 em *Xanthomonas axonopodis* pv. *citri*, resultou em baixos
315 níveis na produção de polissacarídeo extracelular, redução da taxa de crescimento,
316 diminuição da motilidade pulsante, drástica redução da virulência e desenvolvimento
317 das lesões em folhas de citrus (Ahmad, Askora, Kawasaki, Fujie and Yamada 2014b).
318 Muitos bacteriófagos já demonstraram potencial para o controle de diversas
319 fitobactérias economicamente importantes, incluindo, *Xanthomonas* spp.,

Pseudomonas spp., *Erwinia* spp., *Ralstonia* spp. e *Streptomyces* spp. (Balogh, Jones, Iriarte and Momol 2010).

Ralstonia solanacearum, causadora da murcha bacteriana, é uma bactéria Gram-negativa, não formadora de esporos, aeróbia e em forma de bastonete, classificada no filo Proteobacteria, classe, Betaproteobacteria (Guarischi-Sousa *et al.*, 2016). É amplamente distribuída e veiculada pelo solo e água contaminados (Peeters *et al.*, 2013), o que dificulta o manejo e controle da doença. Possui grande importância econômica e ampla distribuição geográfica, podendo afetar mais de 50 famílias de plantas, dentre elas alguns cultivos economicamente importantes tais como a batata, beringela, tomate, banana e tabaco (Yamada, 2013). Essas bactérias também são capazes de colonizar hospedeiros alternativos, geralmente plantas não cultivadas, de forma assintomática em infecções latentes, dificultando ainda mais o seu controle (Genin & Denny, 2012) porque possibilita sua sobrevivência e incidência mesmo em rotações de culturas.

A infecção causada por *Ralstonia solanacearum* se inicia quando as bactérias entram num hospedeiro suscetível colonizando os espaços intercelulares do córtex radicular e parênquima vascular. Colonizam o xilema e se espalham para as partes superiores da planta, causando murcha. A murcha bacteriana é uma doença complexa e envolve múltiplos fatores de virulência, como por exemplo, o EPS, uma grande camada exopolissacarídica ácida rica em nitrogênio que aumenta a velocidade e extensão da infecção. Os sintomas observados são principalmente devidos à restrição do fluxo de água através do xilema, devido à obstrução causada pela multiplicação bacteriana. Outro fator de virulência importante são enzimas que degradam a parede

da célula da planta, como enzimas pectinolíticas que fragmentam a pectina em oligômeros, facilitando o movimento bacteriano através de regiões ricas em pectina, tais como feixes vasculares (Yamada, 2013). *Rasltonia* spp apresenta uma alta variabilidade genética, ampla gama de hospederios e sobrevive no solo por longos períodos. Essas características do patógeno fazem com que o controle químico e genético sejam pouco eficientes (Allen et al., 2005; Wang et al., 2005; Yuliar et al., 2015).

Nos últimos anos, diversos vírus infectando *Rasltonia* spp. tem sido descritos no continente Asiático, e tem sido proposta a utilização de alguns destes vírus para o controle da murcha bacteriana (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).

Vários vírus caracterizados que infectam *Rasltonia* spp. são da família *Inoviridae,* gênero *Inovirus*. Os vírus que pertencem ao gênero *Inovirus* são vírus filamentosos, não envelopados, os filamentos possuem 7 nm de diâmetro e de 700 a 2000 nm de comprimento, com genoma de DNA fita simples, circular e polaridade positiva de tamanho aproximado de 4.5 a 8 Kb (KING et al., [s.d.] in ICTV, 2011). Esses vírus podem integrar no genoma do hospedeiro, se replicarem no citoplasma como um elemento extracromossomal sem a formação de partículas, ou ainda se multiplicarem com a formação de partículas liberadas da célula infectada por extrusão sem causar lise (Rakonjac *et al.*, 2011; Ilyina, 2015; Mai-Prochnow *et al.*, 2015). Como estes vírus coexistem com seus hospedeiros, eles são capazes de mediar alterações nos fenótipos bacterianos de várias maneiras e já foi relatado que a virulência de bactérias

patogênicas de plantas e animais foi afetada pela infecção com este tipo de vírus (Askora & Yamada, 2015). Como exemplos, o vírus CTXφ codifica um dos principais fatores de virulência de *V. cholerae*, a toxina da cólera (CT) podendo converter cepas não virulentas em cepas altamente virulentas (Faruque & Mekalanos, 2012), e o vírus φRSM1 de *Ralstonia solanacearum* reduz a virulência alterando vários fatores, entre eles perda de motilidade e diminuição na produção da camada exopolissacarídica (Addy *et al.*, 2012b).

Geralmente, esses vírus possuem uma organização modular, em que genes relacionados são agrupados juntos e esses módulos são geralmente, bem conservados entre as espécies. São descritos três principais módulos estruturais: um módulo para replicação, que contém os genes para replicação por círculo rolante e genes de ligação a DNA; um módulo estrutural que contém os genes para o capsídeo e para adsorção viral; e um módulo de secreção e montagem com os genes para montagem e excreção das partículas. Alguns vírus podem possuir ainda genes acessórios, com funções diversas, que podem ser únicos ou conservados entre alguns vírus (Ilyina, 2015; Mai-Prochnow *et al.*, 2015).

Para se proteger das infeções virais, as bactérias utilizam uma ampla variedade de estratégias, incluindo respostas inatas ou respostas adaptativas, em particular o sistema CRISPR-Cas (Marraffini, 2015). Em *Ralstonia* spp. resistência natural já foi observada na população e a variabilidade de fenótipos observados sugerem o envolvimento de mais de um mecanismo de defesa (Xavier et al., 2017, submetido para publicação).

Os loci CRISPR a os genes Cas (CRISPR-associated) codificam um sisteme imune adaptativo em bactérias que fornece proteção contra DNA exógeno, incluíndo vírus, plasmídeos e elementos genéticos (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). Esse sistema está amplamente distribuído no genoma de *Archaea* [90%] e *Bacteria* [50%] (Grissa et al., 2007a) e sua diversificação permite a classificação em cinco tipos, subdivididos em 16 subtipos de acordo com diferenças no conteúdo e organização dos genes Cas e no módulo de adaptação (Makarova et al., 2015). Uma análise do sistema CRISPR-Cas em genomas de *Ralstonia* spp. depositadas no GenBank mostrou que esse grupo de bactérias possui possui o sistema CRISPR-Cas subtipo I-E, e em baixa frequência o subtipo II-C. A análise funcional mostrou que o sistema possui todos os elementos mínimos necessários para o funcionamento, entretanto não foi possível detectar a aquisição de novos espaçadores no módulo de adaptação. A expressão dos genes Cas também não foi detectada, sugerindo que o sistema CRISPR-Cas em *Ralstonia* spp pode estar inativo, devido a repressão da expressão das proteínas Cas (Xavier, 2016). H-NS possuem um tamanho de aproximadamente 15,5 kDa e são proteínas bem conservadas em *Escherichia coli* e bactérias relacionadas, tem afinidade por regiões de DNA ricas em AT influenciando vários mecanismos como transcrição, dobramento do DNA e evolução do genoma (Grainger, 2016), e já foram descritas como sendo proteínas responsáveis pela repressão do sistema CRISPR-Cas em *E. coli* (Pul *et al*., 2010),

O primeiro capítulo deste trabalho descreve uma nova espécie de vírus do gênero *Inovirus*, que infecta *Ralstonia solanacearum* e modula a patogenicidade desta bactéria. No segundo capítulo é feita uma análise das proteínas H-NS de *Ralstonia*

411 *solanacearum*, e seu papel na regulação dos genes Cas, e consequente modulação da

412 resposta adaptativa mediada por CRISPR-Cas.

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CHAPTER 1

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

Almeida, J.C.F., Xavier, A.S., Cascardo, R.S., Magalhães, L.L., Lopes, C.A. and Alfenas-Zerbini, P. Biological changes during the conversion of the phytopathogenic *Ralstonia pseudosolanacearum* into a commensal bacteria by an inovirus.

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 host cells, 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 inoviruses. In an attempt to elucidate which factors are associated with the phenomenon, we investigated one isolate of *R. solanacearum* (UB-2014), which rapidly lost its ability to cause disease in plants, originally obtained from eggplant showing wilt symptoms. Pathogenicity tests in tomato and eggplant confirmed the loss of patogenicity 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 genome was sequenced and assembled at 6945 nucleotides with a GC content of 61.25%. Phylogenetic analyzes showed that the virus belongs to the family Inoviridae, with typical characteristics present in members of the genus Inovirus, grouping with other Inoviruses φRSS-Type. The isolated virus was tentatively named Ralstonia virus RSIBR1 (Ralstonia solanacearum Inovirus Brazil 1). When RSIBR1 particles were transmitted to GMI1000, an aggressive virus free 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 ability of GMI1000 to inhibit other isolates of *Ralstonia* spp. and altered its susceptibility response during the secondary

infection by Ralstonia virus phiAP1, a lytic virus. 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, assuming 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 is 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). Generally, these viruses have a modular organization, in which related genes are grouped together and these modules are generally well conserved between species. Three major genomics modules are described: a replication module (R module), which contains rolling cycle replication and DNA-binding genes; A structural module (S module) containing morphogenesis and viral adsorption genes; and a secretion and assembly module (A-S module) with the genes for assembly and excretion of the particles. Some virus may also have accessory genes with diverse functions, which may be unique or conserved among some phages (Ilyina, 2015; 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 modulate bacterial phenotypesin various ways (Waldor & Mekalanos, 1996; Chopin *et al.*, 2002; Addy *et al.*, 2012a,b).

Effects on host physiology have been observed during infection by inoviruses in

694 *Xanthomonas campestris* pv. *oryzae* and *X. campestris* pv. *campestris*, resulting in
695 increased virulence in these two pathovars (Kamiunten and Wakimoto 1981; Tseng
696 1990). In *Pseudomonas aeruginosa*, viral infection is associated with reduction of
697 colonies size and interference in biofilm development (Webb *et al.*, 2004; Rice *et al.*,
698 2009). In *Ralstonia* spp.viral infection is associated with modulation of virulence, growth
699 rate and EPS production (Addy *et al.*, 2012a,b; Askora *et al.*, 2014). More direct
700 involvement of filamentous viruses in host virulence is well characterized in *Vibrio*
701 *cholerae*, where the pathogenicity of this bacterium depends of some key virulence
702 factor, including the cholera toxin encoded by a gene on the filamentous virus CTXφ
703 genome (Waldor & Mekalanos, 1996). It is common to find these filamentous viruses
704 integrated into host chromosomes, as prophages (Delbrock, 1946; Rakonjac *et al.*,
705 2011; Mai-Prochnow *et al.*, 2015), in a state alternative to episomal propagation
706 (pseudolysogeny).

