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The *Azospirillum brasilense* Type VI secretion system promotes cell aggregation, biocontrol protection against phytopathogens and attachment to the microalgae *Chlorella sorokiniana*

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Running title: T6SS in microalgae-bacteria association

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† **Dedication:** This study is dedicated to the memory of Dr. Yoav Bashan, leading figure in the field of Plant Growth-Promoting Bacteria (PGPB) for environmental purposes, and founder of the Bashan Institute of Science, USA. Prof. Bashan passed away during the edition of the manuscript.

Summary

The plant-growth promoting bacterium *Azospirillum brasilense* is able to associate with the microalgae *Chlorella sorokiniana*. Attachment of *A. brasilense* increases the metabolic performances of the microalgae. Recent genome analyses have revealed that the *A. brasilense* Az39 genome contains two complete sets of genes encoding Type VI secretion systems (T6SS), including the T6SS1 that is induced by the indole-3-acetic acid (IAA) phytohormone. The T6SS is a multiprotein machine, widespread in gram-negative bacteria, that delivers protein effectors in both prokaryotic and eukaryotic cells. Here we show that the *A. brasilense* T6SS is required for *Chlorella-Azospirillum* synthetic mutualism. Our data demonstrate that the T6SS is an important determinant to promote production of carbohydrates and photosynthetic pigments by the microalgae. We further show that this is likely due to the role of the T6SS during the attachment stage and for the production of IAA phytohormones. Finally, we demonstrate that the *A. brasilense* T6SS provides antagonistic activities against a number of plant pathogens such as *Agrobacterium*, *Pectobacterium*, *Dickeya* and *Ralstonia* species *in vitro*, suggesting that, in addition to promoting growth, *A. brasilense* might confer T6SS-dependent bio-control protection to microalgae and plants against bacterial pathogens.

Significance

Azospirillum brasilense, a plant growth promoting bacterium, increases the metabolic performances of plants and microalgae. Here we show that the *A. brasilense* type VI secretion system (T6SS) is an important determinant of *Azospirillum* attachment to the microalgae *Chlorella sorokiniana*, and hence that a mutant defective in T6SS decreases metabolite production in the microalga. In addition, we show that the T6SS confers antibacterial activity against competitor

bacterial cells, including phytopathogens *in vitro*, suggesting that the T6SS is also an important determinant for bioprotection. This study therefore demonstrates that a secretion system, involved in the secretion of bacterial effectors and toxins, participates in processes that are beneficial to plant and microalgae cells.

Keywords: Attachment; aggregation; Plant Growth Promoting Bacteria (PGPB); *Azospirillum*; bacteria-microalgae interaction; *Chlorella*; Type VI secretion system (T6SS)

Introduction

Azospirillum spp. is one of the best-characterized genera of plant growth-promoting bacteria (PGPB) and can colonize more than 130 plant species in 37 families (Pereg *et al*., 2016). The association of *Azospirillum* spp. with these plants significantly improves growth, development, and in many cases, yield under field conditions (Cassan *et al*., 2020). There is no single mechanism involved in promoting plant growth with *Azospirillum*, but rather a combination of mechanisms in different cases of inoculation. These mechanisms work together or in tandem, and the phenomenon is commonly known as "multiple mechanism theory" (Bashan and de-Bashan, 2010). The production of indole-3-acetic acid (IAA) by *Azospirillum* spp. is one of the main mechanisms proposed for the effect of this bacterial species on plant growth (Bashan and de-Bashan, 2010). The *A. brasilense* Az39 used in this study was isolated in 1982 from surface-sterilized wheat seedlings in Marcos Juarez, Argentina. This strain was selected for inoculant formulation, based on its ability to increase crop yields of maize and wheat under agronomic conditions (Díaz-Zorita and Fernández-Canigia, 2009). Currently, *A. brasilense* Az39 is one of the most used strains for crops such as maize and sorghum in Argentina and South America (Cassan *et al*., 2020).

For decades, *Azospirillum* spp. has been known to associate to roots of higher plants (Bashan and de-Bashan, 2010), such as cereals, tomato, pepper, cotton, and soybean, and with the microalgae *Chlorella sorokiniana* (Levanony *et al*., 1989; Bashan *et al*., 1991; Assmus *et al*., 1995). The first step of the association corresponds to attachment to the root, a critical step because all azospirilla are highly motile bacteria *in vitro* (Bashan and Holguin, 1994; Alexandre *et al*., 2000), in soil (Bashan and Levanony, 1987), and in water (Puente *et al*., 1999). In addition to motility, root colonization also requires the production of surface polysaccharides (Jofre *et al.*, 2004). After attachment, azospirilla colonize the roots. Host colonization usually requires the

delivery of effector proteins by dedicated secretion systems. Up to eleven secretion systems (Types I–XI) have been described so far in gram-negative bacteria (Costa *et al*., 2015). Genome analyses show that *A. brasilense* strains are lacking Type III and Type IV secretion systems. Transcriptomics and bioinformatics analyses show that *A. brasilense* strains Sp245 and Az39 contain gene clusters encoding putative Type VI secretion systems (T6SSs) (Van Puyvelde *et al*., 2011; Rivera *et al*., 2014; Fig. 1; Table S1). The T6SS is a widespread, multi-protein apparatus, found in gram-negative bacteria (Bingle *et al*., 2008; Cascales, 2008). The T6SS is not only distributed in human, animal, and plant pathogenic bacteria where it is involved in virulence and pathogenesis, but also found in commensal and symbiotic PGPB (Bingle *et al*., 2008; Russell *et al*., 2014). In these bacteria, the T6SS participates to intermicrobial rivalry in bacterial and fungal competition, biofilm formation, and establishment of symbiosis (Jiménez-Guerrero *et al*., 2013; Durand *et al*., 2014; Russell *et al*., 2014; Ryu, 2015; Bernal *et al.*, 2017; Trunk *et al.*, 2019; Gallegos-Monterossa and Coulthurst, 2021). At the molecular level, the T6SS acts like a cellular "biological syringe": it assembles a bacteriophage tail-like structure anchored to the cell envelope by a membrane complex (Zoued *et al.*, 2014; Coulthurst, 2019; Cherrak *et al.*, 2019). The tail-like structure consists of an inner tube made of hexamers of the Hcp protein capped by a puncturing VgrG-PAAR complex and wrapped in the TssBC contractile sheath (Zoued *et al*., 2014; Cherrak *et al.*, 2019; Basler, 2015). Effector toxins are loaded onto the VgrG o PAAR proteins or within the Hcp tube (Hernandez *et al.*, 2020; Jurenas and Journet, 2021). Contraction of the sheath propels the inner tube towards the target cell (Basler, 2015; Brackmann *et al.*, 2017). Penetration of the tube into the target cell allows delivery of the effectors (Hernandez *et al.*, 2020; Jurenas and Journet, 2021). While the mechanisms of action and the role of T6SS in animal and plant pathogens have been well described in recent years, it has been less studied in plant symbiotic and associative

bacteria (Jiménez-Guerrero *et al*., 2013; Ryu, 2015; Wu *et al.*, 2018; Wu *et al.*, 2019; Bernal *et al.*, 2018; Borrero de Acuña and Bernal, 2021). In *Rhizobium leguminosarum*, the T6SS affects the attachment, nodulation, and symbiosis processes (Roest *et al*., 1997; Bladergroen *et al*., 2003). In *Pseudomonas fluorescens*, the T6SS is involved in bacterial competition, motility, biofilm formation, and is critical to persist in the rhizosphere microbiome (Decoin *et al*., 2014; 2015; Gallique *et al.*, 2017; Durán *et al.*, 2021). *P. putida* produces three T6SSs, including K1-T6SS that confers antibacterial activity against numerous bacterial phytopathogens, and hence can be used as a biocontrol agent against plant pathogens (Bernal *et al.*, 2017). Finally, *Agrobacterium tumefaciens* was shown to deploy a T6SS to destroy competitors and to increase colonization *in planta* (Ma *et al.*, 2014; Wu *et al.*, 2018; Wu *et al.*, 2019). *A. brasilense* Az39 contains two gene clusters encoding T6SSs. A transcriptomic study of *A. brasilense* Sp245 showed that the T6SS1 gene cluster is induced by indole-3-acetic acid (IAA) (Van Puyvelde *et al*., 2011). The activation of T6SS1 by a phytohormone in *A. brasilense* prompted us to investigate the role of this T6SS in interbacterial competition and its interaction with microalgal cells.

The freshwater unicellular microalgae *Chlorella* spp. can associate with several bacterial species (Imase *et al*., 2008), including *Azospirillum* spp. (Hernandez *et al*., 2009; de-Bashan *et al*., 2015). The *Chlorella-Azospirillum* association is an established model system of synthetic mutualism to study cellular mechanisms during plant-bacteria interaction (de-Bashan and Bashan, 2008; de-Bashan *et al*., 2016). This association significantly promotes the production of carbohydrates, fatty acids, lipids, and pigments of the microalgae, both autotrophically and heterotrophically (de-Bashan *et al*., 2002, 2015; Choix *et al*., 2012a, b; Leyva *et al*., 2014).

Previously, we tested the attachment and mutual aggregation between the microalga *Chlorella sorokiniana* and *Azospirillum brasilense* immobilized in alginate beads to facilitate the

initial stage of formation of this synthetic association (de-Bashan and Bashan, 2008; de-Bashan *et al*., 2015; de-Bashan *et al*., 2016). Attachment of *Azospirillum brasilense* to *Chlorella* spp. cells involve fibrillar connections (Lebsky *et al*., 2001; de-Bashan *et al*., 2011), similar to what was observed during the attachment of *Azospirillum* spp. to cells of higher plants (Bashan *et al*., 1986; Bashan *et al*., 1991). However, we currently lack molecular details on how *Azospirillum* spp. efficiently colonizes microalgae and plants.

