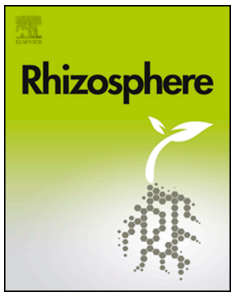

Participation of type VI secretion system in plant colonization of phosphate solubilizing bacteria

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PII: S2452-2198(22)00112-4

DOI: https://doi.org/10.1016/j.rhisph.2022.100582

Reference: RHISPH 100582

To appear in: *Rhizosphere*

Received Date: 13 April 2022

Revised Date: 16 August 2022

Accepted Date: 18 August 2022

Please cite this article as: Lucero, C.T., Lorda, G.S., Ludueña, L.M., Nievas, F., Bogino, P.C., Angelini, J., Ambrosino, M.L., Taurian, T., Participation of type VI secretion system in plant colonization of phosphate solubilizing bacteria, *Rhizosphere* (2022), doi: https://doi.org/10.1016/j.rhisph.2022.100582.

PARTICIPATION OF TYPE VI SECRETION SYSTEM IN PLANT COLONIZATION OF PHOSPHATE SOLUBILIZING BACTERIA

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PARTICIPATION OF TYPE VI SECRETION SYSTEM IN PLANT COLONIZATION OF PHOSPHATE SOLUBILIZING BACTERIA

Abstract.

In mutualistic endophytic bacteria, the type VI secretion system (T6SS) is related to important functions, such as interbacterial competition, stress response, quorum sensing, biofilm formation, and symbiosis. The presence of T6SS in beneficial endophytic bacterial population associated with different plants suggests that it plays an important role in its interaction with the eucaryotic partner. Within plant promoting bacteria, those with phosphate solubilizing activity constitute a group of great relevance to the rhizosphere as they provide phosphorus to plants. Among them, those with endophytic colonization capacity have survival advantages. The aim of this study was to determine whether the T6SS of a native peanut phosphate solubilizing bacterium is involved in its colonization in this legume. Initially, an *in silico* analysis looking for genes related to T6SS in the genome of the *Enterobacter* sp. J49 strain enabled us to identify almost all the *tss* genes, except for the *tssE* gene. A T6SS mutant of the *Enterobacter* sp. J49 strain was obtained by interrupting one of the essential *tss* genes. Then, the *Enterobacter* sp. J49-hcp strain was inoculated on peanut plants to analyze its colonization capacity. In addition, properties associated with endophytic colonization were analyzed, such as the formation of biofilms and the production of pectinase and cellulase enzymes. The results obtained indicated a significant decrease in the epiphytic and endophytic colonization of the mutant with respect to the wild strain. It is possible to conclude that T6SS, although not essential, may participate in bacterial colonization, either by accelerating the infection or by promoting other mechanisms involved in it.

Keywords: T6SS, *hcp* gene, colonization, peanut, *Enterobacter* sp.

1. Introduction

The type VI secretion system (T6SS) is a discovered mechanism in Gram-negative bacteria (Jani and Cotter 2010; Unni et al. 2022) by which cells in close proximity to one another can interact via contact-dependent protein transport from a donor cell to a recipient cell (Alvarez-Martinez and Christie 2009; Hood et al. 2010). Bacteria can use this secretion system to manipulate eukaryotic host cells and/or combat other bacteria that thrive in the same ecological niche (Ho et al. 2013; Gallegos-Monterrosa and Coulthurst 2021). The T6SS, which is frequently encoded by clusters of contiguous genes, is a complex structure composed of 13 conserved proteins and a variable complement of accessory elements (Russell et al. 2014). The genes that code for this secretion system are widely distributed in the genomes of Proteobacteria, in free-living and eukaryotic-associated species, including both pathogens and symbionts of animals and plants (Russell et al. 2014; Bernal et al. 2018). Mutagenesis studies in *Edwardsiella tarda*, *Vibrio cholerae*, *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. tomato have shown that each of the 13 conserved T6SS genes is required for their function (Zheng and Leung 2007; Zheng et al. 2011; Lin et al. 2013; Chien et al. 2020). Structurally, T6SS resembles a phage tail-shaped device made up of a rigid tube of hexameric hemolysin co-regulated protein (Hcp or TssD) rings that are wrapped in a contractile sheath. It is a complex structure in which several proteins are involved to form the envelope (TssB and TssC), arranged in a helical configuration, a tail that expands in the cytosol and starts from the baseplate structure (TssA, TssE, TssF, TssG, and TssK) and the integral membrane complex (TssL, TssM, and TssJ). The Hcp tube is crowned by a trimer of VgrG proteins and a tip called PAAR. The last central component of the T6SS is the ATPase ClpV, which is responsible for the disassembly of the contracted envelope, thus allowing the recycling of its components for subsequent secretion events (Bernal et al. 2018).

Most organisms with T6SS are not pathogenic and are found in marine environments, the rhizosphere, and the soil, or are associated with higher organisms as symbionts or commensals (Bingle et al. 2008; Boyer et al. 2009). The fact that *Pseudomonas syringae* pathovars and *Ralstonia solanacearum* encode T6SS clusters initially suggested a major role for this secretion system in plant colonization and virulence (Sarris et al. 2010). Many bacteria of health importance

possess one or more T6SS; however, the pathogenic relevance of most of these systems is unknown (Silverman et al. 2012). Santos et al. (2020) studied several effectors genes of T6SS of *Agrobacterium tumefaciens* 1D1609 and found that they did not play a role in antibacterial activity probably because of T6SS low expression. Thus, although the T6SS of phytopathogenic bacteria was initially described as a virulence trait, it has recently been shown to be associated with other important functions, such as bacterial interactions and host colonization in a beneficial interaction (MacIntyre et al. 2010; Schwarz et al. 2010; Verster et al. 2017; Bernal et al. 2018; Unni et al. 2022). Indeed, the genes encoding this secretion system were originally reported in the *Rhizobium leguminosarum* (the *imp* genes) (Bladergroen et al. 2003), a nitrogen fixing beneficial symbiotic bacterium associated with legumes. Although still scarce when compared to studies of T6SS in animal pathogens, the analysis of this system is a topic of increasing interest in the phytobacteria field (Bernal et al. 2018).

Type VI protein secretion systems (T6SSs) have been identified in many plant-beneficial bacteria (Bernal et al. 2018; Jiang et al. 2019). Plant growth promoting bacteria are a group of microorganism with interesting traits beasue of their beneficial effects on plant development and health (Bashan and Holguin 1998; Riaz et al. 2021). The promote plant growth by several mechanisms such as nitrogen fixation, solubilization of insoluble soil phosphates, producing phytohormones, and the prevention of diseases caused by phytopathogens (Bashan and De-Bashan 2005). To exert their beneficial effect, these bacteria must be able to colonize plant rhizosphere and tissues efficiently, which implies that they must be competitive in the soil environment (Compant et al. 2010; Sengupta et al, 2017). Most of the T6SS genes described in plant associated bacteria are involved in competition (Allsopp et al. 2020), which may confer an advantage in microbial interaction because they can inhibit the growth of surrounding bacteria (Bernal et al. 2017). The T6SS has been shown to be involved in the interaction with eukaryotic host organisms for a variety of bacteria (Hachani et al. 2016). In *Azoarcus* sp. BH72, a nitrogen fixing endophyte bacterium, that has a functional T6SS, a regulatory protein of this secretory system enhanced plant colonization efficiency (Shidore et al. 2012). In a metagenomic study of

the endophytic population of rice, Sessitsch et al. (2012) observed a high number of genes encoding T6SS components.

In this study, we examined the involvement of T6SS of the *Enterobacter* sp. J49 strain, a peanut endophytic bacterium, in its colonization in this legume. Peanut is a crop of great economic significance worldwide, with a production of over 50M tons in 2018 (Food and Agriculture Organization of the United Nations 2021). Argentina is one of the major producers of this legume in the world (Peanut Argentina Chamber 2018), and approximately 90% of its production is concentrated in the province of Cordoba (Cordoba Cereal Stock-MarketBag Institute 2018). Previous studies demonstrated that in this producing area, peanut rhizosphere harbors a high number of enterobacteria belonging to *Serratia*, *Enterobacter*, and *Pantoea* genera with plant growth promoting traits (Taurian et al. 2010; Anzuay et al. 2013). The *Enterobacter* sp J49 strain is an efficient phosphste solubilizer and a potential P-biofertilizer in microcosmos and field assays (Anzuay et al. 2021; Lucero et al. 2021). Considering that microorganisms possessing a T6SS appear to have a significant competitive advantage within a microbial community (Allsopp et al. 2020) and thus might play an important role in the life style of endophytes, we hypothesized that the T6SS of *Enterobacter* sp. J49 would participate in its colonization in this legume. In addition, T6SS is a relatively less known TSS than other secretion systems; in particular, its importance in plant-associated *Enterobacter* remains almost unexplored.