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

715 Filamentous bacterial viruses (genus *Inovirus*) frequencially appear to be
716 involved in interaction with *Ralstonia* spp., being found with as pseudolysogens or as

717 proviruses in genomes of various bacterial isolates (Yamada, 2013; Askora *et al.*, 2014).
718 Several of them have been well characterized in the Asian continent, including φRSS1,
719 φRSM1, RSM3, RSM4 and PE226 (Yamada *et al.*, 2007; Askora *et al.*, 2009;
720 Murugaiyan *et al.*, 2011). The contrasting effects of these viruses on of these plant
721 pathogens, increasing or reducing bacterial virulence, expose an interesting role for
722 them in the epidemiology of the bacterial wilt in the field.

723 According to Askora and Yamada 2015, the Inoviruses that were found infecting
724 plant pathogenic *Ralstonia spp.* were classified into two groups, φRSS-type and φRMS-
725 type. The representatives of the first group has small particles (1.1 μm) with an
726 approximately 7000 kb ssDNA genome and 11 ORFs. Generally φRSS-type Inoviruses
727 integrate on host chromossome and when compared to Escherichia virus M13, they
728 have an insertion of additional genes within or near the A-S module. RSS1 and RSS3
729 are representatives of this group. The second group, φRSM-type, comprises virus with
730 larger particles (1.5 μm) with a ssDNA genome around 9000 kb and 15 ORFs. Unlike
731 the first group, φRSM-type viruses do not usually integrate on chromossome of their
732 host however for some species integrated forms have been reported (Askora *et al.*,
733 2012). When compared to the genomic sequence of Escherichia virus M13, have five
734 additional genes inserted into the R module, or between the R and S modules are
735 observed. RSM1, RSM3 RS603 are representatives of this group. φRSS-type inoviruses
736 often increase virulence of their hosts and RSM viruses reduce the host virulence.
737 However these differences in effects on their hosts may be primarily linked with the
738 presence of repressor genes and the form of viral spread (integrated or episomal)
739 (Askora & Yamada, 2015).

Here, we isolate and characterize biologically and molecularly, RSIBR1, an inovirus that naturally infects a isolated of *R. solanacearum* that lost virulence and that is also able to infect *R. pseudosolanacearum*, Additionally, we describe significant impacts of viral infection on the modulation of parasitic interaction plant-bacteria and microbial adaptation.

RESULTS

The isolate UB-2014 of *R. solanacearum* (phylotype II) is a non pathogenic isolate that shows abnormal characteristics of growth, including less turbidity and frequent aggregation in the liquid culture (Figure 1A). The identity of the *Ralstonia* spp. isolates was confirmed by PCR (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.*, 2012a,b; 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 ~ 7.0 kb (Figure 2), with expected size for inovirus genome, as reported infecting *Ralstonia* spp. isolates (Askora *et al.*, 2014).

The genome of the viral isolated was completely sequenced by primer walking and the contig was assembled using the genome of the RS603 virus (Van *et al.*, 2014) as reference. The complete genome was assembled in 6945 nucleotides with GC content of 61.25%.and showed 82% of similarity with the RS603 virus. The assembled genome showed the three characteristic modules of the Inovirus: module R, module S and module A-S (Figure 3), although has a genome slightly smaller when compared to

764 inoviruses that infects *Rasltonia solanacearum* with greater genomic similarity, as
765 RSMSuper, RSM3 and RS603. The genome characteristics suggest that the virus
766 belongs to the Inoviridae family, genus *Inovirus*, which multiplies in a pseudo-lysogenic
767 manner, establishing a close relationship with the host. The isolated virus was
768 tentatively named Ralstonia virus RSIBR1 (*Ralstonia solanacearum Inovirus* Brazil 1).

769 The RSIBR1 genome ecodes 12 putative ORFs, organized in the same pattern of
770 others φRSM type, as RS603. The amino acid sequences of all ORFs were analyzed
771 and compared in the UniProt database against other viral proteins. The molar mass and
772 isoelectric point of all proteins were obtained in ExPASy: SIB bioinformatics resource
773 portal, (Artimo, et al, 2012). The predictions for the functions of all proteins, mass and
774 isoelectric point are listed in Table 1.

775 Using the Uniprot database that also compares protein motifs, we have found
776 that RsIBR1 ORF12 has conserved ORF15 motifs of RSM1 and RSM3 viruses that are
777 well characterized and have repressor function of the regulatory protein of various host
778 virulence factors, PhcA (Ilyina, 2015). It is very likely that ORF12 is one of the factors
779 involved in reducing the virulence of the UB-2014 isolate by the Inovirus RsIBR1. The
780 ORF10 was predicted as a putative viral extrusion protein and the ORF11 a putative
781 rolling circle DNA replication initiation protein. In addition, proteins from the three
782 structural modules of the genome were found, and their localization in the genome
783 showed synteny with other inoviruses.

784 Interestingly, we have detected that the genome of RSIBR1 virus is 96% identical
785 to a provirus on the *Ralstonia solanacearum* UY031 chromosome from Uruguay
786 (Guarischi-Sousa *et al.*, 2016), the only difference is the presence of an additional ORF

787 that encodes a serine recombinase/resolvase, probable responsable for integration in
788 the bacterial chromossome (Figure 3). We also found the same in *Ralstonia*
789 *solanacearum EP1* genome, were a copy of the genome of the RS603 was found as a
790 provirus, and the only difference with the RS603 virus is the presence of an additional
791 ORF that codes a serine recombinase/resolvase. It is interesting to note that when the
792 virus is integrated, the phetotype of reduction in bacterial virulence is not observed.

793 In phylogenetic analysis of the complete genome of RSIBR1 and other *Ralstonia*
794 *solanacearum* inoviruses of both, φRSS and φRSM type, inoviruses that infect other
795 species and with *Plectrovirus*, RSIBR1 grouped with other φRSM type viruses, being
796 very close to the RS603, RSM3, RSM1 and RSMSuper (Figure 4). Pairwise sequence
797 comparisons showed that RSIBR1 showed highest nucleotide sequence identity (82%)
798 with RS603 (Figure 5). Since the species demarcation criterion is not clear, and exist
799 different species that show ~87% of identity (RS603, RSM3 and RSM1), we propose
800 that RSIBR1 is a new species from the ΦRSM type group

801 To test if the RSIBR1 could be transmitted to other species of *Rasltonia* spp,
802 VLPs of RSBR1 were incubated with a culture of *R. pseudosolanacearum* GMI1000.
803 Liquid cultures of GMI1000 virus infected (GMI1000 VI), as well as UB-2014, showed
804 less turbidity, overproduction of reddish-brown melanin-like and frequent aggregation
805 (Figure 6A). The analysis of structural proteins (Figure 6B) and of the nucleic acid
806 present in the VLPs (Figure 6C) preparation confirmed the transmission of putative
807 inovirus. Interestingly the overproduction of the reddish-brown melanin-like is
808 accompanied by viral infection because after infection GMI1000 exhibited this property
809 as well as UB-2014, however much less in relation to production in the original host

(Figure 6B). The GMI1000 VI cells were not able to kill plants as observed for the natural host UB-2014 (Figure 6D). Interestingly, GMI1000 VI caused only mild symptoms (Figure 6D), which were reversible in older plants (Figure 6E). Additionally, UB-2014 and GMI1000 VI was reisolated in xylem vessels of plants without symptoms after three months highlights the drastic change in the lifestyle of this pathogen.

To test if the presence of RSIBR1 could affect another biological characteristics as susceptibility to a secondary infection by another virus, GMI1000 VI was infected with with a lytic virus, *Ralstonia* virus phiAP1 (*Phikmvvirus*). In GMI1000 VI is was observed a great reduction in the number of lysis plaques (Figure 7B), showing that RSIBR1 “protect”the cell to a secondary infection by a lytic virus. The effect of the RSIBR1 in the capacity of bacteriocin production was also tested. GMI1000 VI showed a reduction in the capacity of inhibit the growth of the *R. solanacearum* isolates RSB1 and V45, and *R. pseudosolanacearum* V4 (Figure 7B), suggesting that the capacity to produce bacteriocins against *Ralstonia* spp. was impaired by RsIBR1 infection.

DISCUSSION

We report RSIBR1, a new *Inovirus* species 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.

As observed for UB-2014, the *R. pseusosolanacearum* GMII1000 VI, exhibited a virulence decrease, as described for *Ralstonia* spp. infected by the species of inovirus phiRSS0, phiRSM1 and phiRSM3, that is also accompanied by abnormal cell physiology (Addy *et al.*, 2012b).

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 *et al.*, 2015), tolerance to toxic radicals (Fogarty & Tobin, 1996), oxidative stress (Rodríguez-Rojas *et al.*, 2009), extreme cold and hot temperatures (Rosas & Casadevall, 1997), in addition to protection of pathogenic microorganisms from the host immune response (Zughaier *et 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 & Allen, 2009).

The ORF 12 of RSBR1 encodes a protein with conserved motifs with ORF15 of RSM1 and RSM3 viruses that are a supposed virulence repressor-like protein. Since *Ralstonia* spp. infected with RSBR1 lost the capacity to cause disease in tomato plants, but retained the capacity to colonize the plant, it is reasonable to suppose that the ORF 12 encodes a virulence repressor-like protein that is responsible for the phynotype of loss of virulence by UB2014.

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. 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). 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 indicating that in natural environment this infected isolate should be less competitive.

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, and accumulate more evidence about the role of inoviruses in the modulation of the populations of *Ralstonia* spp. not only in Asia but also America.

MATERIAL AND METHODS

Bacterial isolates and growth conditions

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

888 ***Ralstonia complex PCR and phylotyping***
889

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

895 ***Viral propagation and purification***
896

897 To verify if the presence of viruses was related to an avirulence phenotype in the
898 isolate UB-2014, the bacterium was cultivated to induce viral propagation, followed by
899 purification as proposed by Yamada et al. (2007), with some modifications. Two liters of
900 a UB-2014 culture (optical density at 600 nm of 0.2) was incubated for four days at 28°C
901 in static conditions. Intact bacterial cells were lysed with chloroform, shaked at 100 rpm
902 during 30 minutes at 37ºC, and cell debris was removed by centrifugation at 8 000 *g* for
903 5 minutes 4ºC. The supernatant was passed through a 0.22 µm membrane followed by
904 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.