In this study, we show that the *A. brasilense* T6SS1 has a positive effect on growth of the microalgae *Chlorella*. We further demonstrate that a *A. brasilense* strain defective for T6SS1 presents a reduced enhancement of the production of microalgal metabolites such as lipids, carbohydrates, and photosynthetic pigments compared to wild-type *A. brasilense*. Finally, we demonstrate that the *Azospirillum* T6SS provides anti-bacterial activity against *E. coli* and several plant pathogens, including *Agrobacterium tumefaciens*, *Pectobacterium carotovorum* and *Dickeya dadantii*. These data support the idea that the T6SS is required for the establishment of an efficient synthetic mutualism between *Azospirillum* and *Chlorella* and confers protection of the microalgae - and potentially of plants - against pathogens.

Results

Construction of the A. brasilense Az39Δhcp-E mutant

The Hcp protein is an essential component of the T6SS and its encoding gene is present as a single copy in the T6SS1 gene cluster of *A. brasilense* Az39. It is located upstream of the *tssE* gene, which encodes an important component of the T6SS assembly baseplate (Brunet *et al*., 2015) (Fig. 1). Both genes are in tandem in chromid 1 in *A. brasilense* Az39 (GenBank, CP007794.1, AbAz39_p1, WP_051658325.1) and share high identity to those encoded within the genomes of

A. brasilense FP2 (99%), Sp245 (98%), Sp7 (99%), and *Azospirillum* sp. B510 (64%). We used targeted mutagenesis to construct an isogenic mutant of Az39. For this, a genomic fragment overlapping with the *hcp* and *tssE* genes was replaced with a gentamycin-resistant cassette by homologous recombination (Fig. S1). The genomic structure of the wild-type strain and its isogenic Δ*hcp-tssE* (Δ*hcp-E*) mutant was confirmed by PCR using several combinations of primers (Fig. S1).

Phenotypic characterization of the A. brasilense Az39Δhcp-E strain for cell growth, IAA production, motility, cell aggregation and biofilm formation

To characterize the Az39Δ*hcp-E* mutant strain, we measured its growth rate in rich and minimum media, its production of indole-3 acetic acid, and its capacity to swim, swarm and to form biofilm compared to the wild-type strain.

Growth rate - The growth rate and the number of colony-forming units (CFU) of the *A. brasilense* wild-type strain and its *hcp-tssE* mutant were compared in two different media. In both rich (LB) and minimal (MMAB) media, the two strains exhibited similar growth patterns for 80 hours (Fig. S2). Wild-type *A. brasilense* Az39 had the highest number of living cells (4.63×10^9 CFU·mL^{-1} in LB medium, and 1.85×10^9 CFU·mL^{-1} in MMAB) during the exponential growth phase (24 h after inoculation) and fewer cells (0.75×10^9 CFU·mL^{-1} in LB; 0.75×10^9 CFU·mL^{-1} in MMAB) during the stationary growth phase (>36 h after inoculation) (Fig S2). The *A. brasilense* Az39Δ*hcp-E* mutant strain exhibited a similar pattern, with a higher number of cells during the exponential growth phase (2×10^9 CFU·mL^{-1} in LB; 1.6×10^9 CFU·mL^{-1} in MMAB) and fewer living cells during the stationary growth phase (1.5×10^9 CFU·mL^{-1} in LB; 1.06×10^9 CFU·mL^{-1}) (Fig. S2).

Indole-3-acetic acid (IAA) production - In minimum medium, the mutant strain produced significantly less IAA during exponential growth compared to wild-type Az39, with a maximum difference at 24 h of culture (Az39Δ*hcp-E*: 56.77 ± 2.59 $\mu g \cdot mL^{-1}$; Az39: 79.98 ± 2.14 $\mu g \cdot mL^{-1}$), but the accumulation of the phytohormone in the culture medium was restored to wild-type levels during the stationary growth phase (Az39Δ*hcp-E*: 79.82 ± 1.60 $\mu g \cdot mL^{-1}$, Az39: 79.45 ± 4.94 $\mu g \cdot mL^{-1}$), showing a saturation kinetic (Fig. 2).

Swimming, swarming, aggregation and biofilm formation - Previous reports have shown that mutation within T6SS gene clusters confer defects in swimming, swarming or biofilm formation (Aschtgen *et al.*, 2008; de Pace *et al.*, 2010; Decoin *et al.*, 2014; Bouteiller *et al.*, 2020). The Az39Δ*hcp-E* mutant strain exhibited better swimming capacity compared to its parental strain in swim and MMAB culture media (Fig. 3a). By contrast, both strains swarmed at similar rates (Fig. 3b). Cell aggregation was reduced for the Az39Δ*hcp-E* mutant in MMAB and LB culture media with an average reduction of 30% in comparison to the wild-type strain (Table 1). Finally, we observed that the Az39Δ*hcp-E* mutant strain produced ~50 % less biofilm after 48 and 72 h of incubation, indicating a reduction in attachment capacity between cells. After 96 h of incubation, the mutant showed a reduction of 23.15 % of biofilm production compared to the wild-type strain, but this difference was not significant (Fig. 3c). Altogether, these data showed that the *A. brasilense* T6SS1 decreases the ability to swim and confers an increased ability to adhere to abiotic surfaces, at least in the early stages of the attachment phase.

The A. brasilense T6SS provides anti-bacterial activity against competitors, including phytopathogens.

A number of reports have demonstrated that the T6SS is involved in regulating bacterial communities by targeting effector toxins into competing bacteria sharing the same ecological niche (Hood *et al.*, 2010; Wexler *et al.*, 2016; Sana *et al.*, 2016; Anderson *et al.*, 2017; Chassaing and Cascales, 2018; Allsopp et al., 2020; Wood *et al*., 2020; Duran *et al.*, 2021). We therefore asked whether the *A. brasilense* T6SS might confer protection to the microalgae or plants by delivering anti-bacterial toxins into phytopathogens. We performed *in vitro* competition experiments in minimal medium against *Escherichia coli* K-12, the soil bacterium *Ralstonia eutropha* and selected plant pathogens such as *Agrobacterium tumefaciens*, *Pectobacterium carotovorum*, *Dickeya dadantii* and *Ralstonia solanacearum*. After 8 h of co-incubation with wild-type *A. brasilense* Az39 and its isogenic Δ*hcp-E* mutant, surviving prey cells were counted on selective medium. Fig. 4 shows that the wild-type *A. brasilense* strain has T6SS-dependent anti-bacterial activity against all the strains tested as only 7-15% of cells survive against the wild-type *A. brasilense* compared to the Δ*hcp-E* mutant. The T6SS activity was significantly increased in presence of indole-3-acetic acid, previously shown to induce the T6SS gene cluster (Van Puyvelde *et al.*, 2011). In presence of IAA, only 0.1-1% of the prey cells survived co-incubation with wild-type *A. brasilense* cells whereas their recovery was not affected when co-incubated with Δ*hcp-E* mutant cells (Fig. 4). We conclude that the *A. brasilense* T6SS has anti-bacterial activity against several strains and therefore might confer protection to plant and microalgae against bacterial pathogens.

Contribution of the T6SS for C. sorokiniana/A. brasilense association

Microalgae growth - To test the role of the *A. brasilense* T6SS for mutualistic growth of *Chlorella*, we followed the growth of the microalgae alone or in presence of the *A. brasilense* Az39 wild-type

strain or its isogenic Δ*hcp-E* mutant (Fig. 5a). When cultured alone, the *C. sorokiniana* population increased for the first two days and then slowly decreased. After six days, the population reached its lowest numbers (Fig. 5a, open circles). In the presence of the *A. brasilense* wild-type or mutant strains, the *Chlorella* population increased for 4 days and then declined (Fig. 5a, closed squares and triangles, respectively). The growth rate of the microalgae after 4 days with either *A. brasilense* strain was ~3.5 higher than microalgae cultured alone (Fig. 5a). For 6 days, the interaction with both types of bacteria supported larger populations of microalgae, compared to the microalgae that were cultured alone.

Cell aggregation - Chlorella-Azospirillum association and aggregation were probed by fluorescence in situ hybridization (FISH) (Fig. 5b). Aggregates of the two bacterial strains grown with *C. sorokiniana* showed different dynamics throughout the experiment. The initial distribution of cells represents the outset of the microorganism's interaction (Fig. 5b panels a, e, i, m, q). In all cases, the aggregates are increasing in size from day 1 (Fig. 5b panels a-t; Fig. 5c). The difference in aggregates depending on the bacterial strain is evident at day 4. When *C. sorokiniana* was co-cultured with *A. brasilense* Az39 large aggregates composed of bacteria and microalgae were found (Fig. 5b panel d; Fig. 5c). Co-cultures of *C. sorokiniana* with *A. brasilense* Az39Δ*hcp-E* showed aggregates mainly composed of microalgae (Fig. 5b panel h) and significantly smaller than the aggregates formed with the wild-type Az39 strain (Fig. 5c). *C. sorokiniana* alone showed smaller aggregates (5b panel t; Fig. 5c). *A. brasilense* Az39 growing alone displayed aggregates increasing in size (Fig. 5b panels i-l; Fig. 5c); however, *A. brasilense* Az39Δ*hcp-E* showed less aggregation than wild-type, maintaining a similar size of aggregates at days 2 and 4 (Fig. 5b panels m-p; Fig. 5c), in agreement with the defect in cell aggregation observed previously (Table 1).

Accumulation of carbohydrates, lipids and photosynthetic pigments - To better understand the effect of the *A. brasilense* T6SS during mutualistic growth with *C. sorokiniana*, we measured the accumulation of carbohydrates, lipids and pigments in the microalgae alone or in presence of the *A. brasilense* Az39 wild-type strain or its isogenic Δ*hcp-E* mutant (Fig. 6 and 7). Fig. 6a shows that co-culture of *Chlorella* with the wild-type *A. brasilense* Az39 strain significantly increased the accumulation of carbohydrates in the microalgae-bacteria association only at days 1 and 4 of co-culturing, while co-culture with *A. brasilense* Az39Δ*hcp-E* cells had no effect on carbohydrates accumulation (Fig. 6a). Regarding lipids production, co-culture of *C. sorokiniana* with either *A. brasilense* strain significantly improved the production of lipids in the microalgae, although the wild-type induced higher production of lipids at days 1, 2, and 6, while it was higher with the mutant at day 4 (Fig. 6b). The effect of the *A. brasilense* T6SS on microalgae photosynthesis was indirectly measured by quantifying major and auxiliary photosynthetic pigments: chlorophyll *a* and *b*, violaxanthin, lutein and β-carotene (Fig. 7c-f). The accumulation of these photosynthetic pigments followed a similar pattern. After 1 day of co-culture, the presence of *A. brasilense* strains did not improve the synthesis of pigments. However, the presence of the Az39 wild-type strain increased the production of pigments by *Chlorella* on days 2, 4 and 6. By contrast, co-culture of *Chlorella* with the *A. brasilense* Δ*hcp-E* strain did not improve pigment production during the four first days.