This manuscript explore the potential role of type VI secretion system (T6SS) in the colonization and related phenotype of a phosphate solubilizing bacterium. The objectives of this study were to identify the gene cluster of the T6SS of the phosphate solubilizing native peanut endophytic bacterium *Enterobacter* sp. J49 and to determine the role of this secretion system in the colonization ability of the strain and other properties associated with it. By *in silico* analysis, we identified a T6SS gene cluster and then generated a mutant by truncating *hcp*, a core T6SS gene, to disrupt T6SS function. We analyzed the role of this secretion system on plant colonization by inoculating the mutant strain on peanut plants in microcosmos assays, examined the biofilm formation, and measured the activity of plant cell wall degrading enzymes.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and growth conditions

All bacterial strains and plasmids used in this study are described in **Table 1**. *Enterobacter* sp. J49 strain was selected based on its ability to efficiently solubilize insoluble phosphate *in vitro* and promote the growth of peanut, maize, and soybean plants (Anzuay et al. 2017; Lucero et al. 2021). *Enterobacter* sp. J49 and *Enterobacter* sp. J49-hcp were grown on Tryptone Yeast (TY; Tryptone 5 $g.l^{-1}$; yeast extract 3 $g.l^{-1}$; $CaCl_2.6H_2O$ 1.3 $g.l^{-1}$; Beringer 1974) media and maintained in 20% glycerol ($v.v^{-1}$) at -80 °C. The *Enterobacter* sp. J49-hcp strain was supplemented with kanamycin (Km) at a concentration of 50 $\mu g.ml^{-1}$. *Escherichia coli* strains were grown on Luria Bertani (LB; Tryptone 10 $g.l^{-1}$, yeast extract 5 $g.l^{-1}$, NaCl 5 $g.l^{-1}$, Miller 1972). In the biofilm assay, the National Botanical Research Institute's phosphate growth medium was used (NBRIP; glucose 10 $g.l^{-1}$; $Ca_3(PO_4)_2$ 5 $g.l^{-1}$; $MgCl_2.6H_2O$ 5 $g.l^{-1}$; $MgSO_4.7H_2O$ 0.25 $g.l^{-1}$; KCl 0.2 $g.l^{-1}$; $(NH_4)_2SO_4$ 0.1 $g.l^{-1}$; for solid media 15 $g.l^{-1}$ agarose; Mehta and Nautiyal 2001). The images were taken with a Syngene G: BOX image analyzer.

126 **Table 1.** Strains and plasmids used.

	Description	Reference
Strains		
Enterobacter sp. J49	Wild type P-solubilizing isolate. Amp^r	Taurian et al. 2010
Enterobacter sp. J49-hcp	- T6SS disrupted mutant. *hcp*::pKNOCK-Km^r of *Enterobacter* sp. J49	This study
Escherichia coli DH5α	F- φ80d lacZ ΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk- mk+) phoA supE44 λ- thi-1 gyrA96 relA1	Stratagene
E. coli DH5α-pGEM-T-hcp2	*E. coli* DH5α containing the plasmid pGEM-T-*hcp2*	This study
Escherichia coli S17-1	*E. coli* 294 Thi RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Simon et al. 1983
E. coli S17-1-pKNOCK-hcp2	*E. coli* S17-1 containing the plasmid pKNOCK-*hcp2*	This study
Plasmids		
pGEM-T	Cloning vector, Amp^r, *lacZ*	PB-L®
pGEM-T-hcp2	Cloning vector, Amp^r, *lacZ* + 193 bp from the *hcp* gene of *Enterobacter* sp. J49	This study
p-KNOCK-Km	Cloning vector, Km^r	Alexeyev 1999
p-KNOCK-hcp2	Cloning vector, Km^r + 193 bp from the *hcp* gene of *Enterobacter* sp. J49	This study

127

128

129 **2.2. Bioinformatic analyses**

130 Specific T6SS genes were searched in the genome of *Enterobacter* sp. J49 by computer analysis.
131 Available *tss* sequences were searched in the NCBI gene bank database, and a forced alignment
132 was performed with the genome sequence of the peanut native (NZ_MWPY00000000; Ludueña
133 et al. 2019) strain using the blastn and blastp (NCBI) tools.

134

135 **2.3. Site-directed mutagenesis to obtain the *Enterobacter* sp. J49 deficient in the synthesis of**
136 **the Hcp protein (J49-hcp)**

137 To obtain an Hcp deficient strain, site-directed mutagenesis of the *hcp* gene of *Enterobacter* sp
138 J49 was carried out. Initially, a fragment of 190 bp of the *hcp* gene was obtained by PCR by

employing the set of primers hcp2F/hcp2R (**Table 2, Figure S1** - supplementary material). The amplification product obtained was purified using the QIAquick Gel Extraction Kit (QIAGEN) and sent to the Macrogen laboratories (Korea) for sequencing. The ligation of the 190 bp *hcp* gene fragment to the cloning vector pGEM-T was carried out using the pGEM-T Ligation Kit (PB-L, Productos Bio-Lógicos®) following the manufacturer's instructions. In this way, the pGEM-T-hcp2 construct was obtained and used to transform *E. coli* DH5α chemically competent cells (Ausubel et al. 1995). The white colonies that showed growth in LB medium with ampicillin were selected to verify, by colony-PCR, that they contained the gene fragment of interest by using the hcp2F/R primers **(Table 2)**. Subsequently, for the extraction of the recombinant plasmid containing the fragment of interest, the PURO|PLASMID KIT (PB-L, Productos Bio-Lógicos®) was used following the instructions recommended by the manufacturer. The obtained construct was digested with the restriction enzyme *Eco*RI (Promega) and the restriction product was run by horizontal electrophoresis on 0.8% agarose gel. The *hcp2* gene band with sticky ends was excised and purified using the QIAquick Gel Extraction Kit (QIAGEN) and cloned into the pKNOCK-Km^r plasmid previously dephosphorylated and cut with the same restriction enzyme *Eco*RI. The construct pKNOCK-hcp2 obtained was used to transform chemically competent cells of *E. coli* S17-1. The presence of pKNOCK-hcp was verified by colony-PCR using the hcp2F/R primers **(Table 2)**.

The site-directed mutation was performed by biparental conjugation of the plasmid pKNOCK-hcp2 contained in the donor *E. coli* S17-1-pKNOCK-hcp2 strain to the *Enterobacter* sp. J49 strain (Amp^r). As a consequence of the biparental mating, simple recombination occurred through the *hcp2* fragment, and the entire plasmid was inserted into the bacterial chromosome, interrupting the gene of interest. The selection of the transconjugant strains was carried out in LB medium supplemented with Amp and Km (100 $\mu g.ml^{-1}$ and 50 $\mu g.ml^{-1}$, respectively).

The primer pairs hcpF/hcpR and pKNOCK-F/pKNOCK-R **(Table 2)** were used at the end of the experiment to determine the presence of the pKNOCK-hcp2 insert as well as its orientation in the

genome of *Enterobacter* sp. J49 strain. Four combinations of these primers were used: hcpF/pKNOCK-F; hcpF/pKNOCK-R; hcpR/pKNOCK-F; hcpR/pKNOCK-R.

In addition, to verify that the mutant strain was isogenic with respect to the wild strain, fingerprinting was performed using the ERIC-PCR technique according to the methodology of de Bruijn (1992). The primer pairs hcpF/hcpR and hcp2F/hcp2R were designed from the complete sequence of the *hcp* gene obtained from the genome of the *Enterobacter* sp. J49 strain (Ludueña et al. 2019) using WedPrimer and AmplifX software.

To determine whether the mutation affected growth kinetics, a growth curve was performed in LB medium containing Amp (100 $\mu g.ml^{-1}$) or Amp (100 $\mu g.ml^{-1}$) and Km (50 $\mu g.ml^{-1}$) for the wild *Enterobacter* sp. J49 strain and for the J49-hcp transformants, respectively. To evaluate whether the mutation affected the solubilizing phosphate capacity of the *Enterobacter* sp. J49 strain, a qualitative estimation was first carried out in Petri dishes with NBRIP medium. The formation of a translucent halo around the colonies indicated the ability to solubilize phosphate. In addition, quantification of solubilized phosphate was performed. For this purpose, 1 ml aliquot (OD660nm=0.3) of an overnight culture of each bacterium grown in LB medium was transferred to an Erlenmeyer containing 60 ml of NBRIP medium with the corresponding antibiotics. Cultures were incubated at 28 °C with agitation (200 rpm), and at 24 h a 1 ml aliquot was aseptically taken to quantify the P solubilized by the bacteria. The aliquot was centrifuged at 10,000 rpm for 12 min for the bacteria and the insoluble P to settle. Soluble P was determined in the supernatant by the colorimetric technique of Fiske and Subbarow (1925) adapted to small volumes.

187 **Table 2.** Specific primers designed and used for the site-directed mutation of the *Enterobacter*
188 sp. J49 strain.

Pair of primers		SEQUENCE	Reference
hcp2	F	5´ TCCTGTTTACCAAGGAAATCG 3´	This study
	R	5´ TCCTTGATGTCGTGCATTT 3´	
Hcp	F	5´ TGGCAATCCCAGTTTATCTG 3´	This study
	R	5´TTCGTTCCAGGAATCGGAAT 3´	
pKNOCK	F	5´GGTTTAACGGTTGTGGACAA 3´	Alexeyev 1999
	R	5´ATGTAAGCCCACTGCAAGCTA 3´	

189
190
191 **2.4. Bacterial plant colonization assays**
192 Endophytic and epiphytic colonization was analyzed by isolating bacterial cells from internal and
193 external tissues of the leaves, stems, and roots of peanut plants inoculated with *Enterobacter* sp.
194 J49-hcp and *Enterobacter* sp. J49 wild type strains in a microcosm assay. Seeds of *Arachis*
195 *hypogaea* L. (var. Granoleico) were surface disinfected and pregerminated in Petri dishes
196 containing moist cotton and Whatman N°1 filter paper and incubated at 28 °C in darkness.
197 Pregerminated seeds were transferred to pots of 330 ml of volume, containing a sterile sand and
198 vermiculite mix (2:1). The pots were supplemented with 0.2% $Ca_3(PO_4)_2$ (Rivas et al. 2007) as
199 the only source of phosphorus (P). Seven-day-old seedlings were inoculated individually with 3
200 ml of each bacterial culture (~10^9 CFU.ml^{-1}) in the root crown. Uninoculated plants were used as
201 control treatments. Plants were grown in a microcosm with temperature conditions that ranged
202 between 21 and 29 °C and a photoperiod with light cycles of 16 h day/8 h night. The plants were
203 watered alternately with Hoagland solution without P (Hoagland and Arnon 1950) and water.
204 Experiments were performed with six replicates for each treatment. Peanut plants were harvested
205 at 12 days post-inoculation, and epiphytic and endophytic bacterial cells were isolated following
206 the method described by Kuklinsky-Sobral et al. (2004). The plants of each treatment were
207 sampled at harvest and fresh tissue was used. In all cases, the aerial and root tissues were weighted
208 separately.