Full length genome sequence

The viral DNA was amplified by RCA, according to Inoue-Nagata, 2004.

The DNA amplificated was cleaving reaction for three different enzymes (ApaI, EcoRI, and PstI) (Sambrook & Russell, 2011).

According to yet Sambrook & Russell (2011), the mix inserts corresponding to the enzymatic cleavage product was used for binding in vector pBluescript KS. E. coli cells DH5α were transformed and cloned based on Inoue et al, 1990. The clones obtained were completely sequenced by primer walking (Macrogen, South Korea).

Sequence and genome analysis

The quality of the sequences obtained being evaluated using the Chromas lite 2.1 program (Chromas-lite.software.informer.com/2.1/). Obtaining contigs and

assembling complete genome being performed using Geneious R8.1 (Biomatters Ltd., Auckland, New Zealand). The annotation of the ORFs was performed using the ORFinder tool and the genomic sequence obtained was compared to sequences deposited in the NCBI database (www.ncbi.nlm.nih.gov/) tool using the using BLAST algorithms (Altschul et al., 1990; Altschul et al., 1997). For the phylogeny, a maximum likelihood tree was constructed in the MEGA 7.0 program and a similarity matrix was made by the SDT v. 1.0 (Muhire et al., 2013).

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 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 an isolate of *R. pseudosolanacearum* (phylotype I); CFBP2957 is a isolate of *R. solanacearum* (phylotype II).

Figure 2. Estimation of RSIBR1 genome length / RCA concatamer cleavage, and a simple genomic map with the cleavage sites of the enzymes used.

Figure 3. Genomic organization of episomal and integrated form of RSIBR1. RS603, fRSM1 and fRM3 virus are another *Inovirus*. The arrows represent the direction of the transcription of ORFs or genes and the colors represent the modules of organization.

Figure 4. Phylogenetic neighbor-joining tree constructed using full-length genome. The orange lines represent Ralstonia Inoviruses (dashed outline) and other related Inoviruses and as blue lines or another genus of the Inoviridae family, Plectrovirus.

Figure 5. Similarity matrix between RSIBR1 and nearby species.

Figure 6. 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 7. 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).

Gene	Predicted protein	Genomic coordinates	Protein lenght (aa)	Mol. Mass (Da)	Protein pI	Homologs (a) & Motifs	E-value
ORF01	Putative Single-stranded DNA binding protein	1…327	108	11416.05	8.82	B5UAR2 Uncharacterized protein [Ralstonia phage RSM3] & W6CMZ9 Single-stranded DNA binding protein [Ralstonia phage 1 NP-2014]	1.2e-63; 8.9e-56;
ORF02	Uncharacterized protein	393...582	59	6864.95	6,08	A0A097ZIG1 Uncharacterized protein_ORF02 [Ralstonia phage RS603]	3e-30
ORF03	Putative minor coat protein	582...860	92	9784,43	4,65	B5UAR5 Uncharacterized protein [Ralstonia phage RSM3] & E5F069 Putative minor coat protein [Ralstonia phage PE226]	5.3e-31; 5.8e-11
ORF04	Uncharacterized protein	860...1069	69	7718.42	12,48	B5UAR6 Uncharacterized protein [Ralstonia phage RSM3]	3.8e-37
ORF05	Uncharacterized protein	1069...1278	69	7567,08	3,43	A0A097ZIG2 Uncharacterized protein_ORF05 [Ralstonia phage RS603]	3.3e-49
ORF06	Putative structural protein	1281...1529	82	8786,06	4,23	B5UAR8 Uncharacterized protein [Ralstonia phage RSM3] & Q6UAZ6 Putative minor coat protein [Ralstonia phage p12J]	1.3e-62; 4.9e0
ORF07	Putative structural protein	1532...1763	72	6945,23	10,18	B5UAR9 Uncharacterized protein_ORF08 [Ralstonia phage RSM3] & P68674 Capsid protein GP [Xanthomonas phage phiLF] protein motifs: two transmembrane	1.5e-45; 2.8e-3

						domain discovered using TMHMM and Phobius	
ORF08	Putative Termination of virion assembly protein	1825...3354	509	51046,15	5,65	YP_009103103.1 unnamed protein product_ORF08 [Ralstonia phage RS603] & W6CLR8 Termination of virion assembly protein [Ralstonia phage 1 NP-2014] & YP_002290960.1 hypothetical protein RSM3_ORF09 [Ralstonia phage RSM3] protein motifs: six transmembrane domain discovered using TMHMM and Phobius	0.0; 3.5e-13; 0.0
ORF09	Uncharacterized protein	3367...3696	109	11735,73	4,04	YP_009103105.1 unnamed protein product _ORF09 [Ralstonia phage RS603] & YP_002290962.1 hypothetical protein RSM3_ORF10 [Ralstonia phage RSM3] protein motifs: three transmembrane domain discovered using TMHMM and Phobius	2.9e-67; 2.3e-66
ORF10	Putative viral extrusion protein	3771...5018	415	46161,39	10,39	YP_009103107.1 unnamed protein product_ORF10 [Ralstonia phage RS603] & Q4LAU Putative zonula occludens toxin [Stenotrophomonas phage phiSMA9] & G1P_BPPHL - Isoform G11P of Gene 1 protein [Xanthomonas phage phiLf] protein motifs: one transmembrane	0.0; 2.4e-22; 7.5e-7

						domain discovered using TMHMM and Phobius	
ORF11	Putative rolling circle DNA replication initiation protein	5536...6453	305	34583,32	9,48	YP_009103107.1 unnamed protein product_ORF12 [Ralstonia phage RS603] & API74114.1 Rep protein [Ralstonia solanacearum] & W6CM31 rolling circle DNA replication initiation protein_ORF01 [Ralstonia phage 1 NP-2014]	0.0;0.0;0.0
ORF12	Putative viral transcriptional repressor	6463…6753	96	10586,36	9,72	WP_020957343.1 lambda repressor-like, dna-binding; protein [Ralstonia solanacearum] R9TE55 Regulatory protein [Vibrio phage VFJ] & YP_009103108.1 unnamed protein product_ORF13 [Ralstonia phage RS603] & A7Y8H7- C repressor [Pseudomonas phage MP22]	3e-63; 2e0; 2.5e-46; 4.2e0

Table 1. Functional genomic annotation of Ralstonia virus RSIBR1, a novel Inovirus infecting Ralstonia spp.

Isolates	Source[a]	Host	Origin[b]	Phyl-seq[c]	Inhibition zone (cm)[d] GMI1000 VF
***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]
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
V43	INPA	Scarlet eggplant	AM/North	ND	-
V45	INPA	Cucumber	AM/North	II/ND	2,25
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.

Viruses name	NCBI acession	Genome size (kbp)	Host	Origin	Reference
Ralstonia virus RS603	NC_025454.1	7.68	*Ralstonia solanacearum*	Japan	Van TT, et al. 2014
Ralstonia virus RSM1	NC_008574.2	9	*Ralstonia solanacearum*	Japan	Kawasaki,T, et al, 2007.
Ralstonia virus RSM3	NC_011399.1	8.93	*Ralstonia solanacearum*	Japan	Askora A, et al. 2009
Ralstonia virus RSMSuper	AB981170.1	8.9	*Ralstonia solanacaerum*	Japan	Askora A, et al. 2014
Ralstonia virus 1 NP-2014	NC_023586.1	8.46	*Ralstonia solanacearum*	France	Popgeorgiev N, et al. 2014
Ralstonia virus PE226	NC_015297.1	5.48	*Ralstonia solanacearum*	Korea	Murugaiyan S, et al. 2011
Ralstonia virus p12J	NC_005131.2	7.12	*Ralstonia solanacearum*	United States of America	Kim S,et al.2003
Ralstonia virus RS611	AB931172	6.38	*Ralstonia solanacearum*	Japan	Van T.T.B., et al. 2014
Ralstonia virus RSS-TH1	LC066596	7.27	*Ralstonia solanacearum*	Thailand	Kawasaki T,et al. 2015
Ralstonia virus RSS30	NC_021862.1	8.58	*Ralstonia solanacearum*	Japan	Kawasaki T, et al. 2013
Ralstonia virus RSS1	NC_008575.1	6.66	*Ralstonia solanacearum*	Japan	Kawasaki T, et al. 2007
Ralstonia virus	NC_019548.1	7.29	*Ralstonia*	Japan	Addy HS, et

RSS0				*solanacearum*		al. 2012
Propionibacterium virus B5	NC_003460.1		5.8	*Propionibacterium freudenreichii.*	France	Chopin MC, et al. 2002
Pseudomonas virus Pf1	NC_001331.1		7.35	*Pseudomonas aeruginosa*	American Type Culture Collection	Hill DF, et al. 1991
Pseudomonas virus Pf3	NC_001418.1		5.83	*Pseudomonas aeruginosa*	Canada	Luiten RG, et al. 1985
Vibrio virus VFJ	NC_021562.1		8.56	*Vibrio cholerae*	China	Wang Q, et al. 2013
Vibrio phage VSK	NC_003327.2		6.88	*Vibrio cholerae*	India	Basu N, et al. 2001
Vibrio virus Vf33	NC_005948.1		7.97	*Vibrio parahaemolyticus*	Japan	Chang B, et al. 1998
Vibrio virus VCY-phi	NC_016162.1		7.1	*Vibrio cholerae*	United states of America	Xue H, et al. 2012
Vibrio virus KSF-1phi	NC_006294.1		7.11	*Vibrio cholerae*	Bangladesh	Faruque SM, et al.2005
Enterobacteria virus If1	NC_001954.1		8.45	*Escherichia coli*	United States of America	Hill D.F., et al. 2008
Enterobacteria virus fd	NC_025824.1		6.41	*Escherichia coli*	Japan	Beck E, et al. 1978
Enterobacteria virus M13	NC_003287.2		6.41	*Escherichia coli*	Germany	van Wezenbeek PM, et al. 1980
Acholeplasma virusMV-L1	NC_001341.1		4.49	*Acholeplasma virus*	United States of America	Jaeger M, et al. 2000
Spiroplasma virus 1-R8A2B	NC_001365.1	8.27		*Spiroplasma citri virus*	France	Renaudin J, et al. 1990