Discussion

In this study, we provide the first characterization of a Type VI secretion system from the plant-growth promoting bacterium *Azospirillum brasilense* (Fig. 7). We show that the *A. brasilense* T6SS1 is involved in attachment to the microalgae *Chlorella sorokiniana,* participates to the formation of an efficient algae/bacterium consortium, and in the production of the IAA phytohormone. Through its role on attachment and IAA levels, which exert major changes on growth and the metabolism of the microalgae the T6SS indirectly allows increased lipid metabolism in the microalgae. We also reveal that the *A. brasilense* T6SS confers protection against bacterial phytopathogens. This study therefore demonstrates that the T6SS is an important beneficial determinant of *Azospirillum*/*Chlorella* interaction by mediating attachment, improvement of the metabolic performances and bio-control (Fig. 7).

We have shown that the T6SS participates to biofilm formation on abiotic surfaces and helps attachment of *Azospirillum* to *Chlorella* cells and formation of *Chlorella* clusters. In addition, we observed a role of the T6SS in IAA production. Attachment and IAA production are key in *Azospirillum*–plant cell interactions (Bashan and de-Bashan, 2010). Attachment of *Azospirillum* to cells is a major, active and complex phenomenon (Bashan, Levanony and Klein 1986; Gafni *et al*. 1986). The initial interaction of *Azospirillum* spp. with plant cells it mainly mediated by attachment of the bacterium, which employs a variety of piliated appendages at the cell surface to promote stable interactions between the eukaryotic and prokaryotic cells (Pereg *et al*., 2016); Attachment between bacteria and microalgae was previously observed (de-Bashan *et al*., 2011, 2015, 2016), but the question remains on whether this attachment is significant for the development of this association by creating additional massive microalgae-bacteria aggregates. Active attachment to plants and subsequent colonization were observed with the roots of cereals, tomato, pepper, cotton and soybean (Bashan *et al.* 1989; Bashan, Singh and Levanony 1989;

Bashan, Levanony and Whitmoyer 1991; Levanony *et al*. 1989; Assmus *et al*. 1995; Pereg Gerk, Gilchrist and Kennedy 2000). The initial step of plant root colonization by *Azospirillum* spp. necessitates flagella and exopolysaccharides to create very stable interaction between the eukaryotic and prokaryotic cells (Pereg, de-Bashan and Bashan 2016). These structures are also needed for adhesion to non-living substrates (Bashan and Levanony 1988; Bashan and Holguin 1993). The polar flagellum of *A. brasilense*, which is primarily used for swimming, is involved in the initial attachment process of the bacteria to wheat roots (Croes *et al.* 1991). Then the attachment process involves two distinct steps. The first stage is fast and corresponds to a weak adhesion and is mediated by host cell surface hydrophobicity, charges and lectins (Castellanos, Ascencio and Bashan 1998, 2000). The second stage takes longer time but the attachment is irreversible. This stage is mediated by extracellular surface polysaccharides and mucigel-like substances (Gafni *et al.* 1986; Bashan and Levanony 1988; Bashan, Levanony and Whitmoyer 1991; Levanony and Bashan 1991; Michiels *et al.*, 1989; Puente *et al*. 1999; Pereg Gerk, Gilchrist and Kennedy 2000; Lerner *et al*., 2009). Other determinants of *Azospirillum* attachment are the *flcA* regulator (Pereg Gerk *et al*. 1998) that is also involved in stress response and carbohydrate and nitrogen metabolism (Hou *et al*. 2014), chemotaxis pathways (Bible, Russell and Alexandre 2012; Pereg, de-Bashan and Bashan 2016), Cpa pili (Wisniewski-Dye *et al.* 2011) as well as genes related to the surface properties of *Azospirillum*, such as *noeJ* (mannose-6-phosphate isomerase) and *noeL* (GDP-mannose 4,6-dehydratase) and pathways involved in lipopolysaccharide core processing (dTDP-rhamnose biosynthesis pathway; Jofre et al., 2004) or energy taxis (Greer-Phillips, Stephens and Alexandre 2004). Although the T6SS might be a new molecular determinant of the adhesion of Azospirillum to microalgae cells, it remains to define whether the effect of the T6SS mutation on adhesion is due to defect in flagellar synthesis or expression of attachment genes. While cross-talk

between the T6SS and flagellar genes have been demonstrated in the *P. fluorescens* PGPB (Bouteiller et al., 2020), the first hypothesis is unlikely as we have shown here that deletion of the *hcp-tssE* genes does not affect swarming rates and increases swimming rates. The second hypothesis merits investigation, as previous studies have shown that the inactivation of the T6SS may affect expression of fimbrial genes in pathogenic *E. coli* strains (de Pace *et al*., 2010).

Previous studies have shown that *A. brasilense* enhance the growth and induce an increased accumulation of carbohydrates, starch (Choix *et al.,* 2012a, b), fatty acids, total lipids (Leyva *et al*., 2014; Peng *et al.,* 2020) and pigments (de-Bashan *et al.* 2002; Peng *et al.,* 2020) in *Chlorella* spp. The effect has been mostly attributed to the IAA production by the bacteria (de-Bashan and Bashan, 2008; de-Bashan *et al*., 2011, 2015; Peng *et al.,* 2020). Since the concentration of IAA produced by the wild-type and T6SS *A. brasilense* strains is similar at the times of the experiments with the microalgae (after 30 hours), the reduction of lipids and photosynthetic pigments induced by the T6SS mutant can be attributed to its lower attachment capacity. It was previously shown that attachment is not essential for C and N compounds transfer between individual cells of *C. sorokiniana* and *A. brasilense* (de-Bashan *et al*., 2016); however, the metabolites produced by *Azospirillum*, might be transient and probably a close cell proximity is required to achieve a more effective mass transfer (Peng *et al*., 2020).

We have also shown that mutation in the *A. brasilense* T6SS affects IAA production. IAA, as a phytohormone, has no important role in bacterial metabolism (Spaepen and Vanderleyden, 2011), but is widespread among PGPB (Duca *et al*., 2014) and increases plant growth. The production of IAA during the interaction between *Azospirillum* spp. and *Chlorella* spp. and its effect on growth was reported several times (de-Bashan *et al*., 2008; Palacios *et al*., 2016a, b, Peng *et al*., 2020). The role of the T6SS in *Azospirillum*/plant interactions suggests that the expression of this cluster

and/or the activity of the machine is responsive to environmental or plant cues. It would be therefore interesting to determine what are the environmental signals in addition to IAA (Van Puyvelde *et al.*, 2011), and to identify the regulators that control the expression of this T6SS gene cluster.

The role of the *A. brasilense* T6SS in plant/bacterium interactions is supported by the observation that T6SS genes are up-regulated in presence of the IAA phytohormone (Van Puyvelde *et al.*, 2011). However, contrarily to T2SS or T3SS present in plant-associated bacteria, the *A. brasilense* T6SS is not deployed for pathogenic purposes. Interestingly, T6SS gene clusters are conserved in proteobacterial and bacteroidetes genomes, including non-pathogenic bacteria such as gut symbiots (Wexler *et al.*, 2016), soil and marine bacteria. The presence of a T6SS in these strains suggests that the T6SS could be used for promoting commensal or mutualistic relationships between bacteria and eukaryotes (Jani and Cotter 2010; Konovalova, Petters and Søgaard-Andersen 2010; Jiménez-Guerrero *et al*. 2013). Indeed, the *Rhizobium leguminosarum* bv. *trifolii* T6SS has a role in the nodulation process by blocking the colonization/infection process of non-host peas (Roest *et al*. 1997; Bladergroen *et al*. 2003). Whereas it has been proposed that RbsB is a T6SS effector delivered by *R. leguminosarum* into pea cells (Bladergroen *et al*. 2003), the effector(s) delivered by the *A. brasilense* T6SS into plant/algae cells remain to be identified.

Finally, our data show that the T6SS provides antagonistic activities to *A. brasilense* against a number of bacterial strains including phytopathogens such as *Agrobacterium*, *Pectobacterium*, *Dickeya* and *Ralstonia in vitro*. This demonstrates that, in addition to promoting growth, *A. brasilense* likely confers protection against harmful pathogens in a T6SS-dependent manner. Effectors with DNase, phospholipase and peptidoglycan hydrolase activities have been shown to be delivered into bacterial cells (Russel *et al.*, 2014; Jurenas and Journet, 2021). The *A. brasilense*

T6SS effector repertoire needs to be determined. It has been shown that the effector genes are usually located within the T6SS gene cluster or within *hcp*/*vgrG* islands scattered in the genome (Durand *et al.*, 2014). Indeed, the *vgrG* gene encoded within the cluster is fused to an additional domain resembling *Bacillus subtilis* YwqJ, a potent toxin with putative deaminase activity, and is followed by a gene resembling its cognate YwqK antitoxin (Brantl and Müller, 2019; Kobayashi, 2021) (Fig. 1).

In summary, the *Chlorella* spp.–*Azospirillum* spp. association has been used as a model system to study aggregation, metabolic, and molecular effect occurring during plant-bacteria interaction (de-Bashan and Bashan, 2008). The present work extends the potential to study a molecular mechanism occurring between a bacterium and a microalga, specifically by showing that a T6SS mutant is also deficient in attachment capabilities and produces less microalgal aggregation. This T6SS mutant, while promoting the growth of microalgae similar to its parental wild-type *A. brasilense* strain also impacts lipid, and pigment metabolism. This study has thus far broader impacts than observing interactions between microalgae and bacteria *in vitro*, by demonstrating that a widespread secretion system, known almost exclusively from studies of pathogenesis and antibacterial competition, is participating in processes that are beneficial to the plant/microalga cells.

