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Interspecific cooperation: enhanced growth, attachment and strain-specific distribution in biofilms through *Azospirillum brasilense-Pseudomonas protegens* co-cultivation

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One sentence summary: Cooperative interaction between *Azospirillum brasilense* and *Pseudomonas protegens* enhances growth and produces structured mixed biofilms.

Editor: Kendra Rumbaugh

ABSTRACT

Plant-growth-promoting bacteria belonging to *Azospirillum* and *Pseudomonas* genera are major inhabitants of the rhizosphere. Both are increasingly commercialized as crops inoculants. Interspecific interaction in the rhizosphere is critical for inoculants aptness. The objective of this work was to evaluate *Azospirillum* and *Pseudomonas* interaction in mixed biofilms by co-cultivation of the model strains *Azospirillum brasilense* Sp245 and *Pseudomonas protegens* CHA0. The results revealed enhanced growth of both strains when co-cultured in static conditions. Moreover, Sp245 biofilm formed in plastic surfaces was increased 2-fold in the presence of CHA0. Confocal microscopy revealed highly structured mixed biofilms showing Sp245 mainly on the bottom and CHA0 towards the biofilm surface. In addition, *A. brasilense* biofilm was thicker and denser when co-cultured with *P. protegens*. In a colony–colony interaction assay, Sp245 changed nearby CHA0 producing small colony phenotype, which accounts for a diffusible metabolite mediator; though CHA0 spent medium did not affect Sp245 colony phenotype. Altogether, these results point to a cooperative interaction between *A. brasilense* Sp245 and *P. protegens* CHA0 in which both strains increase their static growth and produce structured mixed biofilms with a strain-specific distribution.

Keywords: plant-growth-promoting-bacteria; biofilm structure; colony phenotype

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INTRODUCTION

The rhizosphere constitutes a complex and dynamic environment. As an outcome of co-evolution, interaction between rhizospheric microorganisms involves morphological changes and adjustments in secondary metabolism (Philippot *et al.* 2013). In consequence, the fitness of bacteria is largely determined by their capabilities to bear up secondary metabolites released by other rhizospheric organisms.

Biofilms, defined as matrix-enclosed bacterial population adhered to each other and/or to surfaces, constitute a microniche where the exchange of metabolites between bacterial populations is favored. The biofilm mode of growth has several advantages for soil bacteria compared to the planktonic lifestyle. Biofilm provides protection against desiccation and toxic compounds. Furthermore, an organization of multispecies bacterial consortia into biofilms may enable the coexistence of species that would otherwise outcompete each other and facilitate synergistic interactions and gene transfer (for reviews, see Burmølle, Hansen and Sørensen 2007; Karatan and Watnick 2009; López, Vlamakis and Kolter 2010; Karunakaran *et al.* 2011). Recent findings suggest that when both competitive and cooperative genes are transferred, cooperators are favored because their transfer increases allelic assortment (Dimitriu *et al.* 2014).

Plant-growth-promoting rhizobacteria (PGPR) enhance growth of many terrestrial crop plants by a wide variety of mechanisms (Lugtenberg and Kamilova 2009; Hayat *et al.* 2010; Bhattacharyya and Jha 2012). *Azospirillum brasilense* improves crop growth mainly by a direct effect on the plant, through the production of several phytohormones such as indole acetic acid (Tien, Gaskins and Hubbell 1979; Ona *et al.* 2005) and nitric oxide (Creus *et al.* 2005; Molina-Favero *et al.* 2008). Conversely, fluorescent pseudomonads, other major inhabitants of the rhizosphere, display mostly indirect beneficial effects on plant growth (Kloepper *et al.* 1980). *Pseudomonas* strains produce a wide range of compounds with antimicrobial activity, being 2,4-diacetylphloroglucinol (DAPG) one of the most studied (Keel *et al.* 1990). These PGPR are currently commercialized as inoculants containing single or combined strains (i.e. *A. brasilense* and *Pseudomonas fluorescens*) (Bhattacharyya and Jha 2012). PGPR efficiency depends considerably on their capabilities to survive and establish effective root colonization. Therefore, interaction with other bacteria in the rhizosphere constitutes a critical bottleneck in PGPR aptness. An important concern is that pseudomonads may secrete antibiotics that can negatively affect *A. brasilense* proliferation in mixed inoculants (Combes-Meynet *et al.* 2010; Couillerot *et al.* 2011). It has been reported that, *in vitro*, *A. brasilense* Cd strain was sensitive to the addition of the synthetic antimicrobial metabolite DAPG, which induced carotenoids accumulation, formation of poly-β-hydroxybutyrate-like granules, cytoplasmic membrane damage and growth inhibition (Couillerot *et al.* 2011). The major rhizosphere inhabitants, *A. brasilense* and fluorescent pseudomonads, are expected to be found in mixed consortia in nature. Therefore, studying their interaction is of particular interest for agronomical applications in inoculants technology. The aim of this work was to assess whether interspecific interaction between the models strains *A. brasilense* Sp245 and *P. protegens* CHA0 results in altered static growth, attachment to polystyrene surfaces and strain distribution within mixed biofilms.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Fluorescent derivatives of strains *Azospirillum brasilense* Sp245 and *Pseudomonas protegens* CHA0 were obtained by introducing plasmid pMP2444 (Gm^R, P_{lac}-*egfp*) into Sp245 by biparental mating (Arruebarrena Di Palma *et al.* 2013) or plasmid pME7134 (Tc^R, P_{lac}-*dsRed*) by electroporation. Plasmid pME7134 was constructed as follows. A 0.7-kb *Sma*I-*Hind*III fragment from pDsRed.T3_S4T containing the *dsred*.T4_S4T gene (Sörensen *et al.* 2003) was cloned under the control of the P_{lac} promoter into pME6552 (Wenner *et al.* 2014). Following this, a 1.5-kb *Mlu*I-*Hind*III fragment from the resulting plasmid containing the P_{lac}-*dsred*.T3_S4T fusion was subcloned into pME6031 (Heeb *et al.* 2000) to give pME7134. This reporter vector provides bright red fluorescence and can be used in a wide variety of Gram-negative bacteria.