For the isolation of epiphytic bacteria, the different plant tissues were individually placed in a 250 ml Erlenmeyer flask containing 8 g of glass beads and 20 ml of physiological solution (NaCl 0.9%). They were stirred at 150 rpm at 28 °C for 45 min. One ml suspension was transferred into a tube with 9 ml of physiological solution. Serial dilutions were made and 10 µl were seeded in triplicate in Petri dishes with LB medium supplemented with the corresponding antibiotics. Cell count was performed using the microdrop technique (Somasegaran and Hoben 1994) and the results were expressed as $CFU.g^{-1}$ of plant tissue.

For the isolation of endophytic bacteria, epiphytic bacteria were removed by surface disinfection with successive washes with 70% ethanol for 1 min, 3% sodium hypochlorite for 5 min, 70% ethanol for 30 s, and finally 4 washes in sterile distilled water. To control the disinfection process, aliquots of sterile distilled water used in the final wash were plated on Petri dishes with LB medium. After surface disinfection, the tissue was macerated with 10 ml of physiological solution and 1 ml was transferred to 50 ml conical tubes containing 9 ml of physiological solution. Serial dilutions were made and 10 µl in triplicate were seeded in plates containing LB medium to determine the number of endophytic bacteria expressed as $CFU.g^{-1}$ of plant tissue.

The effect of the inoculation of the *Enterobacter* sp. J49-hcp strain on the promotion of peanut plant growth with respect to the wild type strain was evaluated by measuring aerial and root length, aerial and root dry weight, and P content of the aerial part (Jackson 1973).

2.5 Determination of properties associated with colonization

2.5.1 Bacterial biofilm formation in different culture media

A biofilm formation assay was performed following the methodology proposed by O'Toole and Kolter (1998). First, 200 µl of an overnight culture ($O.D._{620nm}$=0.01) of the wild type and J49-hcp mutant strains grown in TY or NBRIP medium were added to each well of 96-well polystyrene microplates. A total of 18 replicates per treatment were performed. The plates were incubated at 36 °C for 24 h. Planktonic cells were then gently homogenized and bacterial growth was determined by measuring O.D. at 600 nm. Wells were washed with 200 µl of PBS (phosphate

236 buffered saline) and stained for 15 min with 200 µl of 0.1% crystal violet solution (CV) in 5%
237 ethanol. Each well was then rinsed with water. The dye retained in the adhered cells was
238 resuspended with 200 µl of ethanol:acetone (80:20) solution and incubated at room temperature
239 for 30 min. The O.D. at 570 nm of the recovered CV was determined using an Epoch ™
240 Microplate spectrophotometer. The amount of biofilm produced, based on the number of bacteria
241 contained in each well, was estimated by determining the Biofilm Index (BI) from the following
242 formula:

243 $$BI = O.D._{570} / O.D._{600}$$

244

245 **2.5.2. Production of plant cell wall-degrading enzymes: pectinase and cellulase activity**
246 **analysis**

247 For quantitative determination of extracellular constitutive pectinase activities, wild type and J49-
248 hcp strains were grown in TY culture medium at 28 °C in agitation (150 rpm). A sample was
249 taken at 24 h and centrifuged at 10,000 rpm at 4 °C for 10 min in HITACHI CR 22G refrigerated
250 centrifuge. Pectinase enzymes were analyzed by measuring polygalacturonase (PG) and pectin
251 lyase (PL) activities. For the PG activity, the methodology described by Sunnotel and Nigam
252 (2002) was followed, with some modifications. Briefly, 100 µl of 1% polygalacturonic acid
253 solution (PGA) in 0.05M acetate buffer (pH 4.5) were added to 500 µl of bacterial supernatant.
254 The mixture was incubated at 40 °C for 10 min and 400 µl of DNS solution (3,5-dinitrosalicylic
255 acid 2%; NaOH 2.8%; Na-K tartrate 13.3%) was added, incubated at 100 °C for 15 min, and then
256 brought to a final volume of 5 ml with milli-Q water. Absorbance was measured at 530 nm and a
257 standard curve for galacturonic acid was performed in a concentration range of 5 to 100 µM. A
258 unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1
259 µmol of PGA per minute.
260 PL activity was analyzed by following the methodology described by Sunnotel and Nigam (2002)
261 with modifications. For this purpose, 5 ml of 1% pectin solution in 0.05M Tris-HCl buffer (pH
262 8.5) was added to 1 ml of supernatant, and the mixture was brought to a final volume of 10 ml

with distilled H_2O and incubated at 40 °C for 2 h. Further, 0.6 ml of 9% $ZnSO_4$ and 0.6 ml of 0.5M NaOH were added to the mixture, which was subsequently centrifuged at 3,000 rpm for 10 min. Then, 3 ml of 0.04 M thiobarbituric acid and 2.5 ml of 0.1N HCl and 0.5 ml of distilled H_2O were added to 5 ml of the supernatant obtained. The mixture was placed in a bath at 100 °C for 30 min, cooled at room temperature, and measured at 550 nm in a Spectrum SP-1102 spectrophotometer. One unit of PL activity was defined as the amount of enzyme that caused a change in absorbance of 0.01 under this condition.

Extracellular induced cellulase (CL) activity was measured following the methodology described by Ariffin et al. (2006) with modifications. Both strains were grown in CMC medium (KH_2PO_4 1 g.l-1, K_2HPO_4 1.145 g.l-1, $MgSO_4.7H_2O$ 0.4 g.l-1, $(NH_4)_2SO_4$ 5 g.l-1, $CaCl_2.2H_2O$ 0.05 g.l-1, $FeSO_4.7H_2O$ 1.25 µg.l-1, carboxymethyl cellulose 10 g.l-1, pH 7) at 28 °C with agitation. A sample was taken at 15 h and centrifuged as described above. Then, 1 ml of supernatant was added to 1 ml of 1% CMC solution in citrate buffer (sodium citrate/citric acid 0.05M, pH 4.8) and incubated at 40 °C for 30 min. Further, 1.5 ml of DNS solution (3,5-dinitrosalicylic acid 2%; NaOH 2.8%; Na-K tartrate 13.3%) was added and incubated at 100 °C for 15 min. After that time, absorbance was measured at 575 nm. A glucose standard curve was performed in a concentration range of 20 to 400 µM. One unit of cellulase activity was expressed as 1 µmol of glucose released per ml of enzyme per minute.

2.6. Statistical analysis

Data, previously controlled to comply with the assumptions of normality and homoscedasticity (Sokal and Rohlf 1984), were analyzed using the software INFOSTAT (Di Rienzo et al. 2018). Variables were analyzed by one-way analysis of variance (ANOVA) using the different bacterial strains (mutant and wild type) as factors. A comparison of means was conducted using the protected test of Fisher (i.e., LSD), with a significance level of 0.05.

3. Results

290

291 **3.1. Identification of the components of the type VI secretion system in the genome of**
292 ***Enterobacter* sp. J49**

293 From the *in silico* analysis, it was possible to identify almost all the *tss* genes described in the
294 literature in the genome of the strain under study, except for the *tssE* gene. Based on this
295 identification, we performed the T6SS operon of *Enterobacter* sp. J49 (**Table 3**; **Figure 1**). The
296 24 genes of the operon, 13 correspond to T6SS components, two are T6SS associated proteins,
297 and the remaining nine are hypothetical proteins. The arrangement of the genes indicated that
298 they are in tandem. The first two genes of the T6SS cluster were *tss*B and *tss*C, followed by *tss*K
299 and *tss*L. The *hcp* gene was found in the sixth position followed by *tss*H and *tss*I. The other genes
300 of the T6SS were found downstream of a group of hypothetical proteins.

301

302 **Table 3:** Genes of the type VI secretion system (T6SS) identified in the genome of the

303 *Enterobacter* sp. J49 strain.

	Order in the operon	Gene (bp)	Product (aa)	Characterization according to NCBI/genDB
➔	1	*tss*B (501)	TssB (166)	type VI secretion system contractile sheath small subunit [*Enterobacter*] TssB
➔	2	*tss*C (1542)	TssC (515)	type VI secretion system contractile sheath large subunit [*Enterobacter*] TssC
➔	3	*tss*K (1350)	TssK (449)	type VI secretion system baseplate subunit TssK
➔	4	*tss*L (690)	TssL (229)	type IV/VI secretion system protein TssL (DotU)
	5	*omp*A (1680)	OmpA (559)	OmpA family protein [*Enterobacter*]
➔	6	*hcp*/*tss*D (492)	Hcp/TssD (163)	Type VI secretion system tube protein Hcp
➔	7	*tss*H (2673)	TssH (890)	Type VI secretion system ATPase TssH
➔	8	*tss*I (2358)	TssI (785)	Type VI secretion system tip protein VgrG [*Enterobacter*] TssI
	9	*hp* (2700)	Hp (899)	Hypothetical protein
	10	*hp* (861)	Hp (286)	Hypothetical protein
	11	*hp* (516)	Hp (171)	Hypothetical protein
	12	*hp* (492)	Hp (163)	Hypothetical protein
	13	*hp* (168)	Hp (55)	Hypothetical protein
	14	*hp* (477)	Hp (158)	Hypothetical protein
➔	15	*pdp** (264)	PaarP (87)	PAAR domain-containing protein
	16	*hp* (1209)	Hp (402)	Hypothetical protein
➔	17	*tss*M (3372)	TssM (1123)	Type VI secretion protein VasK(TssM)
	18	*hp* (138)	Hp (45)	Hypothetical protein
	19	*hp* (108)	Hp (35)	Hypothetical protein
➔	20	*tss*A (1608)	TssA (535)	Type VI secretion system protein TssA
➔	21	*tss*F (1770)	TssF (589)	Type VI secretion system baseplate subunit TssF
➔	22	*tss*G (1083)	TssG (360)	Type VI secretion system baseplate subunit TssG
➔	23	*tss*J (525)	TssI (174)	Type VI secretion system lipoprotein TssJ [*Enterobacter*]
	24	*rhs* (423)	Rhs (139)	secretion protein Rhs, partial [*Enterobacter* sp. J49]

304 * name assigned in this work.