Experimental procedures

Microorganisms and growth conditions

The unicellular microalga *Chlorella sorokiniana* Shih. et Krauss (UTEX 2714, University of Texas, Austin, TX; formerly *C. vulgaris* UTEX 2714, Bashan *et al*. (2016)) was used in this study. *C. sorokiniana* was axenically grown at 27 ± 2 °C, with stirring at 120 rpm under a light intensity

of 60 µmol photon·m^{-2}·s^{-1} in sterile C30 minimal medium (composition (in g·L^{-1}): KNO_3 (25), $MgSO_4 \cdot 7H_2O$ (10), KH_2PO_4 (4), K_2HPO_4 (1), $FeSO_4 \cdot 7H_2O$ (1), and (in µg·L^{-1}): H_3BO_3 (2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.11), $CuSO_4 \cdot 5H_2O$ (0.09), and $NaMoO_4$ (0.021) at pH 5.25), and transferred to new medium every 7 days. Periodically, the cultures were checked for purity and absence of bacterial contamination on nutrient agar (#N9405, Sigma-Aldrich, St Luis, MO). The wild-type *Azospirillum brasilense* Az39 strain (Instituto de Microbiología y Zoología Agrícola del INTA-IMyZA, Castelar, Buenos Aires, Argentina, strain Az39 (WDCM31), Rivera *et al*. (2014)) and *its isogenic* Az39Δ*hcp-E* mutant (this study, see construction below) were grown in BTB medium containing gluconic acid as a carbon source (Bashan, Trejo and de-Bashan 2011) for 18 h, at 35 ± 2 °C and stirred at 120 rpm, or in MMAB minimal medium (composition (in g·L^{-1}): K_2HPO_4 (3.0), NaH_2PO_4 (1.0), NH_4Cl (1.0), $MgSO_4 \cdot 7H_20$ (0.3), KCl (0.15), $CaCl_2 \cdot 2H_20$ (0.01), $FeSO_4 \cdot 7H_20$ (0.0025), sodium malate (5.0), biotin (0.005) and microelements) at 28°C.

Construction of the A. brasilense Az39Δhcp-E mutant

Genomic analysis of the Type VI secretion system in A. brasilense Az39 - Using the genome sequence of *A. brasilense* Az39 (Rivera *et al.* 2014), two complete sets of genes encoding putative T6SSs were detected and analysed by RAST (Aziz *et al*. 2008), KEGG (Kanehisa *et al*. 2012), SecReT6 (Li et al., 2015) and BastionHub (Wang et al., 2021). The T6SS1 gene cluster, which was studied here, is shown in Fig. 1 and T6SS proteins are listed in Table S1. The gene cluster encodes all the core-component genes, *tssA-tssM*, required to assemble a fully functional T6SS (Cascales, 2008).

Az39Δ*hcp-E mutant construction* - The *hcp* and *tssE* genes were simultaneously deleted by homologous recombination. Fragments upstream and downstream of the *hcp-tssE* region were

amplified by PCR from genomic DNA using primer pairs Az39-5′HcpFW/Az39-B-5′HcpRV and Az39-B-3′HcpFW/Az39-3′HcpRV, respectively (Table S2) cloned into the pGemT-Easy vector (Promega, Madison, WI) and sequenced. The upstream fragment was inserted into the downstream fragment-containing plasmid, using *BamH*I, and *Pst*I restriction enzymes to yield the pGemT-Hcp-UD vector. Then, a gentamicin-resistance cassette (Gm^R), excised from pME3280a (Zuber *et al*., 2003) with *Xho*I, was inserted in reverse orientation into the *BamH*I site of pGemT-Hcp-UD after blunting ends with T4 DNA polymerase (New England Biolabs, Ipswich, MA). The resulting upstream-Gm^R–downstream construction was finally sub-cloned into pK18mobSacB (Schäfer *et al*., 1994), using *Pst*I and *Sph*I to obtain pK18-Hcp-UGD. This suicide plasmid, carrying the mutagenesis cassette, was mobilized into *A. brasilense* Az39 by biparental mating with *Escherichia coli* S17-1 as the donor (Simon *et al*., 1983). Double recombinant clones, resistant to gentamicin and sensitive to kanamycin, were recovered without the need for *sacB* counter selection. To confirm the correct replacement of the targeted genome region, PCR was used with several primer combinations, and the amplified fragments were visualized by TAE-agarose electrophoresis. The phenotypes of the mutant were analyzed in three independent clones (C1, C2, and C3) to exclude the presence of additional mutations (Fig. S1). Fig. S1 describes the genomic arrangement of the wild-type, the two possible simple recombinants (R1 or R2), and the double recombinant (R1+R2) of *A. brasilense* Az39. The site of annealing and the product length of the different diagnostic primers (P1, P2, P3, P4, P5, P6, GmR, and GmF) on the four possible arrangements are indicated. The inserted table indicates the PCR products expected for each strain, and the agarose gel shows the actual results of the PCR. A PCR was performed using P3 and P6 primers that uniquely anneal on the genomic region that flanks the modified region.

Strain characterization

Bacterial Growth - Growth curves of *A. brasilense* Az39 and mutant strain Az39Δ*hcp-E* were determined by measuring the turbidity of cell suspensions (OD_{595}) and cell number ($CFU.mL^{-1}$) over time. Sample of 0.5 mL of an overnight culture (OD_{595} = 1.0, ~10^9 $CFU \cdot mL^{-1}$) were inoculated to 200-mL Erlenmeyer flasks containing 50 mL of Luria-Bertani medium (LB) or MMAB minimal medium (Vanstockem *et al*., 1987) supplemented with 100 $\mu g \cdot mL^{-1}$ of L-tryptophan. Cultures were incubated at 37 °C and constant stirring at 180 rpm for 80 h. Turbidity of cell suspensions was spectrophotometrically measured at 595 nm over time (ZelTec ZL5000P, Zel Technologies, Hampton, VA) at 5 h intervals. At the same time, cell counts were performed on LB agar plates supplemented with Congo red (1.5%, v:v), after incubation for 72 h at 37 °C.

Indole-3-acetic acid production - Pure cultures of *A. brasilense* Az39 and its Δ*hcp-E* derivative were cultivated in LB and MMAB media, supplemented with L-tryptophan, at 37 °C, under constant stirring at 180 rpm for 48 h corresponding to stationary stage of growth of this species. Cultures were centrifuged at 15,650 × *g* for 10 min and cells were discarded. Quantification of IAA was performed by spectrophotometry (Glickmann and Dessaux, (1995) and confirmed by HPLC (Rivera *et al*., 2018). Briefly, aliquots of 1000 µL of bacterial culture were centrifuged at 11,300 × *g* for 10 min. Subsequently, samples were filtered (0.2 µm), and 500 µL of supernatant were mixed with 500 µL of Salkowski´s reagent (7.9 M H_2SO_4 and 12.5 $g \cdot L^{-1}$ $FeCl_3$) and gently shaken in inverted position at least 10 times. Samples were incubated in the dark for 30 min and the absorbance at 530 nm was measured. An aliquot of filtered supernatants was injected with a final volume of 20 µL in an HPLC Waters 600-MS device (Waters Inc., USA) equipped with an U6K injector and C18 reverse phase column (Purospher STAR RP C-18 3 mm, Lichrocart 55-4)

heated at 30°C, coupled to a system with UV-VIS Waters 486 detector (Waters Inc., USA) set at 265 nm. The elution was performed with a mixture of ethanol: acetic acid: water (Et-OH/H-Ac/H20) (12: 1: 87) as mobile phase at a flow rate of 1 mL·min-1 at 30°C. The retention time for IAA was 10.1-10.3 minutes and the quantification was performed by integration of the peak area corresponding to the retention time (RT) using an integration software (Waters Inc. USA). The IAA concentration was expressed in $\mu g \cdot mL^{-1}$

Motility - Motility was assayed under different concentrations of agar-agar: 1 µL of bacterial culture in late exponential phase was placed in the center of Petri dishes containing minimal swim motility medium (Atkinson *et al*., 2006) and MMAB medium as previously described (Vanstockem *et al*., 1987). Minimal swim motility agar plates contained ($g \cdot L^{-1}$) tryptone (10.0) and NaCl (5.0). Both media were prepared with 0.3% (w/v) agar to observe swimming and 0.7% (w/v) agar for determining swarming. The plates were inverted and incubated at 28 ± 2 °C for 72 h to avoid drying the agar at higher temperatures. The diameter of the displacement halo was then measured (Atkinson *et al*., 1999; Hall and Krieg, 1983).

Cell aggregation and biofilm production - Cell aggregation was measured according to Madi and Henis (1989) with modifications (Burdman *et al*., 1998). Briefly, 5 mL of stationary cultures of both strains obtained in MMAB and LB culture medium at 37°C and constant stirring at 180 rpm for 48 hours were transferred to 10 mL capacity conical tubes and allowed to stand for 20 min. Then, turbidity was measured at 540 nm using a Zeltec ZL5000P spectrophotometer (OD_1). The culture was homogenized for 1 min and the turbidity was measured again (OD_2). The aggregation percentage was calculated according to the following equation $\%AP\ (OD_2 - OD_1) \times 100/OD_2$.

Biofilm formation was measured according to O'Toole & Kolter (1998). Briefly, 13 µL of late exponential cultures were inoculated in hemolysis tubes containing 1,300 µL of sterile LB medium and incubated at 37 ± 1°C for 48, 72, and 96 h, without stirring. At each sampling time, the culture medium was carefully removed from the tube by micropipetting, leaving only the biofilm. The biofilm was rinsed three times with 1,300 µL of sterile 0.85 % saline solution. Once the culture medium was removed, the biofilm was stained with 0.1% crystal violet (C3886, Sigma-Aldrich) solution (1,300 µL for 15 min at room temperature, 24 ± 2 °C). The stained biofilm was rinsed three times with sterile distilled water. The biofilm was re-suspended in 1,300 µL of 96% ethanol containing three glass beads (3 mm diam) in each tube and stirred vigorously by a vortex for ~1 min. Finally, absorbance was measured at 560 nm by spectrophotometry (ZelTec ZL5000P, Zel Technologies).

Anti-bacterial competition assay.