Starter single-species cultures of *A. brasilense* Sp245/pMP2444 and *P. protegens* CHA0/pME7134 were grown in Luria-Bertani medium (LB) (Sambrook, Fritsch and Maniatis 1989) at 30°C for 18 h with orbital shaking (100 rpm). When required, final antibiotic concentrations were Gm 25 μg mL^{-1} for *A. brasilense* and Tc 125 μg mL^{-1} for *P. protegens*. For growth, biofilm and colony morphology assays, Nfb-NO_3^--iron-enriched media (Nfb-Fe-NO_3^-) was used according to Döbereiner and Day (1976) with the following modifications: 27.6 mM malic acid, 13.8 mM KNO_3, 0.17 mM $FeCl_3$ and 0.015 mM Fe-EDTA. For preparation of stationary-phase cell-free media, bacteria were grown overnight or for 3 days (overgrown culture) in Nfb-Fe-NO_3^- without antibiotics, pelleted and the supernatant were filter-sterilized (0.22 μm pore size).

Bacterial growth and biofilm quantification

Static growth of Sp245/pMP2444 and CHA0/pME7134 was analyzed in axenic conditions on polystyrene microtiter 96 flat bottom-well plates. Single cultures were initiated with 200 μL of fresh Nfb-Fe-NO_3^- medium containing 10^5 cells mL^{-1} based on optical density (OD_{600}; Spectra MR; Dynex Technologies, 4340 Sullyfield Circle, Chantilly, VA, USA). Mixed cultures were initiated with 5×10^4 cells of each strain in a total volume of 200 μL in order to reach a final concentration of 10^5 total cells mL^{-1}. To test the effect of CHA0 cell-free spent medium on the growth of Sp245, 100 μL of Nfb-Fe-NO_3^- cell filtered media were added to 100 μL of fresh medium containing 5×10^4 Sp245 cells mL^{-1}. The opposite combination was also tested. When necessary, different combinations of bacterial densities were tested varying the initial number of Sp245 cells: 10^4, 10^5 or 10^6 but keeping 10^5 cells of CHA0. Plates were statically incubated for 2 days at 30°C. For total cell growth quantification, biofilms developed in the wells were vigorously pipetted with a sterile tip for mechanical disaggregation and mixing with planktonic cells. OD_{600} and fluorescence (ABI 7500 in fluorimeter mode: Ex: 538 nm, Em: 605 nm for CHA0/pME7134 and Ex: 460, Em: 525 nm for Sp245/pMP2444) were registered. Cells in the biofilm were quantified in the same way after discarding planktonic cells of paralleled wells. Percentage of biofilm-associated cells was calculated as biofilm-associated cells/total cells × 100. Biofilms formed in polystyrene paralleled wells were also quantified by crystal violet dye staining (OD_{550}) and normalized to total bacterial growth (OD_{600}) as previously described (Arruebarrena Di Palma *et al.* 2013).