305 ➔ indicates genes essential for T6SS function.

306

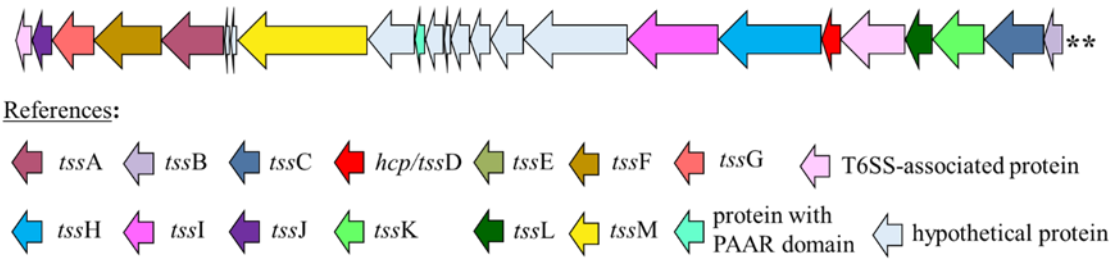


Figure 1: Operon of the type VI secretion system in the *Enterobacter* sp. J49 strain designed based on the bibliographic search and comparison with sequences available in NCBI gene bank. The operon starts at **. The size of the arrows is proportional to the length of the genes (bp).

3.2. Comparative analysis of hcp gene sequence of the *Enterobacter* sp. J49 strain relative to other strains belonging to this genus

Sequence analysis of the *hcp* gene (Accession No. OUC39004.1) indicated that it is 492 bp long. The alignment analysis with *hcp* sequences available in the NCBI Genbank indicated that the *hcp* sequence of *Enterobacter* sp. J49 showed 100% identity with that of *Enterobacter cloacae* P101 **(Table 4)** and 99% with the sequence of this gene of three *E. ludwigii* strains and another *E. cloacae* strain. The forced alignment of this gene with that of *Enterobacter ludwidgii* EN-119 (CP017279.1) and *E. cloacae* EcWSU1 (CP00286.1) showed 85.1% identity with the former (**Table 4**) while no identity was found with the sequence of the *hcp* gene of EcWSU1.

322 **Table 4:** Percentage identity of the *Enterobacter* sp. J49 *hcp* gene sequence against the available
323 sequences of the same gene in the NCBI gene bank and the sequences corresponding to the
324 phylogenetically closest strains.

Strains	Access number	Identity (%)	Coverage (%)
Enterobacter cloacae P101 *	CP006580.1	100	100
Enterobacter ludwigii JP9	CP040527.1	99.19	100
Enterobacter ludwigii JP6	CP040526.1	99.19	100
Enterobacter cloacae complex sp. FDA-CDC-AR_0164	CP028950.1	99	100
Enterobacter cloacae UW5	CP011798.1	99	100
Enterobacter ludwigii EN-119 *	CP017279.1	85.16	100

325 * strains with a higher phylogenetic relationship with *Enterobacter* sp. J49 according to Ludueña
326 et al. (2019).
327
328 **3.3. Endophytic and epiphytic colonization of the *Enterobacter* sp. J49-hcp strain in peanut**
329 **plants (*Arachis hypogaea* L.)**
330 Through ERIC-PCR, the selected mutant was found to be isogenic with respect to the wild type
331 strain **(Figure S2** - supplementary material**)**. In addition, the mutation did not modify the
332 phosphate solubilizing capacity of the strain under study. This result was observed both
333 qualitatively (**Figure 2**) and quantitatively by means of the soluble P value in the supernatant of
334 the J49 and J49-hcp strains (379.42±10.73 and 391.15±10.13 ppm of P, respectively), which did
335 not present significant differences.
336 The results indicated that the mutation of the *hcp* gene produced a significant decrease in the
337 aerial and root epiphytic colonization of *Enterobacter* sp. J49 **(Table 5)**. The J49-hcp strain also
338 showed significantly less endophytic colonization than the wild type strain in aerial tissue.
339

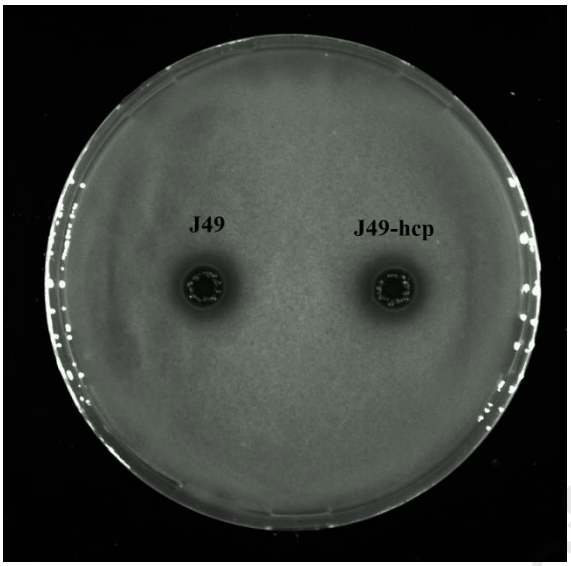


Figure 2: Phosphate solubilization halo by *Enterobacter* sp. J49 (**J49**) and *Enterobacter* sp. J49-hcp (**J49-hcp**) in NBRIP medium.

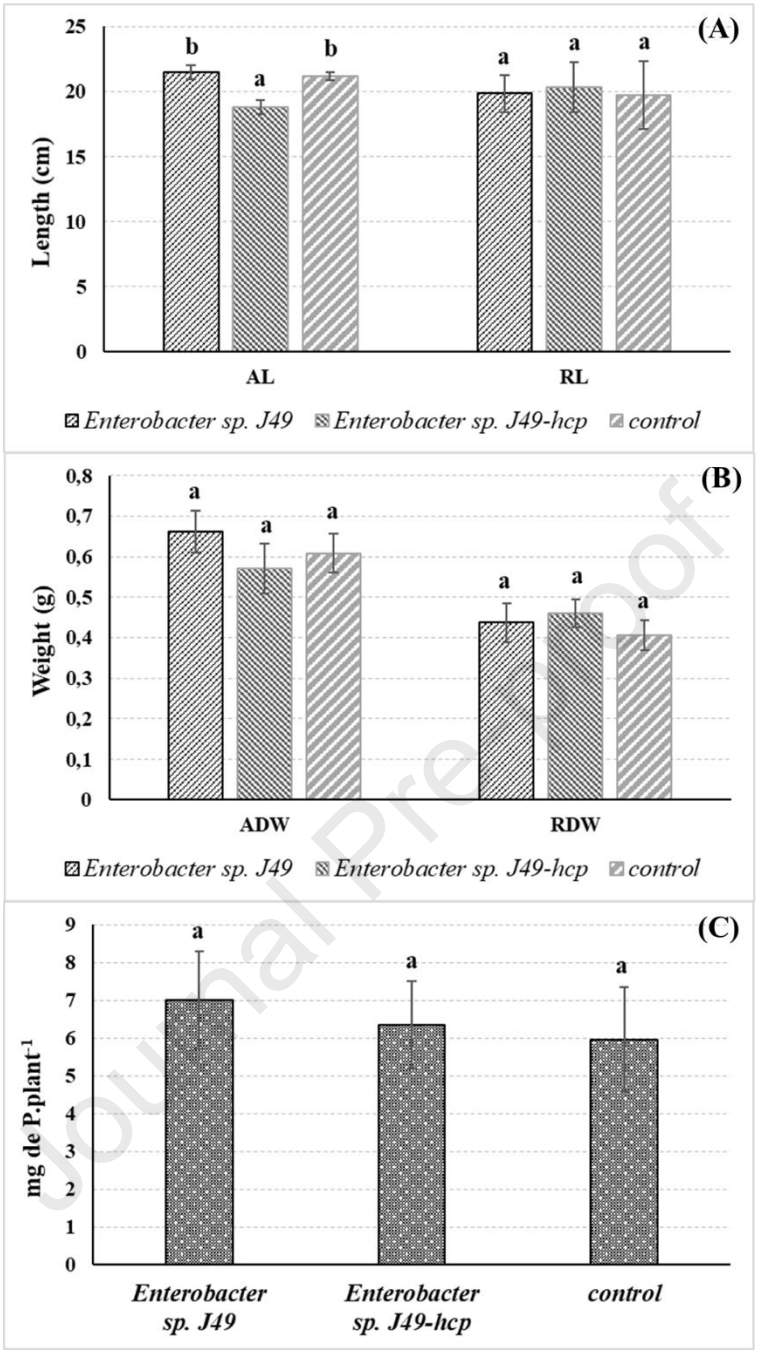
Table 5: Count of CFU.g^{-1} of plant tissue in the endophytic and epiphytic colonization assay of *Enterobacter* sp. J49 and *Enterobacter* sp. J49-hcp strains.

Strains	Endophytes (CFU.g^{-1})		Epiphytes (CFU.g^{-1})	
	Root part	**Aerial part**	**Root part**	**Aerial part**
Enterobacter sp. J49	$1.04.10^4 \pm 4.40.10^3$	$4.99.10^3 \pm 5.05.10^2$ *****	$4.39.10^6 \pm 8.73.10^5$ *****	$8.43.10^4 \pm 2.48.10^4$ *****
Enterobacter sp. J49-hcp	$1.26.10^3 \pm 4.18.10^2$	$1.79.10^3 \pm 4.21.10^2$	$8.61.10^5 \pm 2.50.10^5$	$6.63.10^3 \pm 1.61.10^3$

Data represent mean ± S.E. (n=6). * indicates significant differences between strains for the same tissue ($p<0.05$).