Spontaneous *Agrobacterium tumefaciens*, *Pectobacterium carotovorum*, *Dickeya dadantii*, *Ralstonia eutropha* and *Ralstonia solanacearum* strains resistant to nalidixic acid were obtained after 5 consecutive liquid growth cultures in presence of increasing concentrations of nalidixic acid (2, 5, 10, 20 and 30 µM) and plating on agar plates supplemented with 40 µM nalidixic acid. *Azospirillum brasilense* and nalidixic acid-resistant recipient cells were grown in MMAB supplemented - or not - with 400 µM indole-3-acetic acid (IAA) to an OD_{595} ~1 and concentrated in MMAB to a final OD_{595} of 10. *A. brasilense* attacker cells were mixed with nalidixic resistant recipient cells at a 4:1 ratio and 15-µl drops of the mixture were spotted on dried minimum MMAB or MMAB/IAA agar plates. After incubation for 8 h at 28°C, the spots were scratched off, cells

were re-suspended in LB to an OD_{595} of 0.5, and the surviving recipient cells were counted after platting serial dilutions on LB agar plates supplemented with 40 μM nalidixic acid.

Immobilization of microorganisms in alginate beads

Before immobilization in alginate beads, 10 mL of axenic microalgae culture was inoculated into 90 mL sterile C30 medium and incubated at 27 ± 2 °C, with stirring at 120 rpm under 60 μmol photons·m^{-2}·s^{-1} light intensity for six days. The two strains of *A. brasilense* were grown in BTB culture medium containing gluconic acid as a carbon source (Bashan *et al*., 2011) for 18 h, at 35 ± 2 °C and stirred at 120 rpm. Immobilization procedure was done as described by de-Bashan *et al*. (2015). Briefly, after growth period, each culture was harvested by centrifugation (Hemle Z 200A, Wehingen, Germany) at 2,000 × *g*, washed twice, resuspended in sterile saline solution (0.85% NaCl) and cell density was adjusted to 5×10^6 cells·mL^{-1} for microalgae and ~10^9 CFU·mL^{-1} ($OD_{540\,nm}$ = 1) for bacteria. Then, 40 mL of axenic cultures of *C. sorokiniana* or *A. brasilense* Az39 or *A. brasilense Az39*Δ*hcp-E* were mixed with 160 mL of sterile 1.5% alginate solution (~1200 cP, #05218295, MP Biomedicals, Santa Ana, CA) and stirred for 15 min until the alginate slurry was completely dissolved. Beads (highly homogenous; 3 mm diam) were produced in 2% $CaCl_2$, using automatic bead-forming equipment under constant pressure to ensure uniformity of each bead (de-Bashan and Bashan, 2010). The beads were cured for 30 min in the same 2% $CaCl_2$ solution to improve hardening. They were washed in sterile 0.85% saline solution. This procedure routinely produces ~1×10^6 cells·$bead^{-1}$ for each organism with low variability (de-Bashan *et al*., 2004). For microalgae and bacteria co-immobilized in the same bead, 20 mL of each culture was mixed in 160 mL alginate to form the beads, as previously described. Because immobilization usually reduces the number of *A. brasilense* in beads, the beads were incubated again for 24 h in

10% (v/v) diluted nutrient broth (N7519, Sigma-Aldrich) to reach an initial population of 1×10^6 cells·bead^{-1}.

Experimental conditions to assess the performance of microalgae.

After the second incubation, beads were washed twice with sterile saline solution. In preparation of each experiment, 15 g of beads from each treatment (microorganisms immobilized alone or co-immobilized) were added to 500 mL Erlenmeyer flasks containing 200 mL of synthetic growth medium (SGM) with the following ingredients (in mg·L^{-1}): NaCl (7), $CaCl_2$ (4), $MgSO_4$•$7H_2O$ (2), K_2HPO_4 (217), KH_2PO_4 (8.5), Na_2HPO_4 (33.4), NH_4Cl (191) and incubated under 90 µmol photon·m^{-2} s^{-1} light intensity and stirred at 122 rpm at 29 ± 2 °C for 6 d.

Quantifying microalgae cells

In each experiment for each sampling time, and each experimental replicate, three beads per flask were solubilized in 1 mL of 4% $NaHCO_3$ solution for 20 min at ambient temperature (25 ± 4°C). *C. sorokiniana* cells were counted under a light microscope, using a Neubauer hemocytometer (Bright line counting chamber, Hausser Scientific, Horsham, PA) connected to an image analyser (Image ProPlus 6.3, Media Cybernetics, Silver Spring, MD). Growth rate of *C. sorokiniana* (µ) was defined as: $\mu = (\ln Nt_1 - \ln Nt_0) / (t_1 - t_0)$, where Nt_1 is the number of cells at sampling time, and Nt_0 is the number of cells at the beginning of the experiment, t_1 is sampling time and t_0 the beginning of the experiment (Oh-Hama and Miyachi, 1992).

Analytical methods

Total carbohydrates

For each replicate of each treatment (n = 15), carbohydrates extraction and analysis were done following Choix *et al*. (2012a). Briefly, 1 g of alginate beads were sampled after 1, 2, 4, and 6 d, washed in distilled water, dried at 80 ◦C for 12 h, and ground with a mortar and pestle, which yielded 10 mg samples. These 10 mg ground samples were re-suspended in 5 mL of H_2SO_4 1 M and sonicated for 4 min at 22.5 kHz with an ultrasonic cell disruptor (Misonix, Farmingdale, NY). Carbohydrates were extracted by acid hydrolysis of the sample at 100ºC for 60 min. The quantification was done by the phenol-sulfuric method adapted to microplate (Dubois *et al*., 1956; Masuko *et al*., 2005).

Total lipid analysis

Lipid extraction followed the method of Leyva *et al*. (2014), which was developed for microalgae and based on Bligh and Dyer (1959). Quantification of total lipids was done as previously described (Pande *et al*., 1963).

Pigment analysis

Extraction of pigments (chlorophyll *a* and *b*, violaxanthin, and lutein) was performed with HPLC grade methanol, after dissolving the beads. Identification of pigments used the HPLC method (Vidussi *et al*., 1996). The HPLC system (Agilent 1100; Agilent Technologies, Santa Clara, CA) was equipped with a reverse phase column (Hypersil BDS C8, 5 μm particle size, 100×45 mm; Thermo Scientific, Waltham, MA) and was run isocratically. The mobile phase included two solutions. Solution A was a mixture of 70:30 methanol:1N ammonium acetate. Solution B was 100% HPLC grade methanol. Injection volume was 20 μL; flow rate was 0.5 $mL \cdot min^{-1}$. The detector was a diode array with a wavelength of 190–900 nm, with the capacity to detect at five

fixed wavelengths. Identification criteria for pigments were retention time of the standard pigments (International Agency for ^{14}C determinations, Denmark, DHI-Denmark, PPS-CHLA) and absorbance at 350–750 nm from the diode detector. Results are expressed as $\mu g \cdot g^{-1}$.

Fluorescent in situ hybridization (FISH)

The FISH procedure described by Palacios *et al.* (2019), was used to analyze the patterns of cell aggregation during the interaction of *C. sorokiniana* and *A. brasilense* strains. Approximately 100 images were acquired with a digital camera (Evolution FV Cooled Color; Media Cybernetics). Five random images per treatment were used for quantification and measurement of clusters with an Image ProPlus 4.5 software (Media Cybernetics).

Experimental design and statistical analyses

Two types of experiments were conducted. (a) *In vitro* characterization of the two bacteria, strains were performed in batch culture in four replicates. Each experiment was independently repeated three times. (b) The setup of the experiments of microalgae-bacteria was by batch culturing in Erlenmeyer flasks in five replicates, where one flask served as a replicate. Each of these experiments was independently repeated two times. Each experimental setup contained the following treatments in beads containing: (1) *A. brasilense* Az39, (2) *A. brasilense* Az39Δ*hcp-E*, (3) *C. sorokiniana*, (4) co-immobilization of *C. sorokiniana* with *A. brasilense* Az39, (5) co-immobilization of *C. sorokiniana* with the mutant strain Az39Δ*hcp-E* (n = 20). The results from each treatment from two repetitions were combined. For characterization of the mutant strain, each pair of values at every sampling time were analyzed by Student's *t*-test. For analysis of the effect of *A. brasilense* strains, the data were first analyzed by one-way ANOVA and then by HSD

Tukey's test. Significance was set at $P < 0.05$, using Statistica 6.0 (Tibco Software, Palo Alto, CA), Prism 5.0 (GraphPad Software, La Jolla, CA), and Sigmaplot 13.0 (Systat Software, Chicago, IL).

Disclosure Statement

No potential conflict of interest was reported by the authors.

Author's contribution

Fabricio D. Cassan. Envisioned the project with Luz de-Bashan. Managed the construction of the mutant and its testing in Argentina. Helped with the writing of the manuscript.

Anahí Coniglio. Measured bacterial growth, IAA concentration, biofilm formation and performed swimming and swarming experiments.

Edgar Amavizca. Performed all experiments and FISH analysis related to microalgae-bacteria interaction.

Guillermo Maroniche. Constructed the mutant.

Eric Cascales. Edited the final version.

Yoav Bashan. General supervision of the microalgae-bacteria studies in Mexico. Wrote the initial draft of the manuscript.

Luz E. de-Bashan. Envisioned the project with Fabricio Cassan. Supervised the Mexican part of the project on microalgae-bacteria interaction. Edited the final version.

All authors read and approved the final version of the manuscript.

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Table 1. Aggregation percentage of *A. brasilense* Az39 wild-type and its isogenic Az39Δ*hcp-E* mutant.

Cell aggregation was measured after growth for 48 h in LB or MMAB culture medium. The reduction of the aggregation percentage in the Az39Δ*hcp-E* relative to its parental Az39 strain is indicated below.
* Different letters differ significantly at P<0.05 (Tukey's post-hoc test).