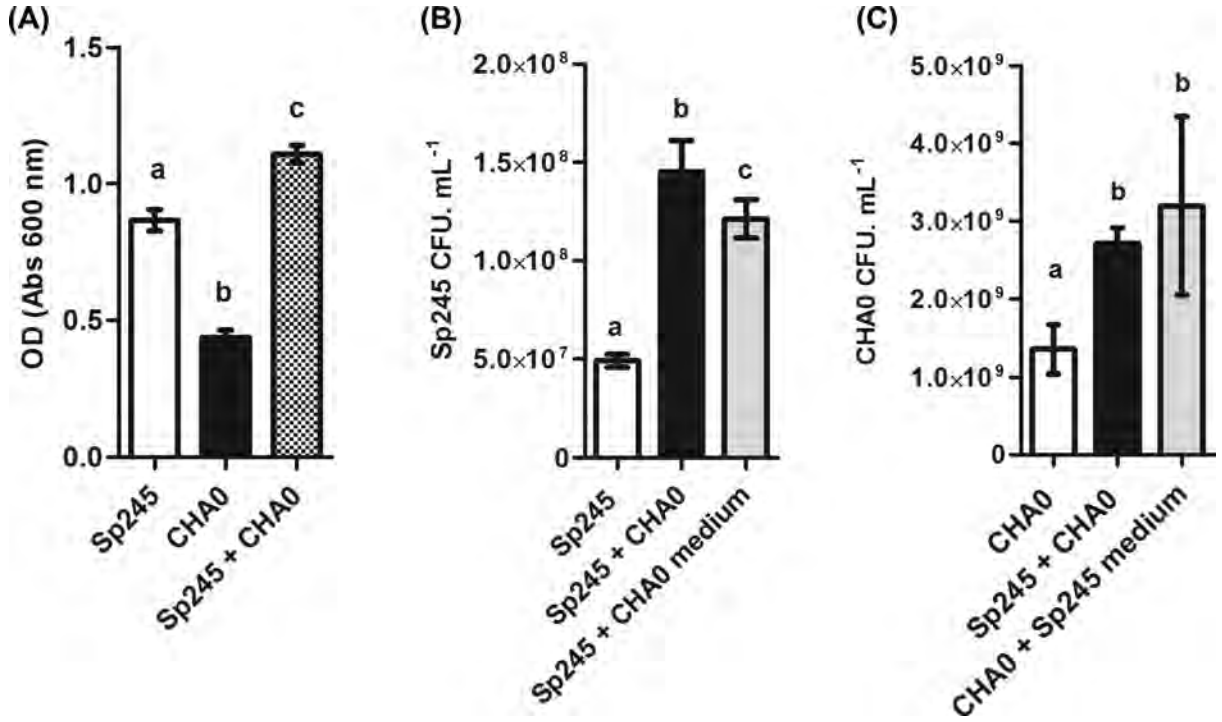

Figure 1. Static co-cultivation enhances growth of *A. brasilense* Sp245 and *P. protegens* CHA0 strains. Total growth (planktonic plus attached cells after disaggregation) of *A. brasilense* Sp245 and *P. protegens* CHA0 strains was analyzed after 2 days of static culture in Nfb-Fe-NO_3^- at 30°C in 96 wells plates. Single cultures were initiated with 10^5 cells and mixed cultures with 5×10^4 cells of each strain. Optical density at 600 nm (**A**) and fluorescence of Sp245 (GFP) (**B**) and CHA0 (dsRed) (**C**) were registered. CFU mL^{-1} was obtained from linear regression with fluorescence values for each strain (Fig. S1). Values are means ± SE of three to nine independent experiments, with three wells replicates each. Results were statistically analyzed by ANOVA and Tukey's Multiple Comparison posttests. Different letters indicate significant differences ($P < 0.05$).

The experimental design consisted of three to nine replicated plates depending on the measured variable, in which three independent starter cultures were each sown in triplicate wells. Results were statistically analyzed by ANOVA and Tukey test. Correspondence between the fluorescence values and the CFU mL^{-1} of bacterial suspensions was determined by linear regression for each strain (Sp245/pMP2444 R square: 0.9869 and CHA0/pME7134 R square: 0.9855; Graphpad Prism 5.03 software). Then, CFU mL^{-1} values were indirectly estimated by measuring fluorescence and interpolating values from the curve (Fig. S1, Supporting Information). In order to ensure the correspondence of fluorescent measurements to CFU mL^{-1}, CFU counts in total and biofilm fractions were analyzed by the drop method on Nfb-Fe-NO_3^- medium supplemented with 25 μg mL^{-1} Gm or 125 μg mL^{-1} Tc for Sp245/pMP2444 or CHA0/pME7134, respectively. Controls to evaluate the level of fluorophore crosstalk were done by registering values of fluorescence (red: Ex: 538 nm Em: 605 nm and green: Ex: 460 nm Em: 525 nm) for different concentrations of CHA0/pME7134. A regression curve between green and red fluorescence (R^2: 0.9905) was done. Correction of crosstalk was made by subtracting the value of green fluorescence obtained from the curve to the fluorescence measured in co-cultures.

Colony morphology experiments

Suspensions of Sp245 and CHA0 obtained from overnight cultures in LB with agitation (100 rpm) were adjusted to 10^8 cells mL^{-1} and 10^9 cells mL^{-1}, respectively. Serial dilutions of each strain were spotted onto Nfb-Fe-NO_3^- agar plates that had been previously inoculated with 1 mL of either CHA0 or Sp245 lawn, or 1 mL of CHA0 or Sp245 cell-free culture filtrate (spent medium).

For the drop assay, 1000 CFUs of Sp245 were spread onto RC agar plate and 10 μL of a suspension of CHA0 (10^9 cells mL^{-1}) or Sp245 (10^8 cells mL^{-1}) were spotted at the center of the plate, and incubated for 5 days at 30°C. When required, plates were incubated for 23 days.

Confocal microscopy

Polystyrene covered slides were placed in test tubes containing 3 mL Nfb-Fe-NO_3^- with 10^5 Sp245/pMP2444 cells, 10^5 CHA0/pME7134 cells or mixed cultures, and statically incubated for 2 days at 30°C. Slides were removed, smoothly washed and the biofilms formed in the liquid–air interface zone were directly observed with a confocal laser scanning microscope Nikon C1, using 40.0x/1.30/0.22 Oil spring-loaded lens. GFP and dsRed proteins were excited at 488 and 543 nm, and detected at 550–650 nm and 650–750 nm, respectively. Images were analyzed with Nikon EZ-C1 Freeviewer.

RESULTS

Bacterial growth is enhanced in static mixed cultures

Interspecific relationships between *Azospirillum brasilense* and *Pseudomonas protegens* were evaluated in single and mixed-species static cultures of Sp245 and CHA0 strains carrying the plasmids pMP2444 (*Gm*^{r}, P_{lac}-*egfp*) and pME7134 (*Tc*^{r}, P_{lac}-*dsRed*), respectively. After 2 days of cultivation, overall bacterial growth was greater when both species grew together (Fig. 1A). Moreover, fluorescence measurements revealed that Sp245 and CHA0 cell numbers exhibited a 3- and 2-fold increase, respectively, when