Spiroplasma virus 1-C74	NC_003793.1	7.77		*Spiroplasma citri virus*	Irak		Renaudin J, et al. 1996
Spiroplasma kunkelii virus SkV1_CR2-3x	NC_009987.1		7.87	*Spiroplasma kunkelii*		United States of America	Davis R.E., et al. 2007
Spiroplasma virus SVTS2	NC_001270.2		6.83	*Spiroplasma citri virus*		United States of America	Sha Y, et al. 2000

Viral isolates used for comparison and analysis with RSIBR1 virus

Primer name	Sequence
M13F	Macrogen
M13R	Macrogen
187aF	CGGAGATACCAAACGTGC
188aR	GTTGTTGAGAGCGAGCG
189aF	GTCTCGACCTTGTGACCAG
189aR	GGGCATGCCAGAGATACCAAAC
192aF	TTATGCTGCGTCGAGTAGGCT
192aR	AGAACGAACAGCGCCATAAC
193aF	TACCAACGACCCAGCATG
193aR	GGCGATGGATACGGTTGCT
181aF	ATGAGCACGAATGTCCAGGC
181aR	CCCTTGACGTATCGGTCA
182aF	ACGAAGCATCTTATGTCCG
183aF	TCTCGATCGCATAGACCAC
184aF	ACATGAGCACGATGTCCAG
181bF	GATAGCGACGAAGACACTGT
181bR	GACTTCAAGCTGTTCGTCTC
182bF	CGTCTTCATTTTCAGGCTTGG
183bF	ATGGTAGGGTGTCCAACC
184bF	GAACAGATCCTCTGAGATCT
187aF	TCTCGACCTTGGGACCAA
188bR	GACTGGCAGGAAGAAATCT
189bF	TTGCGGCCTTTCTGTAGC
189bR	AACCCCTCGTGAACGATCA
192bF	TGCGTGGGTCTCTTGTTC
192bR	TGGCGATGATGGTCAGCTT
193bF	TTGAAGTCGGCCACGTAGTC
193bR	TAGTGCCTGCCCAGCTTTA
181cF	AATCATCTGTGCGCACGGATC
181cR	ACAGCAGTTGCGCATCAC
182cF	TCCAACCGTTAGAGCTACC
182bR	TCTAGCGTCAGGAGTGTC
183bF	TCCTCACGCTCGATCTTG
183bR	GCTAGTGAAAAATGGTCGC
184cF	ACGGAAACGAAACCCCGTAC
184bR	TTGTACTGGCTGCCCTAAGC
185bF	GATTCGTCGTACCACTCGAC
185bR	GCCACTTCTGCGAAATCGAC
186bF	AAGACCTCCCTCTTGCAAGC
186bR	TTTTCCGGAGGTGGTAGGTAC
187b2F	TCATCAGCCATCGCATCCTG
187bR	TTTCCGGAGGTGGTAGGTAC
188bF	AAGACCTCCCTCTTGCAAGC
188cR	GTGTATGGTTTACCTGTCAC
189cF	GCATCAAACATGAATCACCC
189cR	CAGAGCCGACAACGCATT
191bF	GATCTTGCGAACCTCAACGC
191bR	TGCCAGAACATCAGCAACCTC
InoBF1	ATGTCGAACACTCAAAAGCTGACCA

InoBR1	TGTTCGACATGATGGCTCCGACGAA
Ino6008	TCGTCTCCCCCGAACGTTCT
Ino5050	TCGTCTCCCCCGAACGTTCT

Sequences of the primers used to sequence the complete genome of RSIBR1 Inovirus.

Figure 1

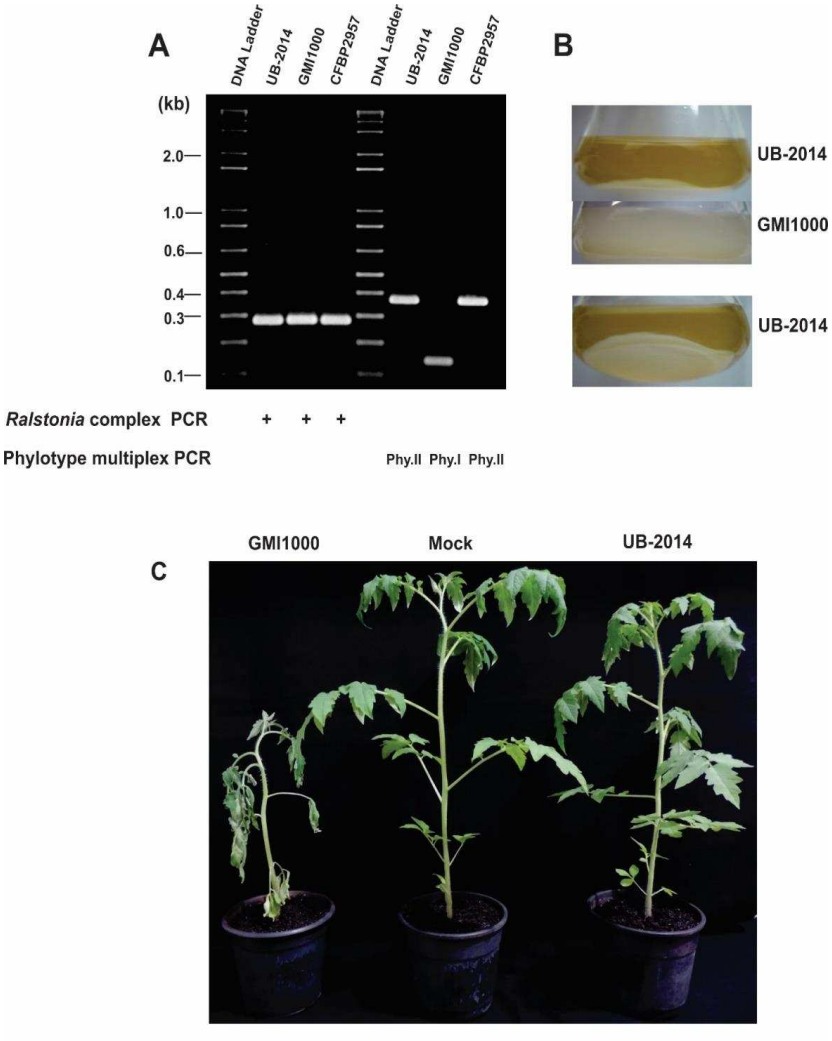

A
DNA Ladder
UB-2014
GMI1000
CFBP2957
DNA Ladder
UB-2014
GMI1000
CFBP2957
(kb)
2.0
1.0
0.6
0.4
0.3
0.1
Ralstonia complex PCR + + +
Phylotype multiplex PCR Phy.II Phy.I Phy.II
B
UB-2014
GMI1000
UB-2014
C
GMI1000
Mock
UB-2014

Figure 2

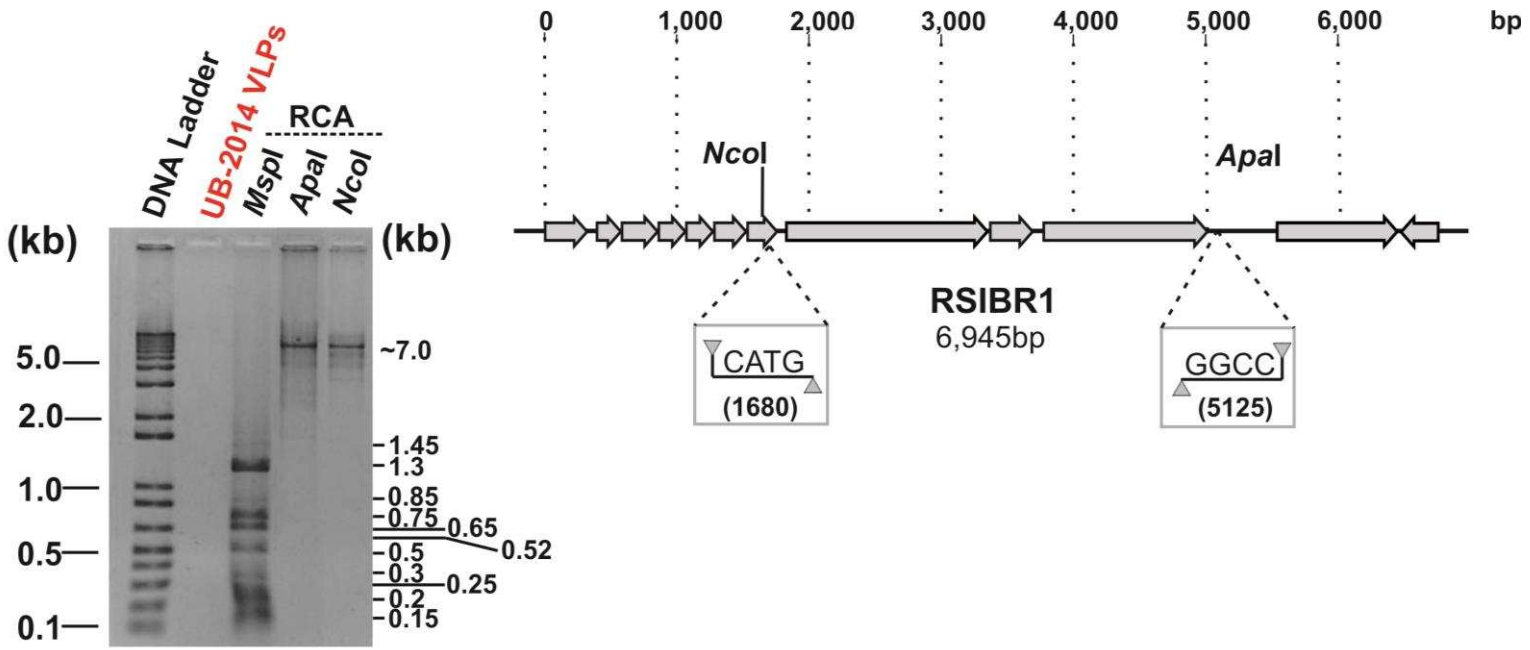

DNA Ladder
UB-2014 VLPs
RCA
MspI
ApaI
NcoI
(kb)
(kb)
5.0
2.0
1.0
0.5
0.1
~7.0
1.45
1.3
0.85
0.75
0.65
0.52
0.5
0.3
0.25
0.2
0.15
0
1,000
2,000
3,000
4,000
5,000
6,000
bp
NcoI
ApaI
RSIBR1
6,945bp
CATG
(1680)
GGCC
(5125)