Strain	Aggregation percentage	
	LB	MMAB
A. brasilense Az39	13.2 ± 2.6 c	26.5 ± 1.4 a
A. brasilense Az39Δ*hcp-E*	8.5 ± 1.1 d	18.2 ± 0.9 b
Reduction of aggregation activity (Az39/Az39Δ*hcp-E*)	35.6%	31.3%

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Figure legends

Fig. 1. Schematic representation of the *Azospirillum brasilense* Az39 T6SS1 gene cluster. T6SS core components are shown in green, while accessory genes are shown in white. Potential effector and immunity genes are shown in blue. The position of the *hcp* and *tssE* gene deletion used in this work is indicated

Fig. 2. Bacterial production of auxins. Expressed as concentration of IAA-like molecules ($\mu g \cdot mL^{-1}$) in MMAB cultures of *A. brasilense* Az39 (●) and Az39Δ*hcp*-E mutant (■). Pair of values at each sampling time sharing the same letter do not differ significantly from each other by Student's *t*-test at $P < 0.05$.

Fig. 3. Swimming and swarming motility, biofilm formation and cell aggregation. Swimming (a) and swarming (b) rates, of *A. brasilense* Az39 and its isogenic Az39Δ*hcp*-E mutant after 72 h incubation at 28 ± 2°C in MMAB or Swim media. The bars represent the mean and SD of growth in diameter (cm) of the swimming and swarming displacement halos, respectively. The percentage of the difference between the mutant and its parental strain is shown on top of the bars. (c) Biofilm production of Az39 and Az39Δ*hcp*-E after 96-h incubation at 28 ± 2°C in LB medium. The bars represent the mean and standard deviation of absorbance at OD_{560nm}. The percentage of the difference between the mutant and its parental strain is shown on top of the bars. (d) Cell aggregation was measured after growth for 48 h in LB or MMAB culture medium. The reduction of the aggregation percentage in the mutant relative to its parental strain is indicated on top of the

bars. Each pair of columns denoted by a different letter differs significantly from each other by Student's *t*-test at $P < 0.05$.

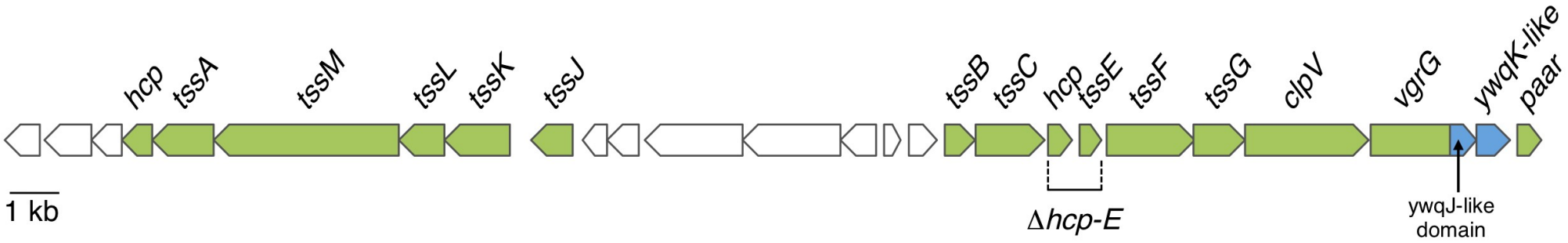
Fig. 4. Antibacterial competition assay. The indicated nalidixic acid-resistant strains were mixed with wild-type or Δ*hcp-E A. brasilense* attacker cells in presence or not of the T6SS gene cluster inducer IAA. The number of surviving recipient cells, counted on nalidix acid plates, is indicated (expressed as $\log^{10}$ of CFU).

Fig. 5. **Effect of co-immobilization of *A. brasilense* strains on *C. sorokiniana* growth and cell aggregation.** (a) The *C. sorokiniana* cell concentration (expressed as 10^5 cells.mL^{-1}) from cultures of the microalgae alone (○) or grown in presence of *A. brasilense* wild-type (■) or Δ*hcp-E* mutant (▲) cells is plotted against time (in days). Cell concentrations along curves having different capital letters differ significantly, using one-way ANOVA and Tukey's post hoc analysis at $P < 0.05$. Cell concentrations at each sampling time that are denoted by different lower-case letters differ significantly, using one-way ANOVA and Tukey's post hoc analysis at $P < 0.05$. Whisker lines represent standard errors (SE). Absence of a whisker line indicates negligible SE. μ = growth rate. (b) Cell aggregation was observed by fluorescence in situ hybridization (FISH) at time 0, 1, 2 our 4 days. (a-d) *A. brasilense* Az39 and *C. sorokiniana* 2714. (e-h) *A. brasilens*e Az39Δ*hcp-E* and *C. sorokiniana* 2714. (i-l) *A. brasilense* Az39 alone. (m-p) *A. brasilense* Az39Δ*hcp-E* alone. (q-t) *C. sorokiniana* alone. All micrographs were taken by epifluorescence microscopy, *C. sorokiniana* was not labeled and appeared in red to orange color, while bacterial strains were labeled with specific probes targeting eubacteria (EUB338Mix FITC) and specific probe for *A. brasilense* (Abras 1420 Cy3) appeared in green to yellow. (c) Size of aggregates created during the

interaction, measured from the results obtained by fluorescence *in situ* hybridization (FISH). Treatments analyzed at each hour denoted by different lowercase letter differ significantly at $P < 0.05$ in one-way analysis of variance, according to Tukey's post hoc analysis. Different capital letter at each day of incubation differs significantly at $P < 0.05$ in Tukey's post hoc analysis.

Fig. 6. Effect of *A. brasilense* co-immobilization on *C. sorokiniana* carbohydrates, lipids, and photosynthetic pigments production. Effect of co-immobilization of wild-type *A. brasilense* Az39 and its isogenic Az39Δ*hcp-E* mutant on accumulation of (a) carbohydrates, (b) total lipids, (c) chlorophyll *a*, (d) chlorophyll *b*, (e) violaxanthin, and (f) lutein in *Chlorella sorokiniana*. Significant differences (one-way ANOVA and Fisher's post hoc analysis at $P < 0.05$) are indicated by different capital letters on top of each columns. Whisker lines represent SE.

Fig. 7. Schematic representation of the roles of *A. brasilense* T6SS1. The *A. brasilense* T6SS1 is involved in *Azospirillum*/microalgae, *Azospirillum*/*Azospirillum* and *Azospirillum*/bacteria interactions. *Azospirillum*/microalgae interaction: the *A. brasilense* (orange cells) T6SS is required for efficient attachment to the microalgae *C. sorokiniana* (green cell) and participates to the increase of the microalgae population and carbohydrates and photosynthetic pigments production. *Azospirillum*/*Azospirillum* interaction: the T6SS is required for biofilm formation and indole-3-acetic acid (IAA, purple triangles) production. IAA positively regulates the expression of the *A. brasilense* T6SS1 gene cluster. *Azospirillum*/bacteria interactions: *A. brasilense* uses its T6SS to eliminate bacteria sharing the same niche (blue cell). By targeting plant pathogens, it confers protection to plants and microalgae.