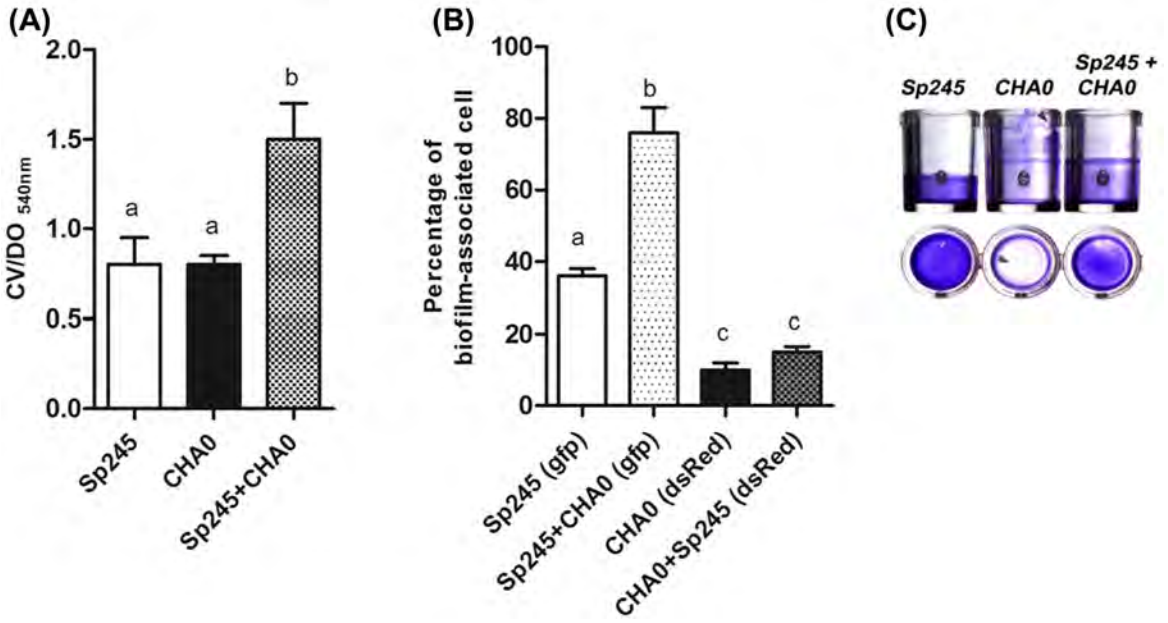

Figure 2. Co-cultivation enhances *A. brasilense* Sp245 biofilm. Biofilm of *A. brasilense* Sp245 and *P. protegens* CHA0 strains was monitored after 2 days of static culture in Nfb-Fe-NO_3^- at 30°C in 96 wells plates. Crystal violet staining of the biofilm was quantified and normalized by the total cell growth (OD550 nm/OD600 nm (**A**). Fluorescence of disaggregated cells was also determined and normalized as percentage of biofilm-associated cells (**B**). Biofilm phenotypes are shown for each condition (**C**). Values are means ± SE of three to nine independent experiments, with wells replicates each. Results were statistically analyzed by ANOVA and Tukey's Multiple Comparison posttests. Different letters indicate significant differences ($P < 0.05$).

co-cultured (Fig. 1B and C). This was highly dependent on the initial number of cells from each strain. If the initial inoculum of Sp245 was 10^6 cells mL^{-1}, the promoting effect on growth was not significant for neither of the species (Fig. S3, Supporting Information). Moreover, CHA0 cell-free spent media was sufficient to exert Sp245 growth promotion, suggesting that a soluble factor released by CHA0 was responsible for Sp245 growth stimulation. Furthermore, Sp245 cell-free stationary-phase supernatants were also able to enhance CHA0 growth (Fig. 1C). No pH changes were detected between single and mixed cultures, so a change in media pH was not responsible for this effect (Fig. S2, Supporting Information). These results suggest that a non-direct bacterial interaction between CHA0 and Sp245 is sufficient to improve the growth of both species.

Bacterial biofilm on artificial surfaces

The amount of biofilm in individual cultures of Sp245 or CHA0 was significantly lower than the amount of biofilm produced in co-cultures (Fig. 2A). Noticeably, whereas Sp245 showed a 2-fold increase in the percentage of biofilm formed in mixed cultures, no difference was observed for CHA0 (Fig 2B). This implies that the overall biofilm increase in mixed cultures was due to a higher proportion of Sp245 cells in the biofilm. The fact that these strains produced biofilm without compromising each other's growth (Fig. 1B and C) suggests that they may occupy different spatial niches within the biofilm. As shown in Fig. 2C, mixed biofilm was localized on the entire surface covered by CHA0 (the liquid–air interface) and by Sp245 (deeper on the wall and the base of the well).

Strain distribution within single- and mixed-species biofilms

The structure and strain distribution within the biofilm developed in the air–liquid interface zone was studied on polystyrene covered slides and observed by confocal microscopy. Single-species Z-projections demonstrated that the Sp245 biofilm was thinner than that formed by CHA0 (Fig. 3C and D). This is in agreement with the location of the biofilm ring observed in microplate experiments using crystal violet staining (Fig. 2C). We also observed during washing steps that the Sp245 biofilm was loosely attached to the surface, while CHA0 single-species biofilm was as tightly attached as the mixed biofilm. CHA0 single species biofilm also showed a higher cell density and homogeneity on the layers closer to the slide (not shown) and a sponge-like structure with channels in the upper layers (Fig. 3C). Surprisingly, CHA0 single species biofilm was much thicker than the mixed-species biofilm, reaching a depth of about 600 μm (25 μm for Sp245 and 200 μm for Sp245+CHA0). As it was previously demonstrated, quantification performed on microtiter plates revealed higher levels of bacterial biofilm in mixed than in single-species cultures. This apparent contradiction might be explained by the fact that Sp245 biofilm formed all over the microplate well (surface, walls and bottom) during static co-culture with CHA0, which would account for the observed increase in total biofilm mass (Fig. 2C). Mixed biofilms were multilayered and complex in structure and showed a particular strain-specific distribution within the biofilm. Sp245 cells were mainly placed towards the slide forming the bottom layers of the biofilm, whereas CHA0 cells were located towards the biofilm surface, in contact with the liquid medium (Fig. 3A and B). Nevertheless, a small fraction of Sp245 cells was also located close to the surface (Fig. 3A and B).