Figure 3

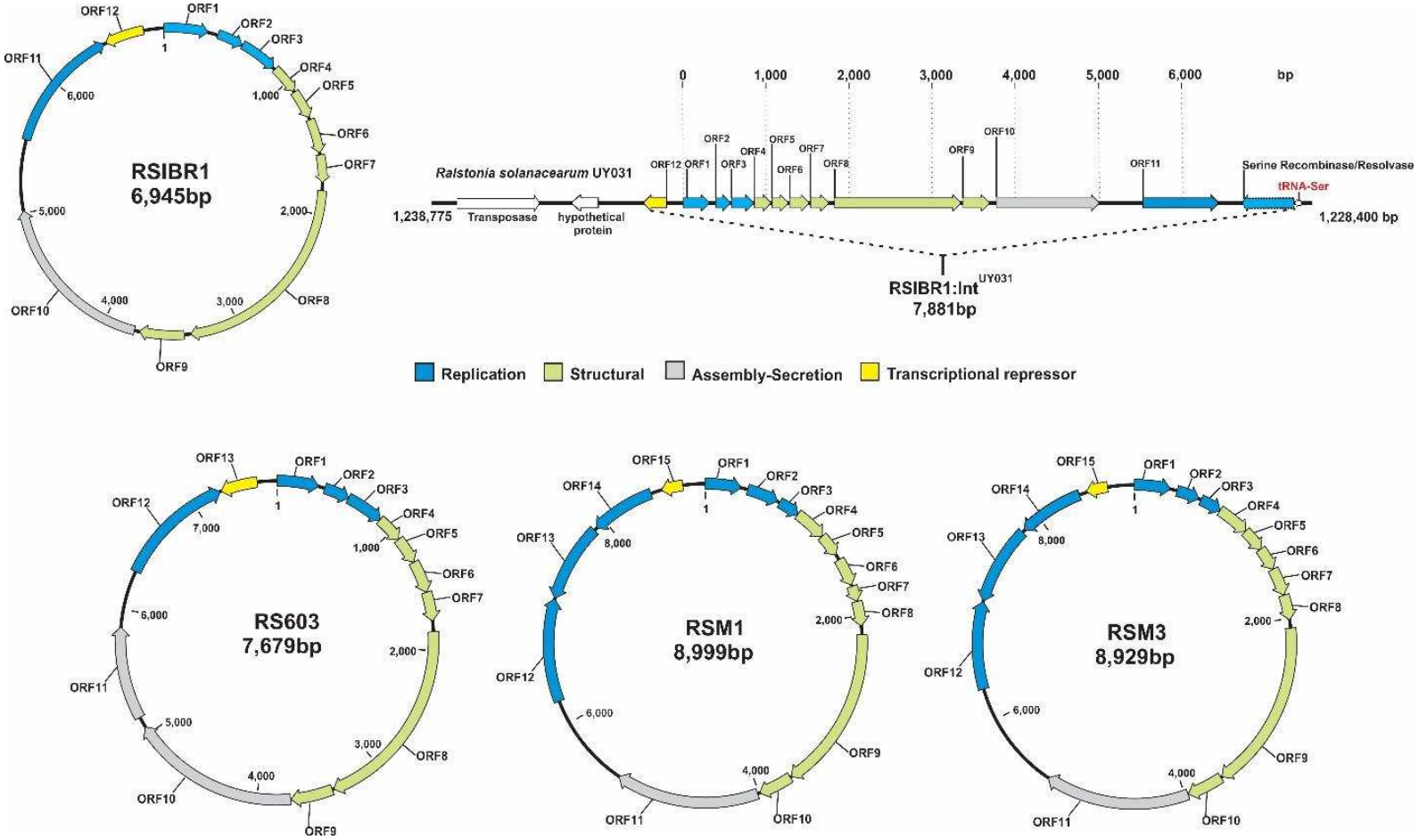

RSIBR1
6,945bp
ORF1
ORF2
ORF3
ORF4
ORF5
ORF6
ORF7
ORF8
ORF9
ORF10
ORF11
ORF12
Ralstonia solanacearum UY031
1,238,775
Transposase
hypothetical protein
Serine Recombinase/Resolvase
tRNA-Ser
1,228,400 bp
RSIBR1:Int UY031
7,881bp
Replication
Structural
Assembly-Secretion
Transcriptional repressor
RS603
7,679bp
ORF13
RSM1
8,999bp
ORF14
ORF15
RSM3
8,929bp

Figure 4

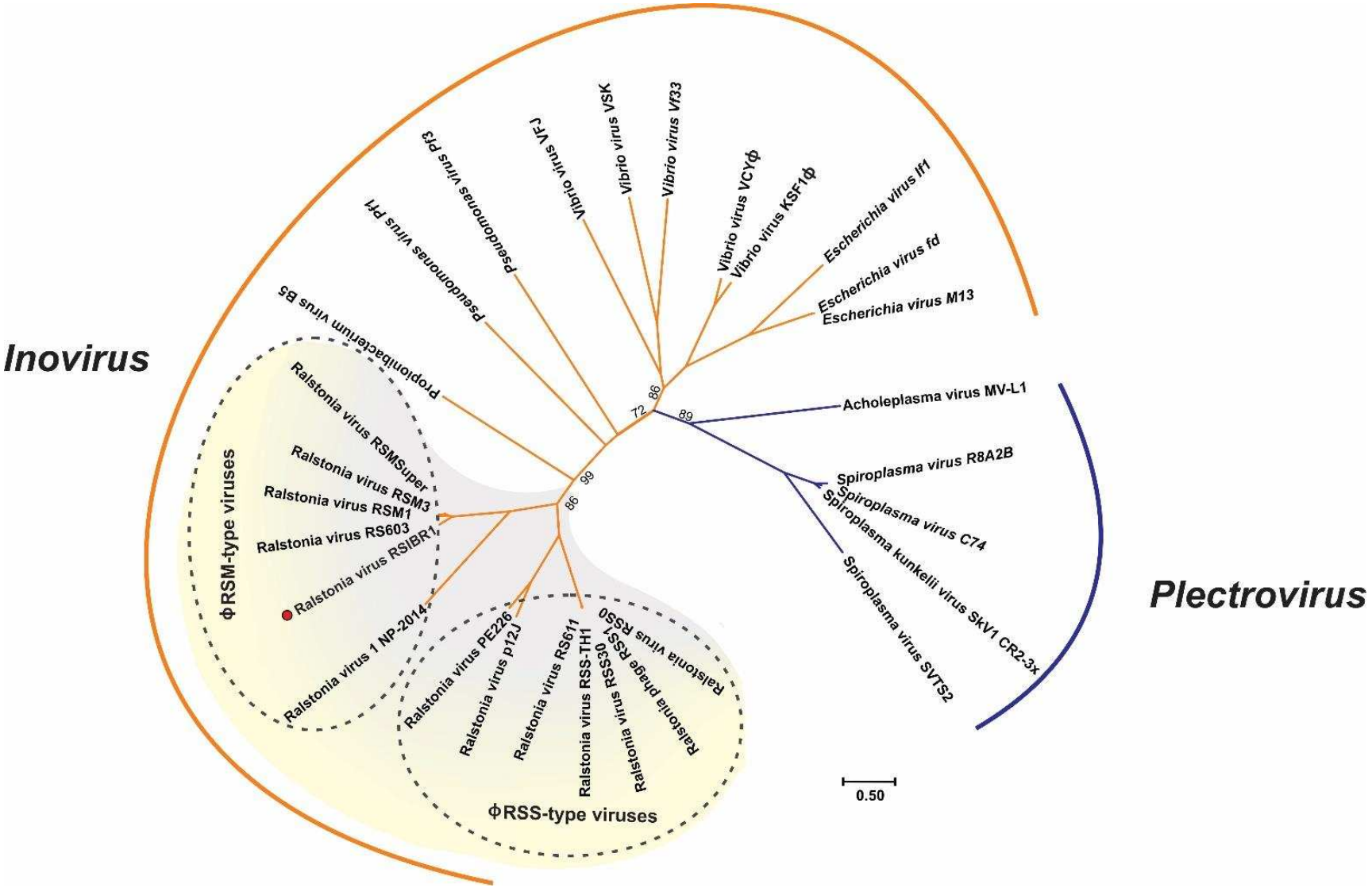

Inovirus
Plectrovirus
Propionibacterium virus B5
Pseudomonas virus Pf1
Pseudomonas virus Pf3
Vibrio virus VFJ
Vibrio virus VSK
Vibrio virus Vf33
Vibrio virus VCYϕ
Vibrio virus KSF1ϕ
Escherichia virus If1
Escherichia virus fd
Escherichia virus M13
Acholeplasma virus MV-L1
Spiroplasma virus R8A2B
Spiroplasma virus C74
Spiroplasma kunkelii virus SkV1 CR2-3x
Spiroplasma virus SVTS2
Ralstonia virus RSMSuper
Ralstonia virus RSM3
Ralstonia virus RSM1
Ralstonia virus RS603
Ralstonia virus RSIBR1
Ralstonia virus 1 NP-2014
ϕRSM-type viruses
Ralstonia virus PE226
Ralstonia virus p12J
Ralstonia virus RS611
Ralstonia virus RSS-TH1
Ralstonia virus RSS30
Ralstonia phage RSS1
Ralstonia virus RSS0
ϕRSS-type viruses
72
86
89
99
86
98
0.50

Figure 5

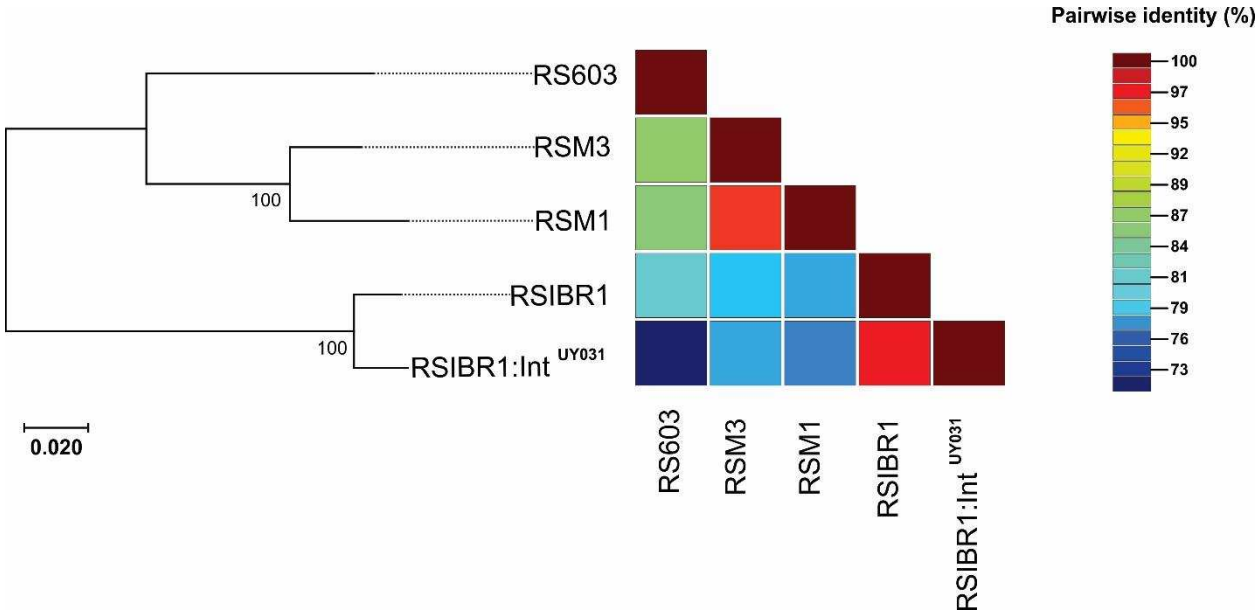

Pairwise identity (%)
100
97
95
92
89
87
84
81
79
76
73
RS603
RSM3
RSM1
RSIBR1
RSIBR1:Int UY031
100
100
0.020
RS603
RSM3
RSM1
RSIBR1
RSIBR1:Int UY031

Figure 6

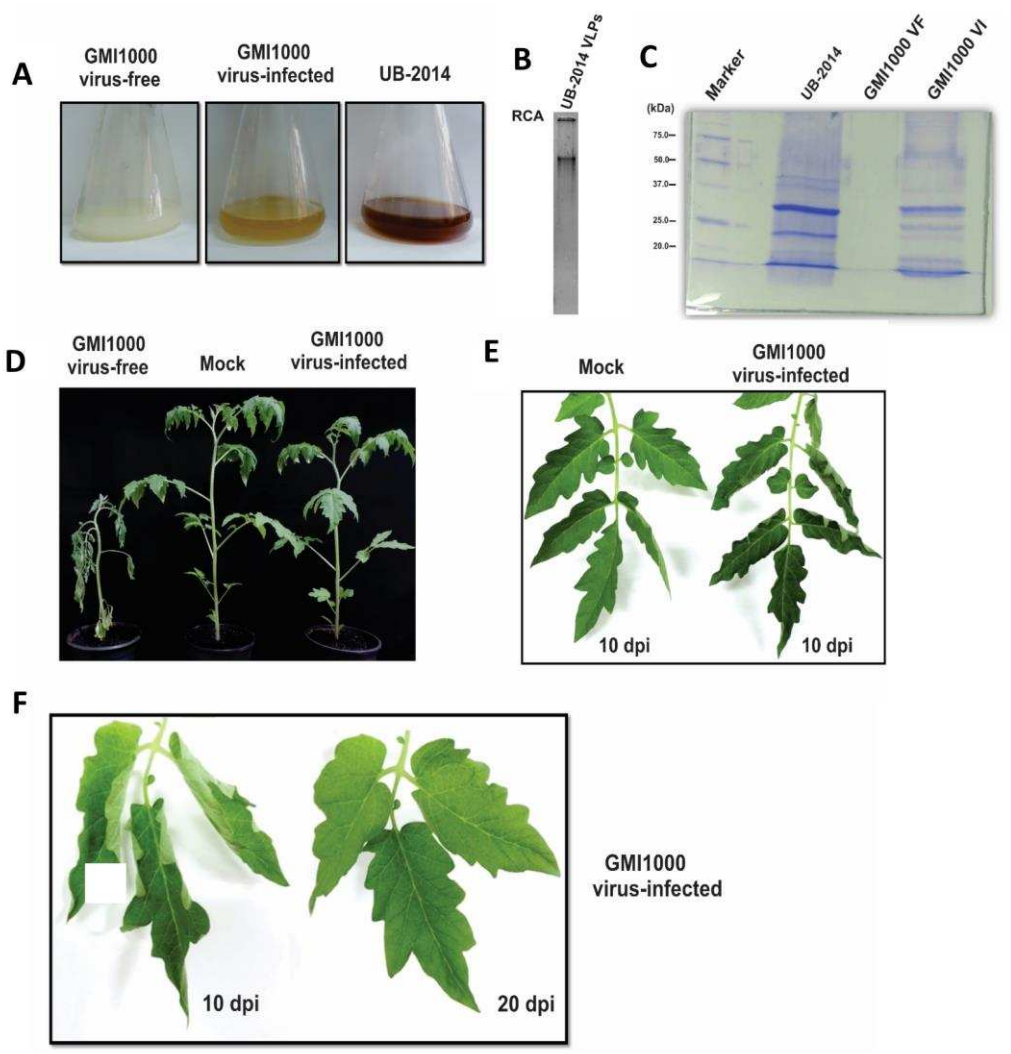
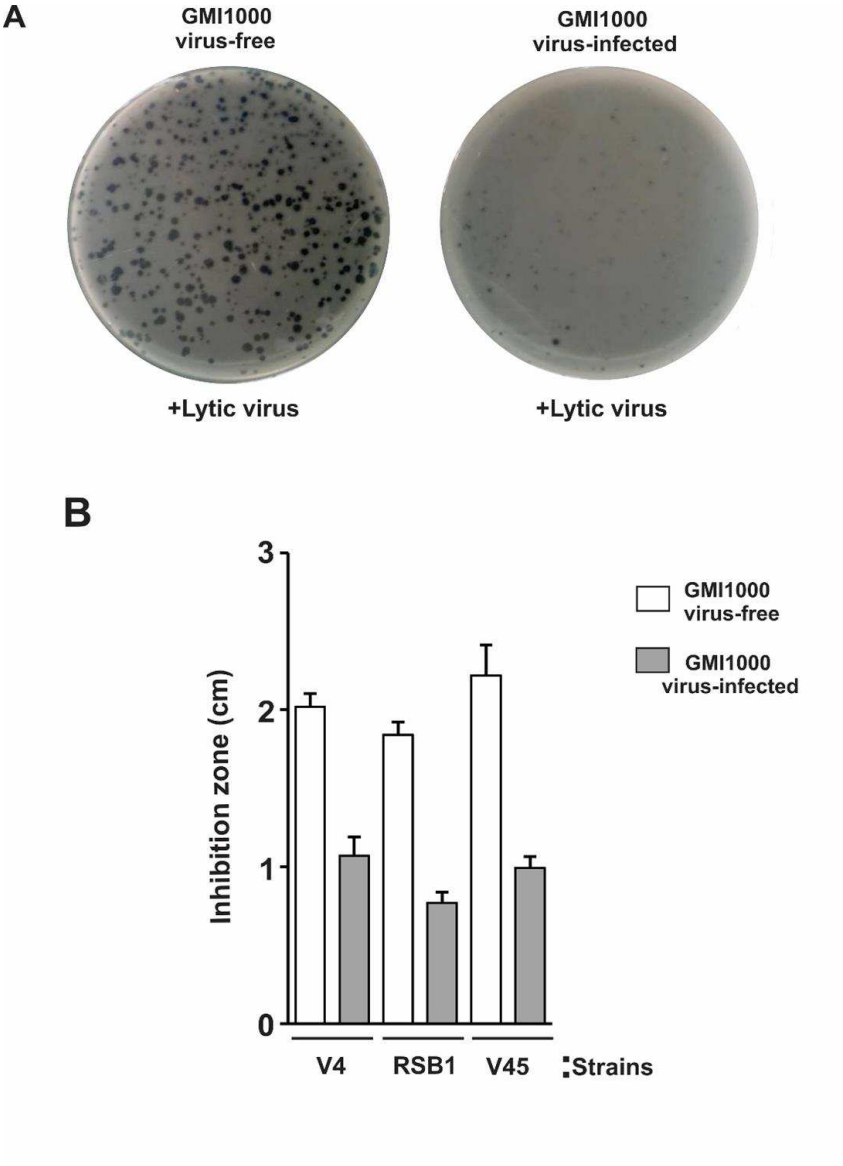

A
GMI1000 virus-free
GMI1000 virus-infected
UB-2014
B
UB-2014 VLPs
RCA
C
Marker
UB-2014
GMI1000 VF
GMI1000 VI
(kDa)
75.0
50.0
37.0
25.0
20.0
D
GMI1000 virus-free
Mock
GMI1000 virus-infected
E
Mock
GMI1000 virus-infected
10 dpi
10 dpi
F
10 dpi
20 dpi
GMI1000 virus-infected

Figura 7


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

CHAPTER 2

Characterization of H-NS proteins and their function in the regulation of the CRISPR-CAS system in *Ralstonia solanacearum*

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CHARACTERIZATION OF H-NS PROTEINS AND THEIR FUNCTION IN THE REGULATION OF THE CRISPR-CAS SYSTEM IN *RALSTONIA SOLANACEARUM*

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Short title: Functional analysis of H-NS proteins in *Ralstonia solanacearum*

Contents category: CRISPR-Cas system regulation

Key words: Bacteria anti-viral defense, transcriptional control, inactive CRISPR, DNA-binding proteins

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

Number of figures: 4

ABSTRACT

H-NS is a small abundant protein that plays an important role in regulating various bacterial genes involved in bacterial adaptation such as osmolarity, temperature and acid shock. H-NS proteins have two domains, N-terminal oligomerization and C-terminal responsible for DNA binding. Some studies have reported the role of H-NS proteins in the repression of the CRISPR-Cas system in *E. coli*. CRISPR loci and CRISPR-associated (Cas) genes code for an inheritable immune adaptive system that provides sequence-specific protection against foreign DNA. We previously carried out a characterization of the Ralstonia spp. CRISPR-Cas system. The results showed that CRISPR-Cas is inactive or in a conditional repression state. Since H-NS proteins could be involved in the repression of the Cas genes, we searched the *R. solanacearum* CFBP2957 for H-NS genes. Three H-NS proteins (H-NS1, H-NS2 and H-NS3), were found in the megaplasmid. The oligomerization domain and the DNA binding domain are well conserved and it was found that operons for Cas genes had the characteristics for H-NS binding. We only detected the expression of H-NS 1 and H-NS3. Knockout studies of the *hn-s* genes in *R. solanacearum* CFBP2957 are currently being conducted in an attempt to reprogram the CRISPR activity, to assess its mode of regulation and functionality.