hcp
tssA
tssM
tssL
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tssB
tssC
hcp
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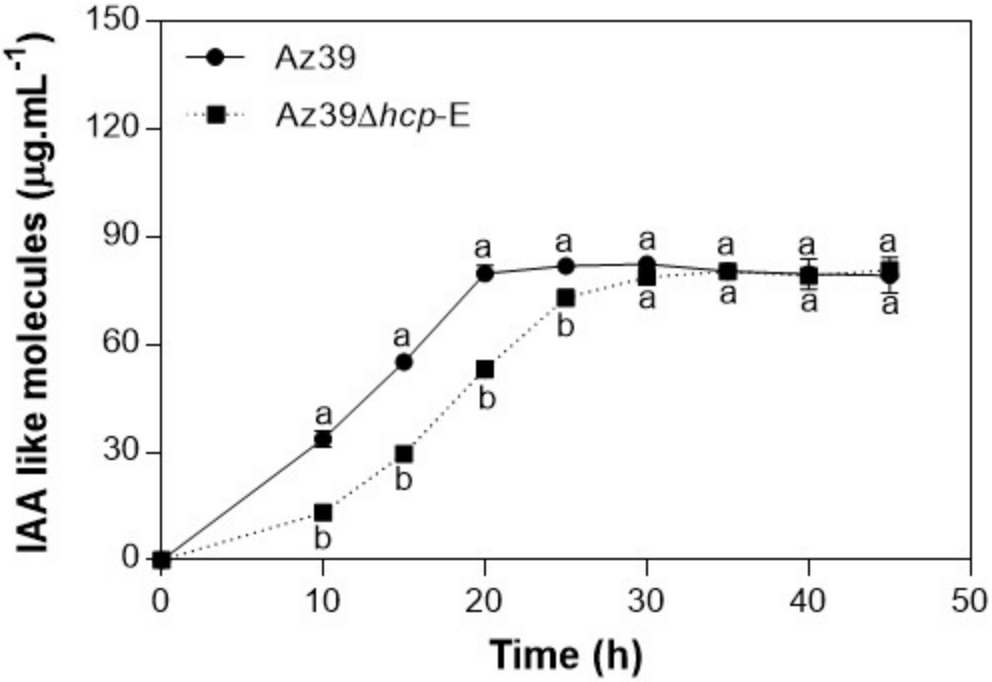
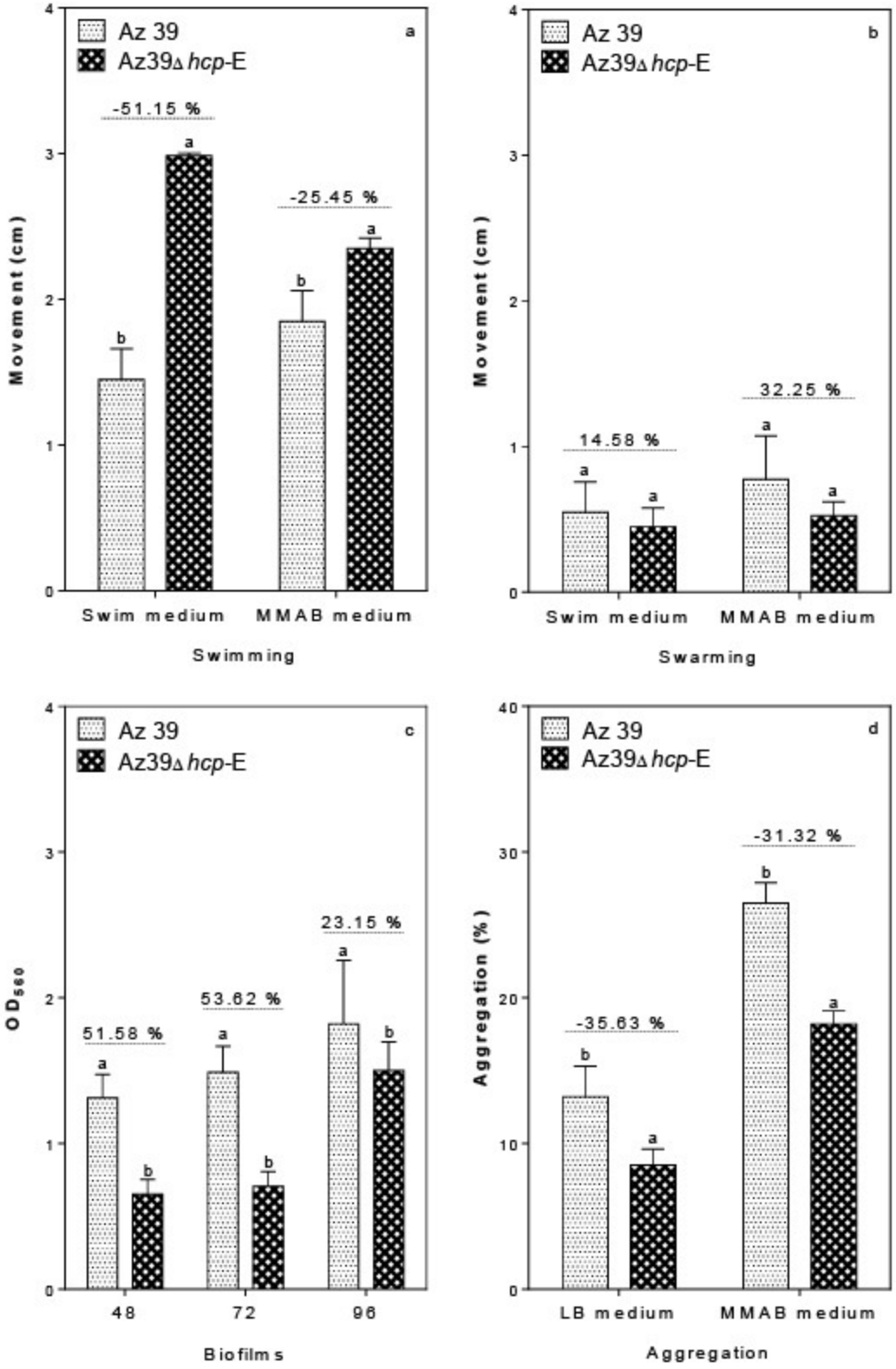
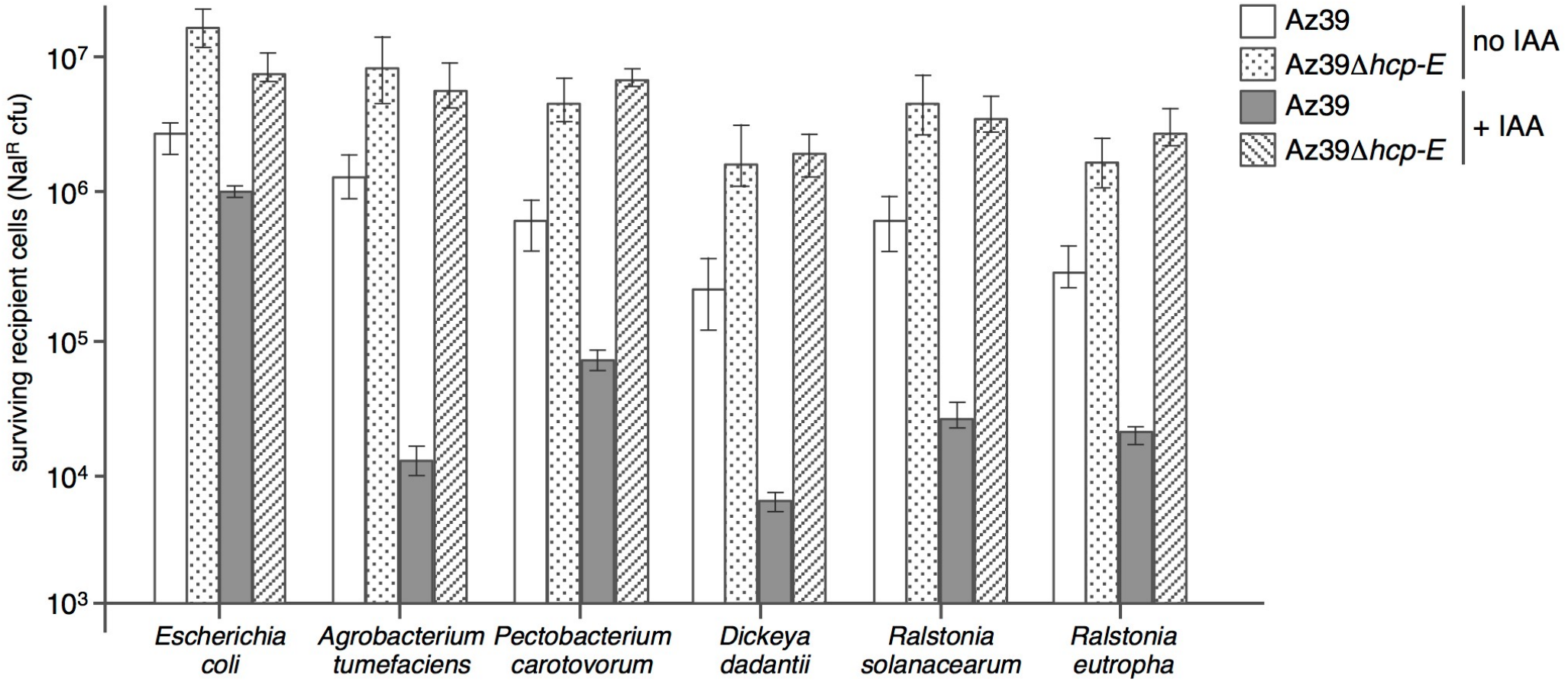
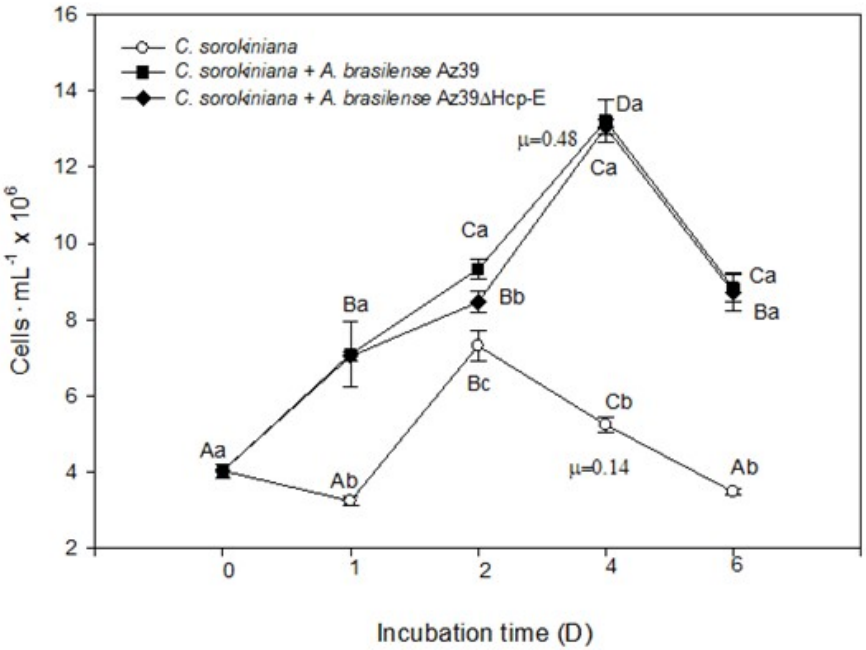
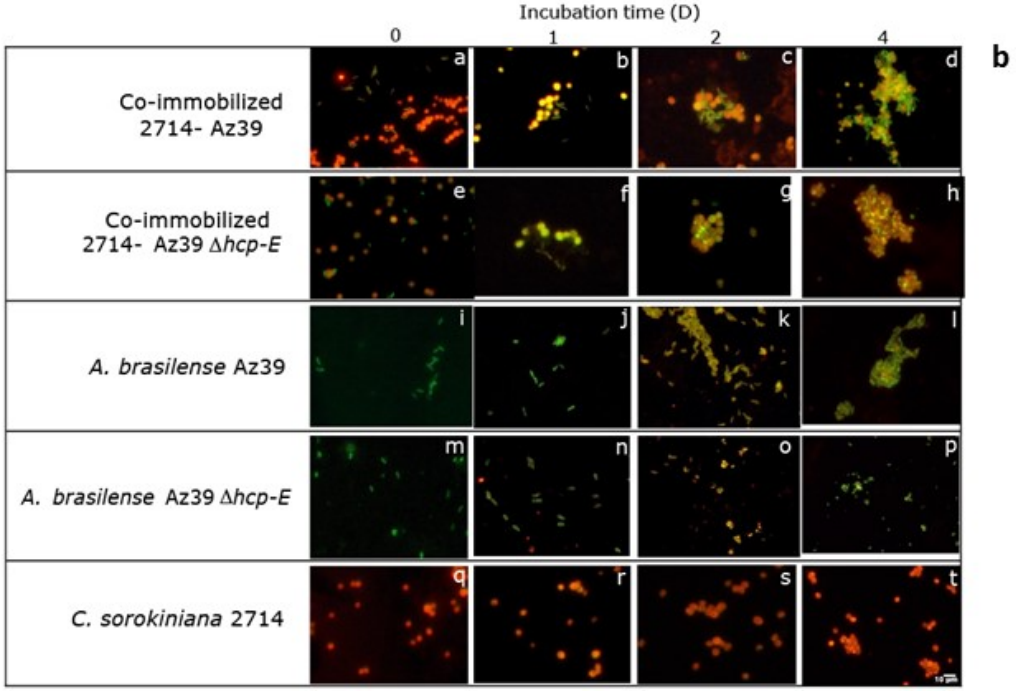
Δhcp-E
ywqJ-like
domain