Sp245 colony diameter is reduced by CHA0

Sp245 colonies were noticeably smaller nearby a CHA0 spot, whereas there was no difference in Sp245 colony diameter close to the control spot (Fig. 4). The diameter of Sp245 colonies was restored at distances higher than 7.5 mm away from the CHA0 spot. This result suggests that a diffusible metabolite produced

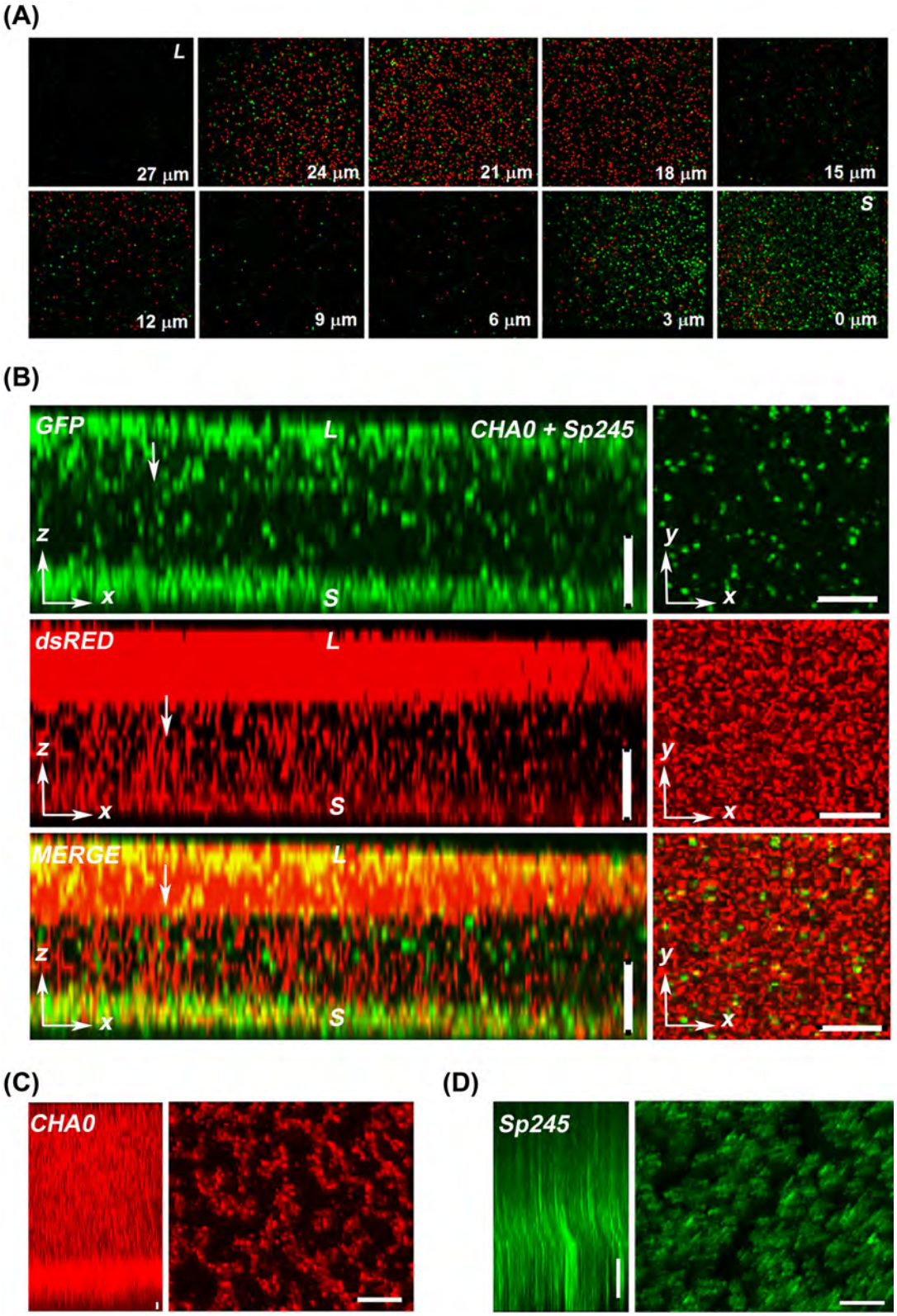

Figure 3. Confocal laser scanning microscopy showing strain distribution within the mixed biofilm located on the air–liquid interface zone. Polystyrene covered slides were placed in 3 mL Nfb-Fe-NO_3^- with 10^5 Sp245 cells, 10^5 CHA0 cells or a combination of both strains and statically cultured for 2 days at 30°C. Fluorescence across the biofilm formed over the slides on the air–liquid interface zone was monitored, reconstruction was done with 3 μm interspace (**A**) and images were analyzed with a Nikon EZ-C1 Freeviewer software. Z-projections (left panels) or XY images (right panels) of Sp245, CHA0 and MERGE images on mixed (**B**) and individual biofilms (**C** and **D**) are shown. White arrows in the z-projection indicate the position of x-y images. Z-projections were reconstructed with 3 μm interspace between images. L: in contact with culture media; S: in contact with slide. Bar scale: 20 μm.