INTRODUCTION

H-NS (histone-like nucleoid-structuring) is a small abundant protein that plays an important role in regulating various bacterial genes, acting as a global negative regulator controling the expression of a large number of genes involved in bacterial adaptation to environmental changes, such as osmolarity, temperature and acid shock (Bouffartigues *et al.*, 2007). H-NS proteins have two domains, N-terminal oligomerization and C-terminal responsible for DNA binding. The first domain has 83 amino acids, four α-helices (α1, α2, α3 and α4) that allow self-association by head-to-head and tail-to-tail. The C-terminal domain has two β-parts (β1 and β2), an α-helix (α5) and a 3_{10} helix. Both domains are well conserved among the H-NS proteins of different bacteria (Grainger, 2016).

Some studies have reported the role of H-NS proteins in the repression of the CRISPR-Cas system in *E. coli* (Pul *et al.*, 2010; Medina-Aparicio *et al.*, 2011). CRISPR loci and CRISPR-associated (*Cas*) genes code for an inheritable immune adaptive system that provides 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 & Sontheimer, 2008).

In a previous work, we demonstrate the presence of canonical CRISPR loci in *Ralstonia* spp. and provided 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, and that the Cas genes are not expressed (Xavier, 2016).

Since the phenotype of CRISPR inactivity can be explained by the expression of the *Cas* operon repressors, in this work we analyze the H-NS proteins presents in *R. solanacearum* CFBP2957 genome. Three H-NS proteins (H-NS1, H-NS2 and H-

NS3) were found in the megaplasmid. 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.

RESULTS AND DISCUSSION

An analysis of the *R. solanacearum* CFBP2957 genome was performed. Three H-ns proteins on the chromosome, H-NS1, H-NS2 and H-NS3, which are present in the megaplasmid were identified (Figure 1A). The H-NS proteins are commonly located on the bacterial chromosome but in *R. solanacearum* CFBP2957 the H-NS proteins are present in the megaplasmid. All three H-NSs found are smaller than H-NS from other bacteria, but all functional domains are present (oligomerization domain and DNA binding domain) and are well conserved (Figure 1B). The domains are much more conserved among the *Ralstonia* spp. when compared to the alignment with bacteria from other families (Enterobacteriaceae and Pseudomonadaceae) (Figure 1B).

A phylogenetic analysis of the amino acid sequences of H-NS proteins of different species showed that H-NS group according to the families of Enterobacteriaceae, Pseudomonadaceae and Burkholderiaceae (Figure 1C). An interesting observation is that a H-NS protein found in a podovirus (EBPR podovirus 1) grouped together with the H-NS of the species of *Ralstonia* spp. being close to the H-NS 1 and 3 of *R. solanacearum* CFBP2957. An alignment between different isolates of *Rasltonia* spp. and EBPR podovirus 1 H-NS was also performed. The H-NS domains of the species of *Ralstonia* spp. are highly conserved with the EBPR podovirus 1 (Figure S1), suggesting a relationship between the H-NS of *Ralstonia* spp. being of viral origin, a mechanism perhaps used by the viruses to scape of the CRISPR-Cas system of its hosts.

In order to find out if the three H-NS proteins were expressed, RT-PCR was performed. Expression of only H-NS 1 and H-NS 3 as detected (Figure 1D). To test if H-NS 2 could be induced by viral infection, the expression of all H-NSs was analyzed in *R. solanacearum* CFBP2957 infected with PhiAP1, a *Phikmvvirus* and only H- NS 1 and 3 were being expressed. Therefore, if the CRISPR-Cas system is being repressed by H-NS proteins, only H-NS 1 and 3 could be performing this role.

Knowing the affinity of the H-NS proteins for regions rich in AT, a silico analysis was performed on the promoters for the CAS proteins, to verify the possibility of being sites for the H-NS. We identified a high AT content in the promoter region and also the analyzes demonstrated that it is a region with curved DNA, two potential characteristics for the H-NS binding sites (Figure 2).

To analyze the role of H-NS 1 and H-NS3 in repress the CRISPR-Cas system, we design two cassets to knockout the *h-ns1* and *h-ns3* (Figure 3). Each cassette contained 400 kb on each side corresponding to the H-NS flanking regions, in place of the sequence for H-NS 1 the sequence was placed for the antibiotic gentamycin and H-NS3 was replaced by the sequence of the antibiotic kanamycin. *R. solanacearum* CFBP2957 transformation is currently being conducted in an attempt to reprogram the CRISPR activity, to assess its mode of regulation and functionality.

METHODOLOGY

H-NS bioinformatics analysis

In silico analyses were performed to locate the position of the H-NS genes and the CRISPR-Cas system using the Geneious program. The sequences were aligned with ClustalX2 and a maximum likelihood tree was constructed in the MEGA 7.0 program. To perform alignments of the H-NS domains we used ClustalW2.

Bend.it Server and DNA Curvature Analysis using the Wedge dinucleotide model to calculate the overall 3D structure of the DNA molecule considering the macroscopic curvature and its angle were used to detect the curvature of the promoter region of the Cas proteins of the CRISPR- Cas. AT content was detected by the Geneious program (Biomatters Ltd., Auckland, New Zealand).

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, non-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). 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 was tested by PCR.

h-ns1 and h-ns3 knockouts

To obtain mutants for the H-NS 1 and H-NS 3 proteins, two cassettes were designed manually based on the genomic sequence of *R. solanacearum* CFBP9595. Each cassette contained 400 kb on each side corresponding to the H-NS flanking regions, in place of the sequence for H-NS 1 the sequence was placed for the antibiotic gentamycin and H-NS3 was replaced by the sequence of the antibiotic kanamycin . The ends of both cassettes contained restriction enzyme sites, *EcoR1*

and *Pst1* for H-NS1 and *BamH1* and *HimdIII* for H-NS3. The cassettes were built by the company GENONE (http://www.genone.com.br/).

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Barrangou R, Fremaux C, Deveau H *et al.*, 2007. CRISPRProvides Acquired Resistance Against Viruses in Prokaryotes. *Science* **315**, 1709–1712.

Bouffartigues E, Buckle M, Badaut C, Travers A, Rimsky S, 2007. H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. *Nature Structural & Molecular Biology* **14**, 441–448.

Flores-Cruz Z, Allen C, 2009. Ralstonia solanacearum Encounters an Oxidative Environment During Tomato Infection. *Mpmi* **22**, 773–782.

Grainger DC, 2016. Structure and function of bacterial H-NS protein. *Biochemical Society Transactions* **44**, 1561–1569.

Marraffini LA, Sontheimer EJ, 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* **322**, 1843–1845.

Medina-Aparicio L, Rebollar-Flores JE, Gallego-Hernández AL *et al.*, 2011. The CRISPR/Cas immune system is an operon regulated by LeuO, H-NS, and leucine-responsive regulatory protein in Salmonella enterica serovar Typhi. *Journal of Bacteriology* **193**, 2396–2407.

Pul Ü, Wurm R, Arslan Z, Geißen R, Hofmann N, Wagner R, 2010. Identification and characterization of E. coli CRISPR-cas promoters and their silencing by H-NS. *Molecular Microbiology* **75**, 1495–1512.

Figure legends:

Figure 1. *In silico* characterization and expression of H-NS proteins in *R. solanacearum* CFBP2957 **(A)** Characterization of CRISPR arrays and *Cas* operon. **(B)** Conservation of H-NS protein domains of different species of bacteria. The amino acids in black are identical and the colored amino acids in gray, although different, have the same properties. The ones that are colorless are not conserved. **(C)** Phylogenetic tree constructed with MEGA software, with the amino acid sequences of the H-NS proteins of various bacteria. **(D)** PCR to demonstrate the expression of H-NS 1, 2 and 3 proteins of *R. solanacearum* CFBP9595.

Figure 2. AT content and DNA curvature demonstrating that the promoters of the CAS proteins are potent sites for H-NS.

Figure 3. Cassettes constructed for *R. solanacearum* CFBP9595 transformation. A) Cassettes cloned in vector pBluescript IISK for for transformation by electroporation. B) Free cassettes for natural transformation

Supplemental figure S1. Conservation of amino acids in H-NS proteins from different species of *Ralstonia.*

Figure 1.