IAA like molecules (µg.mL-1)
150
120
90
60
30
0
Az39
Az39Δhcp-E
a
a
a
a
a
a
a
a
a
a
a
a
b
b
b
b
b
b
0
10
20
30
40
50
Time (h)


a
Az 39
Az39Δ hcp-E
-51.15 %
-25.45 %
Movement (cm)
Swim medium
MMAB medium
Swimming
b
14.58 %
32.25 %
Swarming
c
51.58 %
53.62 %
23.15 %
OD560
48
72
96
Biofilms
d
-35.63 %
-31.32 %
Aggregation (%)
LB medium
Aggregation

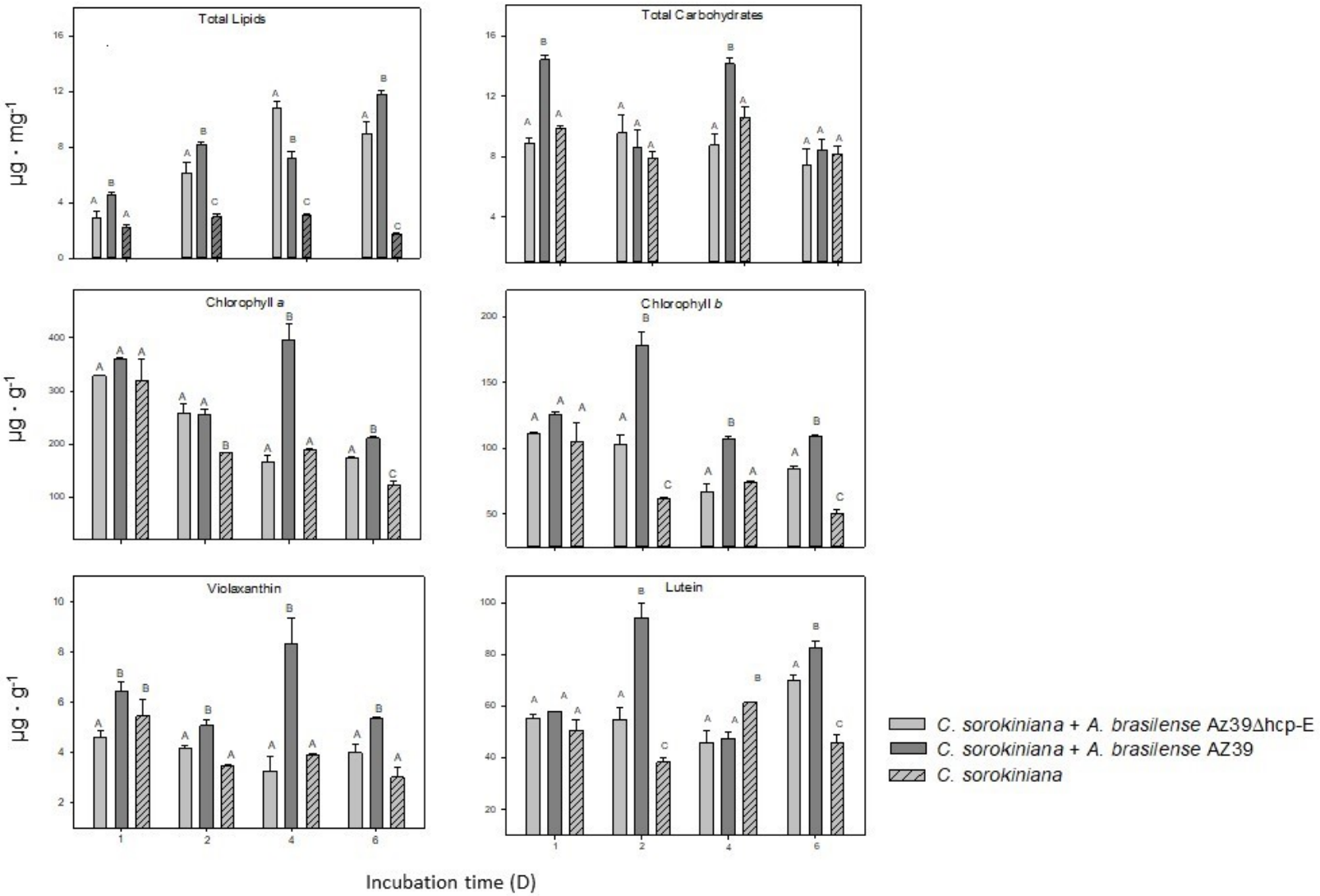

surviving recipient cells (Nal^R cfu)
10^7
10^6
10^5
10^4
10^3
Az39
Az39Δ*hcp-E*
Az39
Az39Δ*hcp-E*
no IAA
+ IAA
Escherichia coli
Agrobacterium tumefaciens
Pectobacterium carotovorum
Dickeya dadantii
Ralstonia solanacearum
Ralstonia eutropha

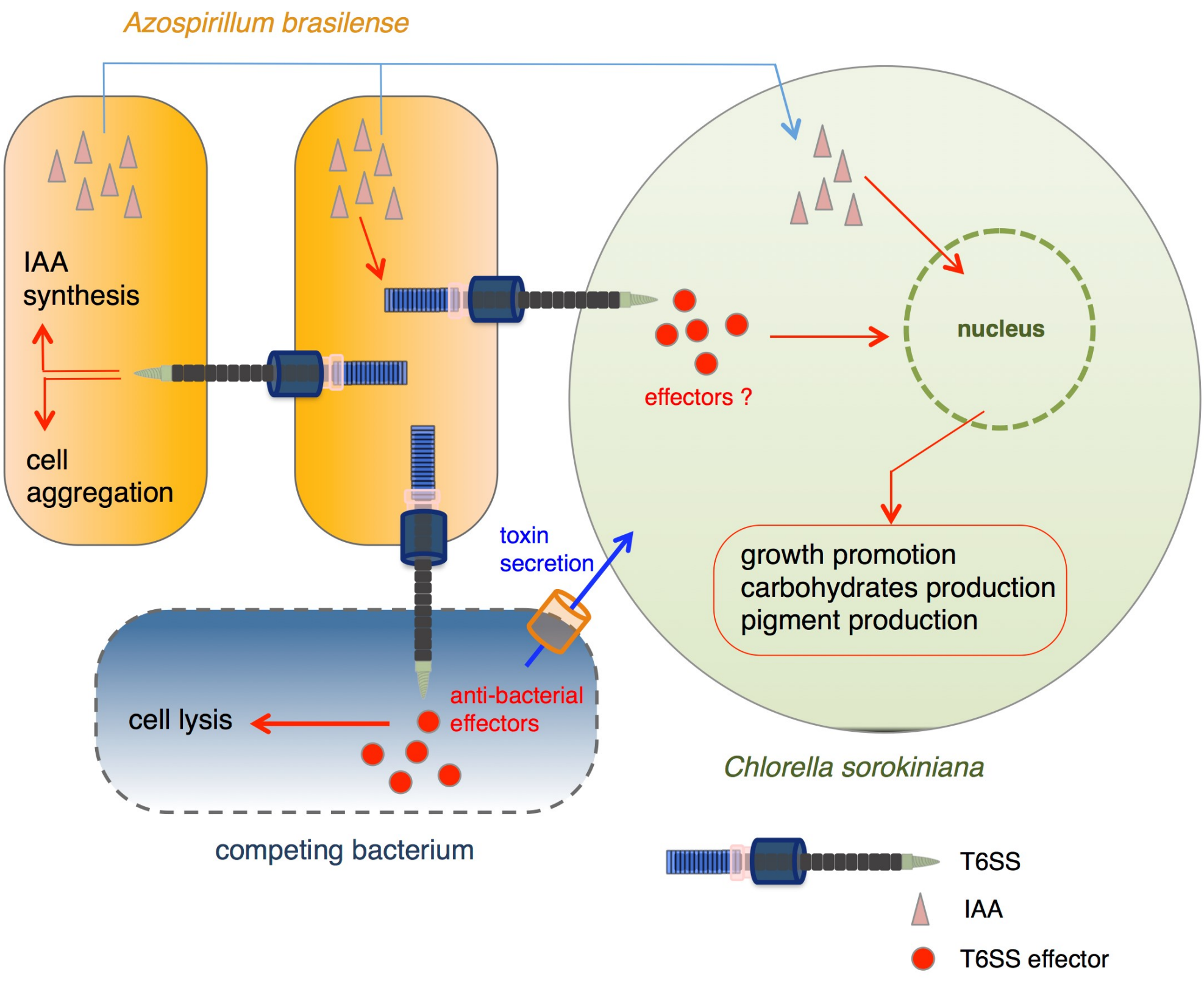




c

Treatments	Aggregate size (μm²) *		
	1 day	2 day	4 day
C. sorokiniana	8.96 ±1.28 aA	44.80 ± 3.91 bA	94.27 ± 7.37 cA
***C. sorokiniana* + *A. brasilense* Az39**	16.99 ±1.70 aC	42.31 ± 8.49 bA	170.44 ± 24.01 cB
C. sorokiniana* + *A. brasilense* Az39Δ*hcp-E	11.22 ± 0.68 aA	39.50 ± 2.28 bA	121.89 ± 2.89 cC
***A. brasilense* Az39**	4.13 ± 0.67 aB	26.40 ± 1.75 bB	36.80 ± 2.29 cD
A. brasilense* Az39Δ*hcp-E	3.41 ± 0.53 aB	16.00 ± 1.22 bC	19.50 ± 2.42 bE


Total Lipids
Total Carbohydrates
Chlorophyll a
Chlorophyll b
Violaxanthin
Lutein
µg · mg-1
µg · g-1
Incubation time (D)
C. sorokiniana + A. brasilense Az39Δhcp-E
C. sorokiniana + A. brasilense AZ39
C. sorokiniana


Azospirillum brasilense
IAA synthesis
cell aggregation
effectors ?
nucleus
growth promotion
carbohydrates production
pigment production
toxin secretion
anti-bacterial effectors
cell lysis
competing bacterium
Chlorella sorokiniana
T6SS
IAA
T6SS effector