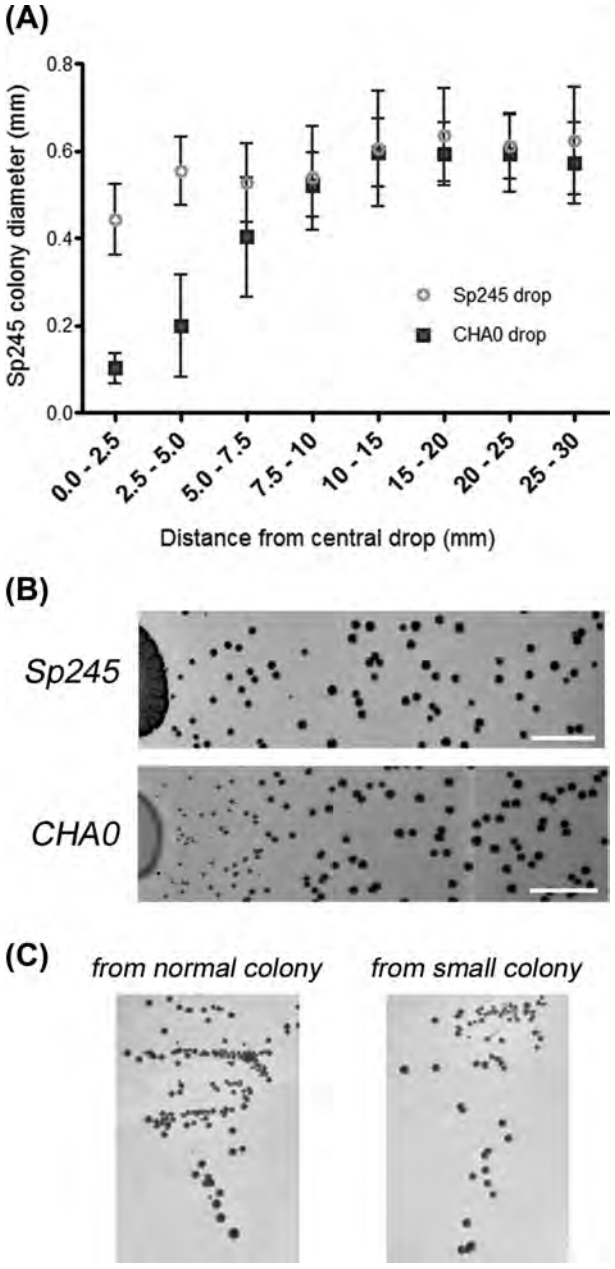

Figure 4. Sp245 colony diameter is reduced by CHA0. A total of 1000 CFUs of Sp245 were spread onto Nfb-Fe-NO_3^- agar plate, and 10 μL of a suspension of CHA0 (10^9 cells mL^{-1}) or Sp245 (108 cells mL^{-1}), was spotted at the center of the plate and incubated for 5 days at 30°C. Diameters of Sp245 colonies were measured (**A**), and colony morphology was analyzed by optical magnifier (**B**). Normal and small colonies phenotypes were streaked on Nbf-Fe-NO_3^- agar plates (**C**). Bar scale: 4 mm.

by CHA0 cells is responsible for the observed phenotype. When plates were cultivated for longer periods (23 days), the small colony phenotype of Sp245 in the proximity of CHA0 spot remained unaltered (data not shown). Small colonies picked from this region of the plate were restreaked onto fresh medium and they displayed a normal sized phenotype (Fig 4C). To determine whether the reduction of Sp245 colony size requires the presence of CHA0 cells, serial dilutions of both strains were placed over a CHA0 or Sp245 strains lawn (1 mL of a 10^6 CFU mL^{-1} suspension), or over their overnight spent cell-free media, and incubated for 3 days at 30°C. Sp245 developed small colonies in the presence of a CHA0 lawn (Fig. 5A and D), while CHA0 colonies remained unaffected when grown over Sp245 lawn (Fig. 5A, inset). However, Sp245 colonies grown in plates with CHA0 cell-free media remained unaffected (Fig. 5A and E). This indicates that the metabolite responsible for the reduction in Sp245 colony size in co-culture with CHA0 in agar plates (Fig. 4A and B) was either absent or in inadequate concentration in this experimental setup. A CHA0 cell-free filtrate from overgrown cultures (3 days, $DO_{600} = 6$) was used to test this possibility; however, Sp245 colonies again exhibited a normal size, discarding a dilution effect (data not shown). Taken together, the results indicate that the development of Sp245 small colonies requires the presence of CHA0 cells in their proximity, or alternatively, a metabolite that is only secreted in cells growing in solid medium and that is induced by the presence of Sp245.