In the plant inoculation assays with the *Enterobacter* sp. J49-hcp strain, peanut seedlings exhibited significantly lower aerial length (AL) than plants inoculated with the *Enterobacter* sp. J49 strain and the uninoculated control treatment **(Figure 3A)**. The aerial dry weight (ADW) of plants inoculated with J49-hcp was lower than that of the other treatment, although not significantly different **(Figure 3B)**. Regarding the parameters measured in the root, no differences were observed between the plants inoculated with the J49-hcp strain and the wild type strain. As to the P content of the aerial part, although lower values were observed in the plants inoculated

356 with the J49-hcp mutant than in the wild type strain, the values were not statistically significant
357 **(Figure 3C)**.
358



359

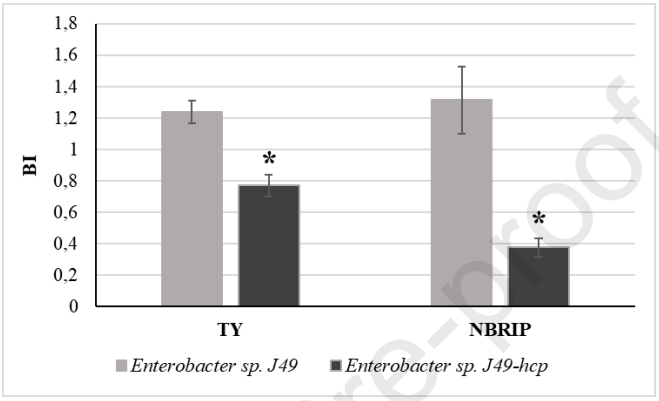
360 **Figure 3:** Aerial length (AL) and root length (RL) **(A);** aerial dry weight (ADW) and root dry
361 weight (RDW) **(B);** P content in the aerial part expressed as mg P.plant^{-1} **(C)** of peanut plants.
362 Treatments: ***Enterobacter* sp. J49**: plants inoculated with the *Enterobacter* sp. J49 strain;
363 ***Enterobacter* sp. J49-hcp**: plants inoculated with the *Enterobacter* sp. J49-hcp strain; **control:**
364 uninoculated plants. Data represent mean ± S.E. (n=6). Different letters indicates significant
365 differences between treatments for each parameter evaluated ($p<0.05$).

366

367 **3.4. Properties associated with bacterial plant colonization**

368 The mutation in the *hcp* gene in *Enterobacter* sp. J49 led to a significant decrease in biofilm
369 formation with respect to the wild type (**Figure 4**). This reduction was observed in both media
370 used, with the lowest BI value occurring in NBRIP medium.

371



372

373 **Figure 4:** Biofilm Index (BI) in TY and NBRIP media. Data represent mean ± S.E. (n=18). *
374 indicate significant differences ($p<0.05$) between strains in each culture medium.

375

376 To evaluate the possible role of T6SS on the production of pectinase and cellulase enzymes, these
377 activities were measured in the J49-hcp strain. Sampling times were determined based on previous
378 results, by searching for the maximum activity (Lucero et al. 2021). The results obtained showed
379 a significant decrease in the constitutive production of PG when the *hcp* gene was mutated in
380 *Enterobacter* sp. J49 strain (**Figure 5**). Regarding the constitutive production of PL and the
381 induced activity of CL, no differences were observed between *Enterobacter* sp. J49-hcp and wild
382 type strains (**Figure 5**).

383

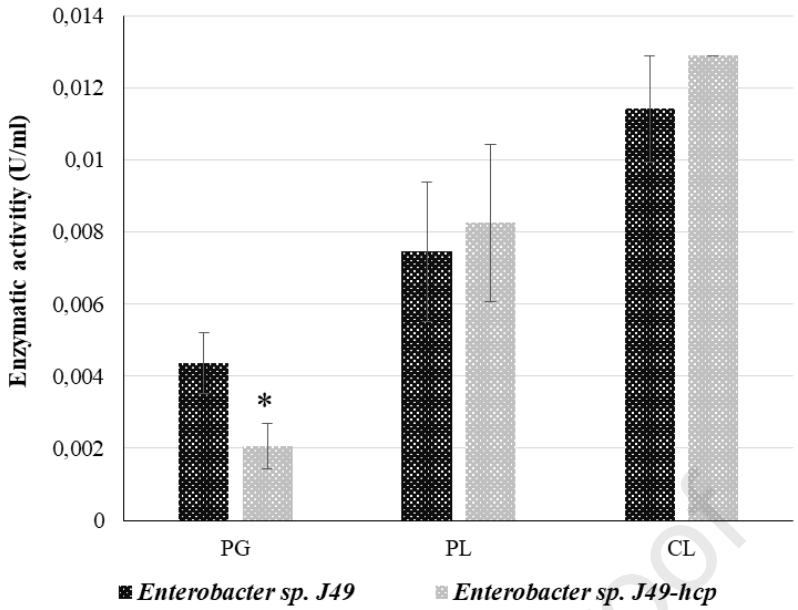


Figure 5: Enzymatic polygalacturonase activity (**PG**), pectin lyase activity (**PL**), and cellulase activity (**CL**) of *Enterobacter* sp. J49 and *Enterobacter* sp. J49-hcp strains at different times of growth. Data represent mean ± S.E. (n= 4). * indicate significant differences ($p<0.05$) between strains in a culture medium.

4. Discussion

There are different types of secretion systems in Gram-negative bacteria that transfer molecules both to the extracellular medium and to the interior of adjacent prokaryotic or eukaryotic cells. They are numbered from I to IX and vary according to the nature of the secretion system and the transport mechanism of the released compound (Journet and Cascales 2016). Thus, for example, type III and IV secretion systems are essential for the release of effector proteins by pathogenic bacteria towards the plant (Green and Mecsas 2016). On the other hand, the type VI secretion system (T6SS) is commonly found in commensal and pathogenic endophytic bacteria and in beneficial bacteria associated with plants (Reinhold-Hurek and Hurek 2011; Bernal et al. 2018). This system is used by many bacteria to engage in social behavior with other microbial communities, which can positively affect the health of their host (Wu et al. 2020). The presence

of T6SS provides several advantages to model systems associated with agriculture, since most of the T6SS described in plant-associated bacteria are involved in interbacterial competition (Allsopp et al. 2020). This suggests that it is involved in the establishment and protection of beneficial plant-associated communities (Coulthurst 2019).

T6SS is widespread in Gram-negative bacteria, and bioinformatic studies have described it as a highly conserved secretion system (Bernal et al. 2018). It has been estimated that approximately 25% of Gram-negative bacteria contain at least one T6SS, most commonly within the α, β, and γ proteobacteria (Pallen et al. 2002; Das and Chaudhuri 2003; Schlieker et al. 2005; Bingle et al. 2008; Boyer et al. 2009; Trunk et al. 2019). Bacterial species, and indeed individual strains within a species, can have anywhere from none to six different T6SSs, with the complement of secreted effector proteins being even more variable (Coulthurst 2019). This secretion system represents a contractile nanomachine that can translocate effector proteins directly to neighboring cells. It is a versatile bacterial structure that can deliver effector molecules to different classes of cells, playing key roles in interbacterial competition and bacterial interactions with eukaryotic cells (Coulthurst 2019). An *in silico* analysis of T6SS gene clusters, showed that a minimum set of 13 genes, termed core components, is required to assemble a functional T6SS (Zoued et al. 2014). To identify the genes involved in the biosynthesis of the structure of the type VI secretion system, its effectors, and associated proteins, a computer search of all its genetic components was performed. We found that the *Enterobacter* sp. J49 strain has all the essential genes for this secretion system, which allows us to infer that it may be functional in this bacterium. In relation to the specific analysis of the *hcp* gene in the *Enterobacter* sp. J49 strain, the highest percentage of identity was that of the same gene corresponding to *Enterobacter cloacae* P101, one of the three bacteria with which *Enterobacter* sp. J49 has the greatest phylogenetic relationship (Ludueña et al. 2019). The forced alignment of the sequence of *hcp* gene of the J49 strain with the other phylogenetically close strains showed a low percentage or no identity. This result is probably due to the fact that the genes involved in this secretion system were acquired by horizontal gene transfer, which is a widely described mechanism in bacteria (Davison 1999; Arber 2000). This phenomenom of gene

transfer between bacteria has been described for other important features, i.e. *luxI/luxR* genes involved in bacterial communication mediated by AHL-type molecules (Wei et al. 2006) and nitrogen fixation *nif* genes (Bohlius et al. 2010).

Different authors have found that the endophytic population associated with different plants has a high number of genes related to T6SS, and thus this system has been proposed to play an important role in plant-microorganism interactions (Pukatzki et al. 2009; Sessitsch et al. 2012). In this study, we focused on the role of this secretion system on the colonization ability of a peanut native efficient phosphate solubilizing bacterium, *Enterobacter* sp. J49. For this purpose, we developed a mutant truncated in *hcp* considering that it is a core gene of T6SS. The Hcp protein is the hallmark of a functional T6SS. Hcp was first identified as a major T6SS-associated protein in *Vibrio cholerae* (Pukatzki et al. 2006). This protein is the structural component of the hexameric ring of the transport channel between the inner and outer membranes of the bacterium (Shrivastava and Mande 2008; Leiman et al. 2009). It is considered a secreted protein with several roles in different bacteria (Williams et al. 1996; Dudley et al. 2006; Hood et al. 2010) including bacterial interaction with host cells (Williams et al. 1996; Pukatzki et al. 2006; Suarez et al. 2008; Wu et al. 2008). This is why the gene that encodes this protein was selected to carry out the mutation and interfere with the correct functioning of T6SS. To evaluate whether T6SS participates in the colonization of the phosphate solubilizing *Enterobacter* sp. J49 strain on peanut, plant inoculation assays were carried out with a mutant strain obtained by a site-directed mutagenesis of the *hcp* gene. The significantly reduced colonization by *Enterobacter* sp J49-hcp suggests that T6SS is involved in this property although it is not essential. It was interesting to observe that both epiphytic and endophytic colonization decreased significantly in the mutant strain. This suggests that T6SS is required, although not exclusively, to infect the inner plant tissues where less bacterial competition is present. Therefore, it is possible to propose that in addition to the bacterial competition advantage that T6SS confers to rhizospheric bacteria, it may also participate in the infection strategy of the bacteria. This finding could be confirmed by future studies of other *tss* mutants.