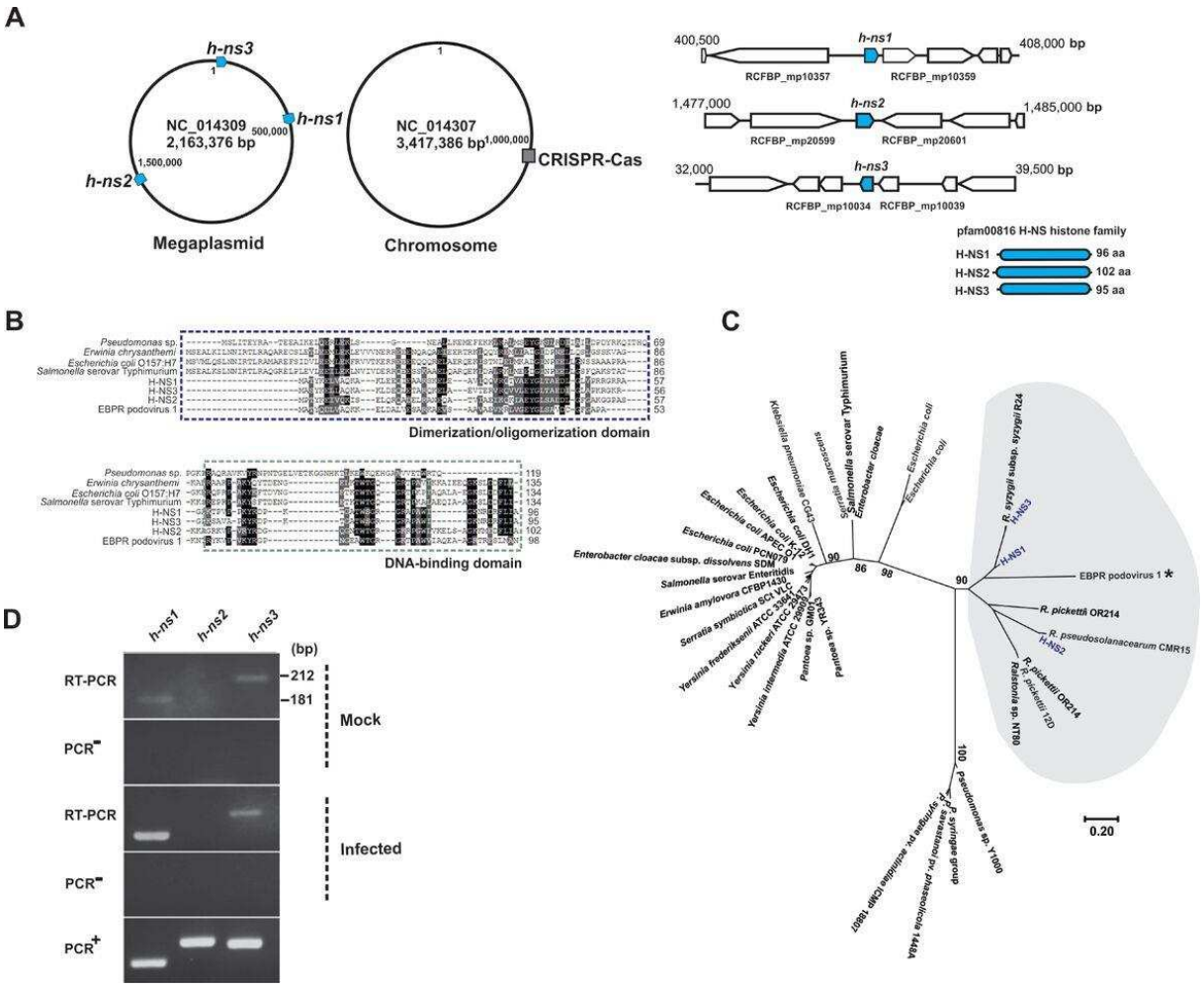

A
h-ns3
h-ns1
h-ns2
NC_014309
2,163,376 bp
NC_014307
3,417,386 bp
CRISPR-Cas
Megaplasmid
Chromosome
400,500
408,000 bp
RCFBP_mp10357
RCFBP_mp10359
1,477,000
1,485,000 bp
RCFBP_mp20599
RCFBP_mp20601
32,000
39,500 bp
RCFBP_mp10034
RCFBP_mp10039
pfam00816 H-NS histone family
H-NS1 96 aa
H-NS2 102 aa
H-NS3 95 aa
B
Dimerization/oligomerization domain
DNA-binding domain
C
D
RT-PCR
PCR-
PCR+
(bp)
212
181
Mock
Infected

Figure 2.

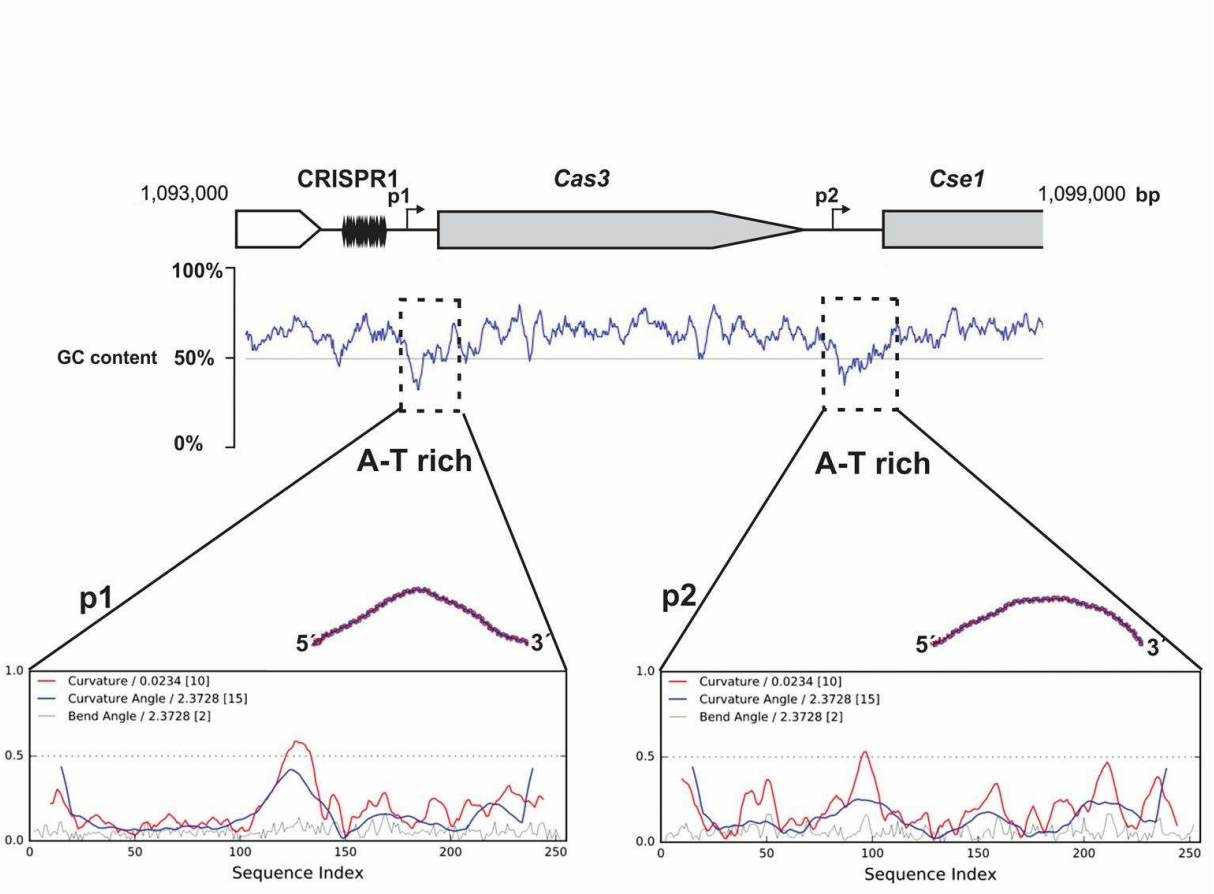
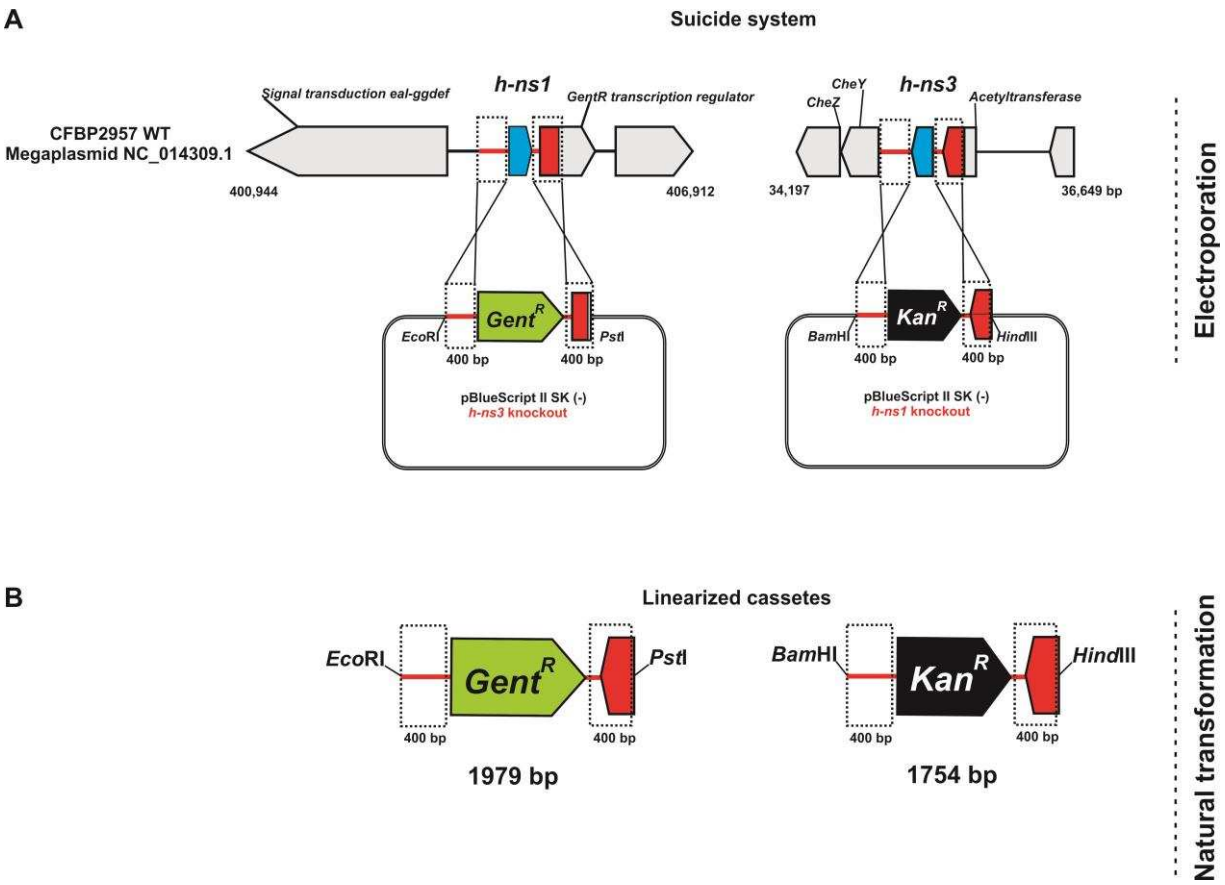

1,093,000
CRISPR1
p1
Cas3
p2
Cse1
1,099,000 bp
100%
GC content 50%
0%
A-T rich
A-T rich
p1
p2
5´
3´
5´
3´
Curvature / 0.0234 [10]
Curvature Angle / 2.3728 [15]
Bend Angle / 2.3728 [2]
Curvature / 0.0234 [10]
Curvature Angle / 2.3728 [15]
Bend Angle / 2.3728 [2]
1.0
0.5
0.0
0
50
100
150
200
250
Sequence Index
Sequence Index

Figura 3.


A
Suicide system
CFBP2957 WT
Megaplasmid NC_014309.1
Signal transduction eal-ggdef
h-ns1
GentR transcription regulator
400,944
406,912
CheY
CheZ
h-ns3
Acetyltransferase
34,197
36,649 bp
EcoRI
Gent R
PstI
400 bp
400 bp
pBlueScript II SK (-)
h-ns3 knockout
BamHI
Kan R
HindIII
400 bp
400 bp
pBlueScript II SK (-)
h-ns1 knockout
Electroporation
B
Linearized cassetes
EcoRI
Gent R
PstI
400 bp
400 bp
1979 bp
BamHI
Kan R
HindIII
400 bp
400 bp
1754 bp
Natural transformation

Supplemental figure S1.