DISCUSSION

PGPR have been studied for their ability to stimulate the growth and health of economically important crops (Lugtenberg and Kamilova 2009; Hayat *et al.* 2010; Beneduzi, Ambrosini and Passaglia 2012; Bhattacharyya and Jha 2012; Drogue *et al.* 2013). However, cell-to-cell communication between PGPR and other rhizosphere-inhabiting microorganisms has received much less attention. Static co-cultivation of strains Sp245 and CHA0 results in a clear interspecific cooperation, as overall growth was enhanced for both species (Fig. 1 and S4, Supporting Information). Moreover, cell-free stationary-phase supernatants from CHA0 and Sp245 cultures were sufficient to exert interspecies growth promotion (Fig. 1C). Bacteria are found to interact synergistically, by either providing nutrients, removing some inhibitory products or stimulating each other through physical or biochemical mechanisms (Burmølle *et al.* 2006). Growth enhancement upon CHA0 and Sp245 interaction can be explained by several mechanisms such as pH changes, exopolysaccharide (EPS) production, microaerophilic conditions and/or release of soluble metabolites that could modify medium redox conditions, among others. However, experiments conducted with spent media (where neither microaerophilic conditions nor EPS production can be determinant factors) also increased interspecies growth (Fig. 1B and C). Thus, these observations support the hypothesis that soluble metabolites released by CHA0 and Sp245 are responsible for this growth stimulation. The secondary metabolite DAPG is one of the most important antimicrobial compounds produced by certain *Pseudomonas* strains and can act as a relevant interspecific signal molecule (Notz *et al.* 2001; Combes-Meynet *et al.* 2010; Yang and Cao 2012; Powers *et al.* 2015). In this regard, subinhibitory concentrations of DAPG promote *A. brasilense* growth *in vitro* (Couillerot *et al.* 2011), suggesting that DAPG can contribute to the observed *A. brasilense* growth promotion. Further experiments with CHA0 strains unable to produce DAPG might be interesting to unravel this issue.

The position of the biofilm in the well was different for both species in single cultures, in agreement with their preferences for O_2 concentration: it was located deep in the well for Sp245, where microaerobiosis is generated, and in the upper part for CHA0, a strictly aerobic microorganism. However, in mixed cultures the biofilm was developed at the same superficial location than for strain CHA0 alone (Fig. 2C). We propose that in co-culture, O_2 consumption by *Pseudomonas* biofilm allowed *Azospirillum* to exploit the generated microaerophilic zones located in the well at an upper location than when cultivated

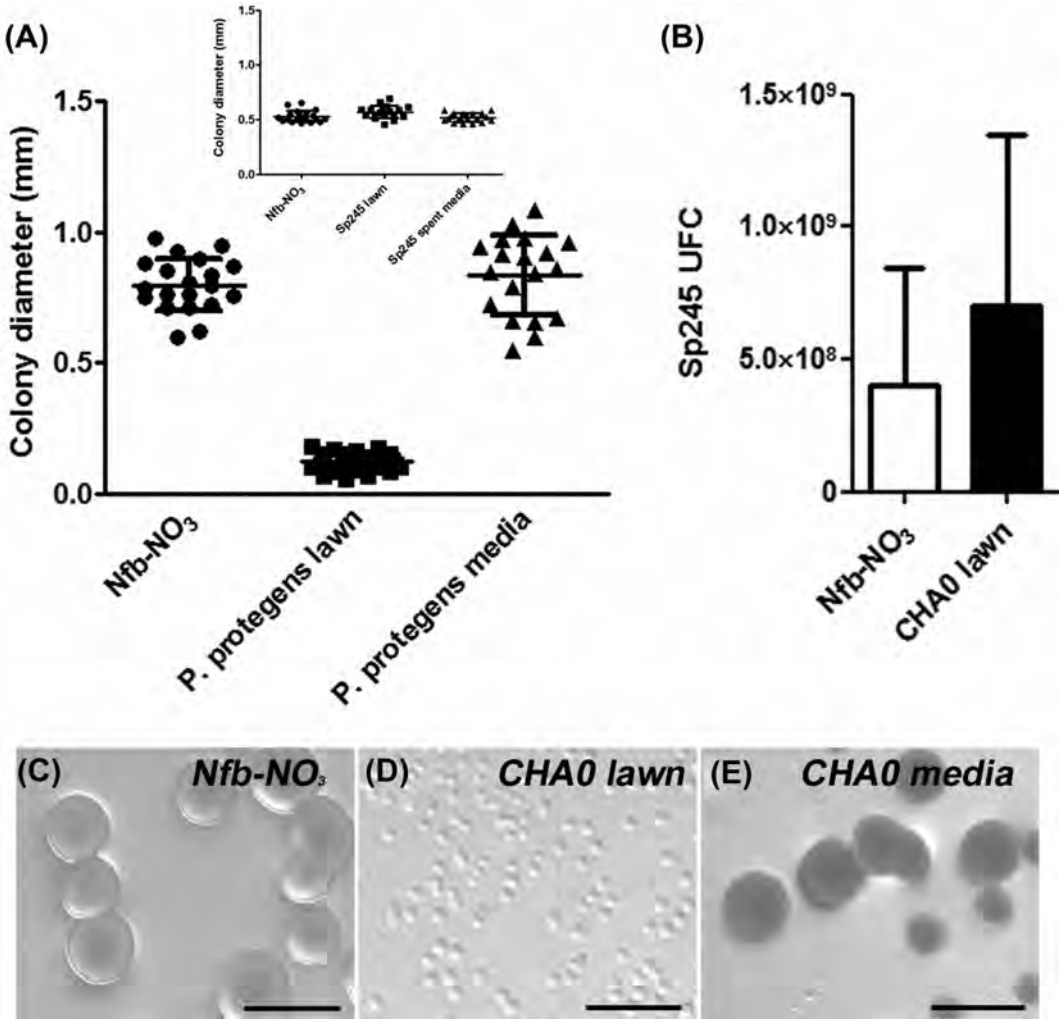

Figure 5. Sp245 colony diameter is not affected by CHA0 stationary-phase supernatants. Serial dilutions of each strain were spotted onto Nfb-Fe-NO_3^- agar plates, over a CHA0 or Sp245 lawn or over cell-free culture filtrates. Sp245 (**A**) or CHA0 (A, inset) colonies diameter were measured, Sp245 CFU mL^{-1} was calculated (**B**) and Sp245 colony morphology was analyzed by optical magnifier (**C–E**). Bar scale: 1 mm.