456 The T6SS allows bacteria to establish themselves in natural habitats, and the impact of the plant
457 environment on this process is notable (Chakraborty et al. 2011; Ma et al. 2014; Shyntum et al.
458 2018; Bellieny-Rabelo et al. 2019). Kapitein and Mogk, (2014) suggested thet this impact require
459 a deeper analysis of T6SS in the natural environment and of how its activities can be modulated
460 by host factors. Mutants of the T6SS of *Paraburkholderia phymatum* were less competitive than
461 the wild type strain in plant assays (de Campos et al. 2017). These authors suggest that T6SS is
462 one of the factors responsible for the success of the infection because it directly inhibited its
463 competitors (*P. phymatum, P. diazotrophica, P. mimosarum, P. sabiae*) in *in vitro* assays.
464 Likewise, in the *Pectobacterium carotovorum* subsp. *brasiliense* strain PBR1692 – Pcb1692, by
465 means of T6SS, it inhibited the members of the *Enterobacteriaceae* when is was inoculated in
466 potato tubers (Shyntum et al. 2019). Although peanut plants inoculated with the wild type
467 *Enterobacter* sp. J49 strain showed an increase in growth parameters and P content, compared to
468 control plants, this was not statistically significant in most cases. This is probably because this
469 effect is seen in later stages of plant development (Lucero et al. 2021). However, it should be
470 noted that at the time when the test was performed, it was possible to observe significantly lower
471 colonization by the J49-hcp mutant in most cases, which could translate into a significant decrease
472 in the aerial length of the peanut. Thus, the decrease observed in the growth parameters analyzed
473 in the plants inoculated with the J49-hcp strain suggests that it is a consequence of a lower number
474 of bacteria colonizing peanut roots. In agreement with these results, Mosquito et al. (2020)
475 observed that the deletion of T6SS from *Kosakonia* spp endophytes significantly decreased plant
476 root rhizosphere and endosphere colonization.
477 Effectors released by T6SS in plant-associated bacteria have been shown to perform various
478 functions such as interbacterial competition, stress response, enzyme production, quorum sensing,
479 biofilm formation, and symbiosis (Whitney et al. 2013; Ryu 2015; Hachani et al. 2016; Cianfanelli
480 et al. 2016; Bernal et al. 2018). Zhang et al. (2014) observed a decrease in biofilm formation and
481 colonization capacity in a TssB minus mutant of *Ralstonia solanacearum*. Similaly, the
482 participation of T6SS in biofilm formation has been described in other bacteria (Enos-Berlage et

al. 2005; Aschtgen et al. 2008). To evaluate the production of biofilm of the *Enterobacter* sp. J49 strain, the TY and NBRIP culture media were used. The culture media was selected based on those in which the strain showed the highest values of biofilm production at 48 h of growth (Lucero et al. 2020). In this regard, the results obtained in the present work demonstrate that the *Enterobacter* sp. J49-hcp strain produced significantly lower biofilm than the wild type strain. In addition, considering that biofilm production is an important colonization trait, it is possible to speculate that the reduced epiphytic and endophytic colonization observed in the J49-hcp strain could have resulted from the reduced biofilm formation. It should be considered that the T6SS could modulate bacterial competence when the bacteria colonize and gain entrance in the plant tissues.

Plant cell walls are formed by the innermost secondary wall composed mainly of cellulose and hemicellulose fibers, and the outermost primary wall is formed by the same fibers combined with pectic substances. The primary walls of two contiguous cells are joined by the middle lamella, which is made up mainly of pectic substances (Carpita and Gibeaut 1993; Jarvis and MacCann 2000). Given that the interior of plants represents a unique habitat, bacterial endophytes are likely to have differential functions with respect to those rhizospheric and epiphytic bacteria (Okunishi et al. 2005; Compant et al. 2010). Among them, the production of hydrolytic enzymes can be a useful tool in endophytic colonization (Verma et al. 2018). Some studies found that endophytes can actively penetrate plant cells by the production and release of cellulolytic and pectinolytic enzymes (Hallmann et al. 1997; Khan et al. 2017; Gupta et al. 2019). T6SS from different bacteria has been shown to secrete two broad families of bacterial cell wall-degrading enzymes, amidases and glycoside hydrolases (Russell et al. 2012). For this reason, the release of enzymes that degrade the plant cell wall may be expected to also be associated with this secretion system. From the results obtained in this study, we observed that, of the three enzymatic groups studied, only PG was negatively affected when the *hcp* gene was mutated. In this sense, Pagel and Heitefuss (1990) observed that PG activity is probably the main determinant in initial tissue infection by the pathogen (*Erwinia carotovora* subsp. *atroseptica*) in potatoes with respect to cellulolytic,

proteolytic, xylanolytic, and pectin lyase enzymes, whose activities were not detected until several hours after the infection. In line with this, it would be plausible to attribute the lower endophytic colonization observed in peanut plants by *Enterobacter* sp. J49-hcp among other factors to lower production of PG enzymes, which are important in the first moments of plant wall degradation.

This study shows the relevance of this secretion system in *Enterobacter* sp. J49 in the colonization of its host plant. Future research will continue in this direction, and to this end, studies will be designed to describe hypothetical effector proteins involved, including their structural prediction, by relating them to the biological role of T6SS in plant colonization. In addition, the evidence found in this study shows the importance of the *hcp* gene in the colonization of peanut plants, and therefore, it would be interesting to infer the incidence of plant root exudates or the presence of other microorganisms on hcp gene expression. For this purpose, real-time quantitative PCR experiments are proposed, because this would be a convenient way to analyze the relative changes in gene expression. Although more work is required to elucidate the functions and mechanisms of the T6SS, these analyses should help us to better understand the mechanistic and biological functions of T6SS, in terms of its involvement in the plant colonization process and interbacterial competition.

5. Conclusions

The results obtained in the present work allow us to infer that the T6SS of *Enterobacter* sp. J49 may participate in the endophytic colonization of peanut. This secretion system may favor colonization by either accelerating or making the infection more efficient, or by promoting other mechanisms involved in it, such as the formation of biofilm and the activity of polygalacturonase enzymes.

Acknowledgments

This research was supported by Facultad de Ciencias Exactas y Naturales de la Universidad Nacional de La Pampa (FCEyN-UNLPam), Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECYT-UNRC), and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT).

References

Alexeyev MF (1999). The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. BioTechniques. 26(5), 824-828.

Allsopp LP, Bernal P, Nolan LM, Filloux A (2020). Causalities of war: the connection between type VI secretion system and microbiota. Cellular microbiology, 22(3), e13153.

Alvarez-Martinez CE, Christie PJ (2009). Biological diversity of prokaryotic type IV secretion systems. Microbiology and Molecular Biology Reviews. 73(4), 775-808.

Anzuay MS, Ciancio MGR, Ludueña LM, Angelini JG, Barros G, Pastor N, Taurian T (2017). Growth promotion of peanut (*Arachis hypogaea* L.) and maize (*Zea mays* L.) plants by single and mixed cultures of efficient phosphate solubilizing bacteria that are tolerant to abiotic stress and pesticides. Microbiological Research, 199, 98-109.

Anzuay MS, Frola O, Angelini JG, Ludueña LM, Fabra A, Taurian T (2013). Genetic diversity of phosphate-solubilizing peanut (*Arachis hypogaea* L.) associated bacteria and mechanisms involved in this ability. Symbiosis. 60(3): 143-154.

Anzuay MS, Viso NP, Ludueña LM, Morla FD, Angelini JG, Taurian T (2021). Plant beneficial rhizobacteria community structure changes through developmental stages of peanut and maize. Rhizosphere, 19, 100407.

Arber W (2000). Genetic variation: molecular mechanisms and impact on microbial evolution. FEMS microbiology reviews, *24*(1), 1-7.

Ariffin H, Abdullah N, Umi Kalsom MS, Shirai Y, Hassan MA (2006). Production and characterization of cellulase by Bacillus pumilus EB3. Int J Eng Technol. 3(1):47-53.