alone. In line with this hypothesis, confocal images showed multilayered mixed biofilms located on the air–liquid interface zone, with Sp245 strain mainly restricted to the bottom of the biofilm in contact with the polystyrene covered slide, and with CHA0 mainly located in contact with culture medium (Fig. 3A and B). Alternatively, the enhanced attachment of Sp245 in co-cultures might be the result of its co-aggregation with CHA0. *Azospirillum* cells aggregate and flocculate under diverse stress conditions and in the presence of various carbon and nitrogen sources (Burdman *et al.* 1998; Joe *et al.* 2009). The ability of *Azospirillum* to co-aggregate with other species endures its tolerance to desiccation, heat and osmotic shock, at different degrees depending on the identity of the species involved (Joe *et al.* 2009). Recently, Ren *et al.* (2015) showed the prevalence of synergistic effects in biofilm formation among isolates from different soils when co-cultured in combinations of four species. Moreover, absolute individual strain cell numbers were significantly enhanced when compared with those of single-species biofilms, indicating that all the individual strains benefit from inclusion in the multispecies community (Ren *et al.* 2015).

The fact that the *A. brasilense* biofilm is thicker (Fig. 2A) and denser (Fig. 3) when co-cultured with *P. protegens* suggests that this particular interspecific relationship could be an adaptation to complex environments where microbial communities coexist. This response may result in an increased ability of *A. brasilense* to colonize roots in the rhizosphere when both microorganisms are present. On the contrary, the outcome of the interaction depends on the species partner, as it has been reported that biofilms of *Bacillus subtilis* are inhibited by *P. protegens* and *P. putida* (Powers *et al.* 2015). To our knowledge, our work is the first to demonstrate a true collaboration between statically co-cultured *A. brasilense* and *P. protegens* that is reflected not only in an increase in the total cell numbers, but also in a distribution of niche occupancy within an interspecies biofilm.

On the other hand, interaction on agar plates revealed that Sp245 colonies are non-pigmented and noticeably smaller nearby a CHA0 spot, but they are unaffected when spread onto agar plates containing a cell-free CHA0 spent medium (Fig. 5A, D and E). The smaller Sp245 colonies around a CHA0 spot cannot be interpreted as slowly-growing colonies since their size remained unaltered even after 23 days of culture (data not shown). An inoculum of Sp245 produced ca. 10^8 CFU mL^{-1} when plated either on fresh media or on a lawn of CHA0 (Fig. 5B). The fact that the number of CFU mL^{-1} of the same Sp245 inoculum is

not diminished, or even showed a statistically non-significant tendency to augment, revealed that CHA0 did not affect Sp245 viability but only its colony phenotype. A nutrient depletion effect cannot be discarded in this experimental approach in which a lawn of CHA0 coexists with colonies of Sp245. Although Nfb-Fe-NO_3^- is a complete rich medium supplemented with nitrate to favor biofilm formation (Arruebarrena Di Palma *et al.* 2013) and with iron to limit siderophores production that can impair Sp245 growth, a different experimental set up in which CHA0 was spotted in the center of the plate showed a radial negative effect on Sp245 colony development (Fig. 4A and B). This undoubtedly accounts for one or more soluble factors that diffuse and affect the size of Sp245 colony. The small non-pigmented colony phenotype is usually observed in pathogenic bacteria under stress conditions. Such variants are called *small colony variants* (SCVs) and have been extensively studied in several genera including *Pseudomonas* (Häußler *et al.* 1999). SCVs constitute a subpopulation of non-pigmented colonies that are ca. 10 times smaller than their counterparts on agar plates and less susceptible to antibiotics (Proctor *et al.* 2006). The observed effect of CHA0 on Sp245 colonies might be a result of exposition to a stressful metabolite that induces a SCV-like phenotypic response. The fact that Sp245 small colonies restore to a normal colony phenotype upon being streaked in fresh medium (Fig 4C) implies that the probability of plasmid loss or genetic rearrangement is negligible in the SCV. Moreover, the dose-dependent SCV phenotype observed in our experiments (Fig 4A and B) is neither consistent with genetic rearrangements. Antibiotic production by *P. protegens* CHA0 is tightly regulated by the post-transcriptional cascade Gac/Rsm. This signal transduction pathway regulates the production of important secondary metabolites for the biocontrol of root pathogens (Lapouge *et al.* 2008). Since the SCV-like phenotypic response of Sp245 might be the result of exposition to stressful metabolites such as those released by CHA0, future studies using CHA0 Gac/Rsm mutants, which are impaired in antibiotic production, could be useful to elucidate the participation of metabolites secreted by CHA0 in the development of Sp245 SCV.

In conclusion, the observations presented here suggest that interspecies co-culture forming mixed biofilms is a favorable way of cooperation, in which the growth of interacting strains is enhanced, and the development of spatially structured biofilms may be a cooperative solution for better exploitation of available resources. The two species used in this work are major inhabitants of rhizosphere and their mutually cooperative interaction could probably prevail in this habitat. There is a need for integrative studies in soil microbiology, and a holistic consideration of the interactions between the various species at play in the rhizosphere would undoubtedly improve management of the rhizosphere microbiota and PGPR performance.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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