563 Aschtgen MS, Bernard CS, De Bentzmann S, Lloubes R, Cascales E (2008). SciN is an outer
564 membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia*
565 *coli*. Journal of bacteriology, 190(22), 7523-7531.
566 Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K (eds) (1995).
567 Current Protocols in Molecular Biology. Wiley Interscience, New York.
568 Bashan Y, De-Bashan LE (2005). Plant growth-promoting. Encyclopedia of soils in the
569 environment, 1, 103-115.
570 Bashan Y, Holguin G (1998). Proposal for the division of plant growth-promoting rhizobacteria
571 into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. Soil
572 Biology and Biochemistry. 30(8), 1225-1228.
573 Bellieny-Rabelo D, Tanui CK, Miguel N, Kwenda S, Shyntum DY, Moleleki LN (2019).
574 Transcriptome and comparative genomics analyses reveal new functional insights on key
575 determinants of pathogenesis and interbacterial competition in *Pectobacterium* and *Dickeya*
576 spp. Appl. Environ. Microbiol. 85(2), e02050-18.
577 Beringer JE (1974). R factor Transfer in *Rhizobiurn Zegurninosarum*. Journal of General
578 Microbiology. 84, 188-198.
579 Bernal P, Allsopp LP, Filloux A, Llamas MA (2017). The *Pseudomonas putida* T6SS is a plant
580 warden against phytopathogens. The ISME journal, 11(4), 972-987.
581 Bernal P, Llamas MA, Filloux A (2018). Type VI secretion systems in plant-associated bacteria.
582 Environmental microbiology. 20(1), 1-15.
583 Bingle LE, Bailey CM, Pallen MJ (2008). Type VI secretion: a beginner's guide. Current opinion
584 in microbiology. 11(1), 3-8.
585 Bladergroen MR, Badelt K, Spaink HP (2003). Infection-blocking genes of a symbiotic
586 *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein
587 secretion. Molecular plant-microbe interactions, 16(1), 53-64.

588 Bolhuis H, Severin I, Confurius-Guns V, Wollenzien UI, Stal LJ (2010). Horizontal transfer of
589 the nitrogen fixation gene cluster in the cyanobacterium Microcoleus chthonoplastes. The
590 ISME journal, 4(1), 121-130.
591 Boyer F, Fichant G, Berthod J, Vandenbrouck Y, Attree I (2009). Dissecting the bacterial type VI
592 secretion system by a genome wide in silico analysis: what can be learned from available
593 microbial genomic resources. BMC genomics. 10(1), 104.
594 Carpita NC, Gibeaut DM (1993). Structural models of primary cell walls in flowering plants:
595 consistency of molecular structure with the physical properties of the walls during growth. The
596 Plant Journal, 3(1), 1-30.
597 Chakraborty S, Sivaraman J, Leung KY, Mok YK (2011). Two-component PhoB-PhoR
598 regulatory system and ferric uptake regulator sense phosphate and iron to control virulence
599 genes in type III and VI secretion systems of *Edwardsiella tarda*. Journal of Biological
600 Chemistry. 286(45), 39417-39430.
601 Chien CF, Liu CY, Lu YY, Sung YH, Chen KY, Lin NC (2020). HSI-II gene cluster of
602 *Pseudomonas syringae* pv. tomato DC3000 encodes a functional type VI secretion system
603 required for interbacterial competition. Frontiers in Microbiology, 11, 1118.
604 Cianfanelli FR, Monlezun L, Coulthurst SJ (2016). Aim, load, fire: the type VI secretion system,
605 a bacterial nanoweapon. Trends in microbiology. 24(1), 51-62.
606 Compant S, Clément C, Sessitsch A (2010). Plant growth-promoting bacteria in the rhizo-and
607 endosphere of plants: their role, colonization, mechanisms involved and prospects for
608 utilization. Soil Biology and Biochemistry, 42(5), 669-678.
609 Cordoba Cereal Stock-MarketBag Institute (2018). http://www.bccba.com.ar/datos-finales-
610 produccion-6381.html
611 Coulthurst S (2019). The Type VI secretion system: a versatile bacterial weapon. Microbiology.
612 165(5), 503-515.

613 Das S, Chaudhuri K (2003). Identification of a unique IAHP (IcmF associated homologous
614 proteins) cluster in *Vibrio cholerae* and other proteobacteria through in silico analysis. In silico
615 biology. 3(3), 287-300.
616 Davison J (1999). Genetic exchange between bacteria in the environment. Plasmid, 42(2), 73-91.
617 de Campos SB, Lardi M, Gandolfi A, Eberl L, Pessi G (2017). Mutations in two *Paraburkholderia*
618 *phymatum* type VI secretion systems cause reduced fitness in interbacterial
619 competition. Frontiers in microbiology. 8(2473).
620 Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat
621 versión 2018. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL
622 http://www.infostat.com.ar
623 Dudley EG, Thomson NR, Parkhill J, Morin NP, Nataro JP (2006). Proteomic and microarray
624 characterization of the AggR regulon identifies a *pheU* pathogenicity island in
625 enteroaggregative *Escherichia coli*. Molecular microbiology. 61(5), 1267-1282.
626 Enos-Berlage JL, Guvener ZT, Keenan CE, McCarter LL (2005). Genetic determinants of biofilm
627 development of opaque and translucent Vibrio parahaemolyticus. Molecular
628 microbiology, 55(4), 1160-1182.
629 Fiske EH, Subbarow Y (1925). The solubilization of phosphate: the action of various organic
630 compounds on dicalcium and tricalcium phosphate. New Zealand J Sci Technol. 33, 436-444.
631 Food and Agriculture Organization of the United Nations (2021). http://www.fao.
632 org/faostat/en/#data/SC
633 Gallegos-Monterrosa R, Coulthurst SJ (2021). The ecological impact of a bacterial weapon:
634 microbial interactions and the Type VI secretion system. FEMS Microbiology Reviews, 45(6),
635 fuab033.
636 Green ER, Mecsas J (2016). Bacterial secretion systems: an overview. Virulence Mechanisms of
637 Bacterial Pathogens. 213-239.

638 Gupta A, Verma H, Singh PP, Singh P, Singh M, Mishra V, Kumar A (2019). Rhizome
639 endophytes: Roles and applications in sustainable agriculture. In Seed Endophytes (pp. 405-
640 421). Springer, Cham.
641 Hachani A, Wood TE, Filloux A (2016). Type VI secretion and anti-host effectors. Current
642 opinion in microbiology. 29, 81-93.
643 Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997). Bacterial endophytes in
644 agricultural crops. Canadian journal of microbiology, 43(10), 895-914.
645 Ho BT, Basler M, Mekalanos JJ (2013). Type 6 secretion system–mediated immunity to Type 4
646 secretion system–mediated gene transfer. Science. 342(6155), 250-253.
647 Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without
648 soil. Circular. California Agricultural Experiment Station. 347(2nd edit).
649 Hood RD, Singh P, Hsu F, et al (2010). A type VI secretion system of *Pseudomonas aeruginosa*
650 targets a toxin to bacteria. Cell host & microbe. 7(1), 25-37.
651 Jackson ML (1973). Soil Chemical Analysis. Prentice Hall of India Private Limited New Delhi
652 (pp.38-82).
653 Jani AJ, Cotter PA (2010). Type VI secretion: not just for pathogenesis anymore. Cell host &
654 microbe. 8(1), 2-6.
655 Jarvis MC, McCann MC (2000). Macromolecular biophysics of the plant cell wall: concepts and
656 methodology. Plant Physiology and Biochemistry, 38(1-2), 1-13.
657 Jiang X, Beust A, Sappa PK, et al (2019). Two functionally deviating type 6 secretion systems
658 occur in the nitrogen-fixing endophyte *Azoarcus olearius* BH72. Frontiers in
659 microbiology, 10, 459.
660 Journet L, Cascales E (2016). The type VI secretion system in *Escherichia coli* and related
661 species. EcoSal Plus. 7(1).
662 Kapitein N, Mogk A (2014). Type VI secretion system helps find a niche. Cell host &
663 microbe. 16(1), 5-6.

664 Khan AL, Shahzad R, Al-Harrasi A, Lee IJ (2017). Endophytic microbes: a resource for
665 producing extracellular enzymes. In Endophytes: Crop productivity and protection (pp. 95-
666 110). Springer, Cham.
667 Kuklinsky-Sobral J, Araújo WL, Mendes R, et al(2004). Isolation and characterization of
668 soybean-associated bacteria and their potential for plant growth promotion. Environmental
669 microbiology. 6(12), 1244-1251.
670 Leiman PG, Basler M, Ramagopal UA, et al (2009). Type VI secretion apparatus and phage tail-
671 associated protein complexes share a common evolutionary origin. Proceedings of the
672 National Academy of Sciences. 106(11), 4154-4159.
673 Lin JS, Ma LS, Lai EM (2013). Systematic dissection of the agrobacterium type VI secretion
674 system reveals machinery and secreted components for subcomplex formation. PloS one, 8(7),
675 e67647.
676 Lucero CT, Lorda GS, Anzuay MS, Ludueña LM, Taurian T (2021). Peanut endophytic phosphate
677 solubilizing bacteria increase growth and p content of soybean and maize plants. Current
678 Microbiology, 78(5), 1961-1972.
679 Lucero CT, Lorda GS, Ludueña LM, Anzuay MS, Taurian T (2020). Motility and biofilm
680 production involved in the interaction of phosphate solubilizing endophytic strains with
681 peanut, maize and soybean plants. Rhizosphere, 15, 100228.
682 Ludueña LM, Anzuay MS, Angelini JG, et al (2019). Genome sequence of the endophytic strain
683 *Enterobacter* sp. J49, a potential biofertilizer for peanut and maize. Genomics. 111(4), 913-
684 920.
685 Ma LS, Hachani A, Lin JS, Filloux A, Lai EM (2014). *Agrobacterium tumefaciens* deploys a
686 superfamily of type VI secretion DNase effectors as weapons for interbacterial competition in
687 planta. Cell host & microbe. 16(1), 94-104.
688 MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S (2010). The *Vibrio cholerae* type VI secretion
689 system displays antimicrobial properties. Proceedings of the National Academy of
690 Sciences. 107(45), 19520-19524.

691 Mehta S, Nautiyal CS (2001). An efficient method for qualitative screening of phosphate-
692 solubilizing bacteria. Current microbiology. 43(1), 51-56.
693 Miller JH (1972). Experiments in molecular genetics. Cold Spring Laboratory Press.
694 Mosquito S, Bertani I, Licastro D, et al (2020). In planta colonization and role of T6SS in two
695 rice Kosakonia endophytes. Molecular Plant-Microbe Interactions, *33*(2), 349-363.
696 Okunishi S, Sako K, Mano H, Imamura A, Morisaki H (2005). Bacterial flora of endophytes in
697 the maturing seed of cultivated rice (*Oryza sativa*). Microbes and Environments, 20(3), 168-
698 177.
699 O'toole GA, Kolter R (1998). Initiation of biofilm formation in *Pseudomonas fluorescens*
700 WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Molecular
701 microbiology. 28(3), 449-461.
702 Pagel W, Heitefuss R (1990). Enzyme activities in soft rot pathogenesis of potato tubers: effects
703 of calcium, pH, and degree of pectin esterification on the activities of polygalacturonase and
704 pectate lyase. Physiological and Molecular Plant Pathology, 37(1), 9-25.
705 Pallen M, Chaudhuri R, Khan A (2002). Bacterial FHA domains: neglected players in the
706 phospho-threonine signalling game?. Trends in microbiology. 10(12), 556-563.
707 Peanut Argentina Chamber (2018). http://www.camaradelmani.org.ar/espanol/wp-
708 content/uploads/2018/CAM_caracterizacion_web.pdfPukatzki S, Ma AT, Sturtevant D, et al
709 (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae*
710 using the *Dictyostelium* host model system. Proceedings of the National Academy of
711 Sciences. 103(5), 1528-1533.
712 Pukatzki S, McAuley SB, Miyata ST (2009). The type VI secretion system: translocation of
713 effectors and effector-domains. Current opinion in microbiology. 12(1), 11-17.
714 Reinhold-Hurek B, Hurek T (2011). Living inside plants: bacterial endophytes. Current opinion
715 in plant biology. 14(4), 435-443.

716 Riaz U, Murtaza G, Anum W, Samreen T, Sarfraz M, Nazir MZ (2021). Plant Growth-Promoting
717 Rhizobacteria (PGPR) as biofertilizers and biopesticides. In Microbiota and Biofertilizers (pp.
718 181-196). Springer, Cham.
719 Rivas R, Peix A, Mateos PF, Trujillo ME, Martínez-Molina E, Velázquez E (2007). Biodiversity
720 of populations of phosphate solubilizing rhizobia that nodulates chickpea in different Spanish
721 soils. In First international meeting on microbial phosphate solubilization (pp. 23-33).
722 Springer Netherlands.
723 Russell AB, Peterson SB, Mougous JD (2014). Type VI secretion system effectors: poisons with
724 a purpose. Nature reviews microbiology. 12(2), 137-148.
725 Russell AB, Singh P, Brittnacher M, et al (2012). A widespread bacterial type VI secretion
726 effector superfamily identified using a heuristic approach. Cell host & microbe, 11(5), 538-
727 549.
728 Ryu CM (2015). Against friend and foe: type 6 effectors in plant-associated bacteria. Journal of
729 Microbiology. 53(3), 201-208.
730 Santos MNM, Cho ST, Wu CF, et al (2020). Redundancy and specificity of type VI secretion
731 vgrG loci in antibacterial activity of *Agrobacterium tumefaciens* 1D1609 strain. Frontiers in
732 microbiology, 10, 3004.
733 Sarris PF, Skandalis N, Kokkinidis M, Panopoulos NJ (2010). In silico analysis reveals multiple
734 putative type VI secretion systems and effector proteins in *Pseudomonas syringae*
735 pathovars. Molecular plant pathology. 11(6), 795-804.
736 Schlieker C, Zentgraf H, Dersch P, Mogk A (2005). ClpV, a unique Hsp100/Clp member of
737 pathogenic proteobacteria. Biological chemistry. 386(11), 1115-1127.
738 Schwarz S, West TE, Boyer F, et al (2010). *Burkholderia* type VI secretion systems have distinct
739 roles in eukaryotic and bacterial cell interactions. PLoS pathogens. 6(8), e1001068.
740 Sengupta A, Gunri SK, Biswas T (2017). Microbial interactions and plant health. In Plant-
741 Microbe Interactions in Agro-Ecological Perspectives (pp. 61-84). Springer, Singapore.

742 Sessitsch A, Hardoim P, Döring J, et al (2012). Functional characteristics of an endophyte
743 community colonizing rice roots as revealed by metagenomic analysis. Molecular Plant-
744 Microbe Interactions. 25(1), 28-36.
745 Shidore T, Dinse T, Öhrlein J, Becker A, Reinhold-Hurek B (2012). Transcriptomic analysis of
746 responses to exudates reveal genes required for rhizosphere competence of the endophyte
747 *Azoarcus* sp. strain BH72. Environmental Microbiology, 14(10), 2775-2787.
748 Shrivastava S, Mande SS (2008). Identification and functional characterization of gene
749 components of Type VI Secretion system in bacterial genomes. PloS one. 3(8), e2955.
750 Shyntum DY, Nkomo N, Gricia AR, Shigange NL, Bellieny-Rabelo D, Moleleki LN (2018). The
751 impact of type VI secretion system, bacteriocins and antibiotics on competition amongst Soft-
752 Rot *Enterobacteriaceae*: Regulation of carbapenem biosynthesis by iron and the
753 transcriptional regulator Fur. bioRxiv, 497016.
754 Shyntum DY, Nkomo N, Shingange N, Gricia AR, Bellieny-Rabelo D, Moleleki LN (2019). The
755 impact of type VI secretion system, bacteriocins and antibiotics on bacterial competition of
756 *Pectobacterium carotovorum* subsp. *brasiliense* and the regulation of carbapenem
757 biosynthesis by iron and the the ferric-uptake regulator. Frontiers in microbiology. 10(2379).
758 Silverman JM, Brunet YR, Cascales E, Mougous JD (2012). Structure and regulation of the type
759 VI secretion system. Annual review of microbiology. 66, 453-472.
760 Simon RU, Priefer U, Pühler A (1983). A broad host range mobilization system for in vivo genetic
761 engineering: transposon mutagenesis in gram negative bacteria. Biotechnology. 1(9), 784-791.
762 Sokal RR, Rohlf FJ (1984). Introducción a la Bioestadística. Editorial Reverté S.A., Barcelona,
763 España, 376 pp.
764 Somasegaran P, Hoben HJ (1994). The handbook for rhizobia: methods in legume rhizobia
765 technology (Springer Verlag: New York).
766 Suarez G, Sierra JC, Sha J, et al (2008). Molecular characterization of a functional type VI
767 secretion system from a clinical isolate of *Aeromonas hydrophila*. Microbial
768 pathogenesis. 44(4), 344-361.

769 Sunnotel O, Nigam P (2002). Pectinolytic activity of bacteria isolated from soil and two fungal
770 strains during submerged fermentation. World J Microbiol Biotechnol. 18(9):835-839.
771 Taurian T, Anzuay MS, Angelini JG, Tonelli ML, Ludueña LM, Pena D, Ibañez F, Fabra A
772 (2010). Phosphate-solubilizing peanut associated bacteria: screening for plant growth-
773 promoting activities. Plant and Soil. 329(1-2), 421-431.
774 Trunk K, Coulthurst SJ, Quinn J (2019). A new front in microbial warfare—delivery of antifungal
775 effectors by the type VI secretion system. Journal of Fungi. 5(50).
776 Unni R, Pintor KL, Diepold A, Unterweger D (2022). Presence and absence of type VI secretion
777 systems in bacteria. Microbiology, 168(4), 001151.
778 Verma SK, Kingsley K, Bergen M, English C, Elmore M, Kharwar RN, White JF (2018).
779 Bacterial endophytes from rice cut grass *(Leersia oryzoides* L.) increase growth, promote root
780 gravitropic response, stimulate root hair formation, and protect rice seedlings from
781 disease. Plant and Soil, 422(1), 223-238.
782 Verster AJ, Ross BD, Radey MC, Bao Y, Goodman AL, Mougous JD, Borenstein E (2017). The
783 landscape of type VI secretion across human gut microbiomes reveals its role in community
784 composition. Cell host & microbe. 22(3), 411-419.
785 Wei JR, Tsai YH, Horng YT, et al (2006). A mobile quorum-sensing system in *Serratia*
786 *marcescens*. Journal of bacteriology. 188(4), 1518-1525.
787 Whitney JC, Chou S, Russell AB, et al (2013). Identification, structure, and function of a novel
788 type VI secretion peptidoglycan glycoside hydrolase effector-immunity pair. Journal of
789 Biological Chemistry, 288(37), 26616-26624.
790 Williams SG, Varcoe LT, Attridge SR, Manning PA (1996). *Vibrio cholerae* Hcp, a secreted
791 protein coregulated with HlyA. Infection and immunity. 64(1), 283-289.
792 Wu CF, Lien YW, Bondage D, et al (2020). Effector loading onto the VgrG carrier activates type
793 VI secretion system assembly. EMBO reports. 21*: e4796.*

794 Wu HY, Chung PC, Shih HW, Wen SR, Lai EM (2008). Secretome analysis uncovers an Hcp-
795 family protein secreted via a type VI secretion system in *Agrobacterium tumefaciens*. Journal
796 of bacteriology. 190(8), 2841-2850.
797 Zhang L, Xu J, Xu J, Zhang H, He L, Feng J (2014). TssB is essential for virulence and required
798 for type VI secretion system in *Ralstonia solanacearum*. Microbial pathogenesis, *74*, 1-7.
799 Zheng J, Ho B, Mekalanos JJ (2011). Genetic analysis of anti-amoebae and anti-bacterial
800 activities of the type VI secretion system in *Vibrio cholerae*. PloS one. 6(8), e23876.
801 Zheng J, Leung KY (2007). Dissection of a type VI secretion system in *Edwardsiella*
802 *tarda*. Molecular microbiology, 66(5), 1192-1206.
803 Zoued A, Brunet YR, Durand E, et al (2014). Architecture and assembly of the Type VI secretion
804 system. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 1843(8), 1664-1673.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: