Serveur Académique Lausannois SERVAL serval.unil.ch

Author Manuscript

Faculty of Biology and Medicine Publication

This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Published in final edited form as:

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

Authors: Pagnussat LA, Salcedo F, Maroniche G, Keel C, Valverde C, Creus CM

Journal: FEMS microbiology letters

Year: 2016 Oct

Issue: 363

Volume: 20

DOI: 10.1093/femsle/fnw238

In the absence of a copyright statement, users should assume that standard copyright protection applies, unless the article contains an explicit statement to the contrary. In case of doubt, contact the journal publisher to verify the copyright status of an article.

serval
serveur académique lausannois

Unil
UNIL | Université de Lausanne
Faculty of Biology and Medicine

CHUV

OXFORD UNIVERSITY PRESS | FEMS Microbiology Letters

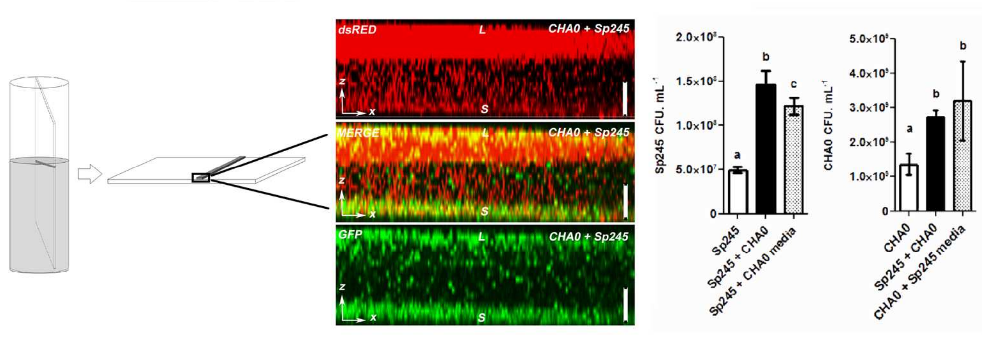
http://mc.manuscriptcentral.com/fems

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

Journal:	*FEMS Microbiology Letters*
Manuscript ID	FEMSLE-16-04-0331.R3
Manuscript Type:	Research Letter
Date Submitted by the Author:	n/a
Complete List of Authors:	Pagnussat, Luciana; Universidad Nacional de Mar del Plata, Fac. Cs. Agrarias Salcedo, Florencia; Universidad Nacional de Mar del Plata, Facultad de Ciencias Agrarias; CONICET, Lab. Bioquimica Vegetal y Microbiana Maroniche, Guillermo; Universidad Nacional de Mar del Plata, Facultad de Ciencias Agrarias; Consejo Nacional de Investigaciones Cientificas y Tecnicas, Lab. Bioquimica Vegetal y Microbiana, FCA Keel, Christoph; Université de Lausanne , Département de microbiologie fondamentale Valverde, Claudio; Universidad Nacional de Quilmes, Departamento de Ciencia y Tecnología Creus, Cecilia; Universidad Nacional de Mar del Plata, Fac. Cs. Agrarias
Keywords:	plant-growth-promoting-bacteria, biofilm structure, colony phenotype
All articles in FEMS Microbiology Letters are published under one of eight subject sections. Please select the most appropriate subject category for your submission from the drop down list:	Environmental Microbiology (Editor: Tim Daniell)

SCHOLARONE™ Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



167x57mm (300 x 300 DPI)

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

Luciana A. Pagnussat[1], Florencia Salcedo[1], Guillermo Maroniche[1], Christoph Keel[2], Claudio Valverde[3], and Cecilia M. Creus[4]*

[1]Universidad Nacional de Mar del Plata (UNMdP), CONICET, FCA, Laboratorio de Bioquímica Vegetal y Microbiana, Balcarce, Argentina.

[2]Universite de Lausanne. Departement de Microbiologie Fondamentale (DMF) Batiment Biophore. Quartier UNIL-Sorge. CH-1015 Lausanne. Switzerland.

[3]Universidad Nacional de Quilmes (UNQ), CONICET, Departamento de Ciencia y Tecnología, Laboratorio de Bioquímica, Microbiología e Interacciones Biológicas en el Suelo, Bernal, Argentina.

[4]Universidad Nacional de Mar del Plata (UNMdP), FCA, Laboratorio de Bioquímica Vegetal y Microbiana, Balcarce, Argentina.

*Corresponding author: Cecilia M. Creus, Laboratorio de Bioquímica Vegetal y Microbiana, UIB Balcarce: FCA, Universidad Nacional de Mar del Plata-INTA, CC 276 (7620), Balcarce, Argentina. Tel. +54-2266439100, Fax +54-2266439101, email: creus.cecilia@inta.gob.ar

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

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

ABSTRACT

Plant growth promoting bacteria belonging to *Azospirillum* and *Pseudomonas* genera are major inhabitants of the rhizosphere. Both are increasingly commercialized as crops inoculants. Inter-specific 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 *A. brasilense* Sp245 and *P. 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.

INTRODUCTION

The rhizosphere constitutes a complex and dynamic environment. As an outcome of co-evolution, interaction between rhizospheric microorganisms involve 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 micro-niche where the exchange of metabolites between bacterial

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

51 populations is favored. The biofilm mode of growth has several advantages for soil bacteria
52 compared to the planktonic lifestyle. Biofilm provides protection against desiccation and toxic
53 compounds. Furthermore, an organization of multispecies bacterial consortia into biofilms may
54 enable the coexistence of species that would otherwise outcompete each other and facilitate
55 synergistic interactions and gene transfer (for reviews see: (Burmølle*, et al.*, 2007, Karatan &
56 Watnick, 2009, López*, et al.*, 2010, Karunakaran*, et al.*, 2011)). Recent findings suggest that
57 when both competitive and cooperative genes are transferred, cooperators are favored because
58 their transfer increases allelic assortment (Dimitriu*, et al.*, 2014).
59 Plant growth-promoting rhizobacteria (PGPR) enhance growth of many terrestrial crop plants
60 by a wide variety of mechanisms (Lugtenberg & Kamilova, 2009, Hayat*, et al.*, 2010,
61 Bhattacharyya & Jha, 2012). *Azospirillum brasilense* improves crop growth mainly by a direct
62 effect on the plant, through the production of several phytohormones such as indole acetic acid
63 (Tien*, et al.*, 1979, Ona*, et al.*, 2005), and nitric oxide (Creus, *et al.,* 2005, Molina-Favero*, et al.*,
64 2008). Conversely, fluorescent pseudomonads, other major inhabitants of the rhizosphere,
65 display mostly indirect beneficial effects on plant growth (Kloepper*, et al.*, 1980). *Pseudomonas*
66 strains produce a wide range of compounds with antimicrobial activity, being 2,4-
67 diacetylphloroglucinol (DAPG) one of the most studied (Keel*, et al.*, 1990). These PGPR are
68 currently commercialized as inoculants containing single or combined strains (i.e. *Azospirillum*
69 *brasilense* and *Pseudomonas fluorescens*) (Bhattacharyya & Jha, 2012). PGPR efficiency
70 depends considerably on their capabilities to survive and establish effective root colonization.
71 Therefore, interaction with other bacteria in the rhizosphere constitutes a critical bottleneck in
72 PGPR aptness. An important concern is that pseudomonads may secrete antibiotics that can
73 negatively affect *A. brasilense* proliferation in mixed inoculants (Combes-Meynet*, et al.*, 2010,
74 Couillerot*, et al.*, 2011). It has been reported that, *in vitro*, *A. brasilense* Cd strain was sensitive
75 to the addition of the synthetic antimicrobial metabolite DAPG, which induced carotenoids

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

76 accumulation, formation of poly-β-hydroxybutyrate-like granules, cytoplasmic membrane
77 damage and growth inhibition (Couillerot*, et al.*, 2011). The major rhizosphere inhabitants, *A.*
78 *brasilense* and fluorescent pseudomonads, are expected to be found in mixed consortia in
79 nature. Therefore, studying their interaction is of particular interest for agronomical applications
80 in inoculants technology. The aim of this work was to assess whether interspecific interaction
81 between the models strains *A. brasilense* Sp245 and *Pseudomonas protegens* CHA0 results in
82 altered static growth, attachment to polystyrene surfaces and strain distribution within mixed
83 biofilms.

84

85 **MATERIALS AND METHODS**

86

87 **Bacterial strains and culture conditions**

88 Fluorescent derivatives of strains *A. brasilense* Sp245 and *P. protegens* CHA0 were obtained by
89 introducing plasmid pMP2444 (Gm^R, P_{lac}-*egfp*) into Sp245 by biparental mating (Arruebarrena
90 Di Palma *et al*. 2013) or plasmid pME7134 (Tc^R, P_{lac}-*dsRed*) by electroporation. Plasmid
91 pME7134 was constructed as follows. A 0.7-kb *Sma*I-*Hind*III fragment from pDsRed.T3_S4T
92 containing the *dsred*.T4_S4T gene (Sörensen *et al*., 2003) was cloned under the control of the
93 P_{lac} promoter into pME6552 (Wenner *et al*., 2014). Following this, a 1.5-kb *Mlu*I-*Hind*III
94 fragment from the resulting plasmid containing the P_{lac}-*dsred*.T3_S4T fusion was subcloned
95 into pME6031 (Heeb *et al*., 2000) to give pME7134. This reporter vector provides bright red
96 fluorescence and can be used in a wide variety of Gram-negative bacteria.
97 Starter single-species cultures of *Azospirillum brasilense* Sp245/pMP2444 and *Pseudomonas*
98 *protegens* CHA0/pME7134 were grown in Luria-Bertani medium (LB) (Sambrook*, et al.*, 1989)
99 at 30ºC for 18 h with orbital shaking (100 rpm). When required, final antibiotic concentrations
100 were Gm 25 μg mL^{-1} for *A. brasilense* and Tc 125 μg mL^{-1} for *P. protegens*. For growth,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

101 biofilm and colony morphology assays, Nfb-NO_3^--iron-enriched media (Nfb-Fe-NO_3^-) was used
102 according to (Döbereiner & Day, 1976) with the following modifications: 27.6 mM malic acid,
103 13.8 mM KNO_3, 0.17 mM $FeCl_3$ and 0.015 mM Fe-EDTA. For preparation of stationary-phase
104 cell free media, bacteria were grown overnight or for three days (overgrown culture) in Nfb-Fe-
105 NO_3^- without antibiotics, pelleted and the supernatant were filter-sterilized (0.22 µm pore size).
106

107 **Bacterial growth and biofilm quantification**

108 Static growth of Sp245/pMP2444 and CHA0/pME7134 was analyzed in axenic conditions on
109 polystyrene microtiter 96 flat bottom-well plates. Single cultures were initiated with 200 µL of
110 fresh Nfb-Fe-NO_3^- medium containing 10^5 cells.mL^{-1} based on optical density (OD_{600}; Spectra
111 MR; Dynex Technologies). Mixed cultures were initiated with 5.10^4 cells of each strain in a
112 total volume of 200 µL in order to reach a final concentration of 10^5 total cells.mL^{-1}. To test the
113 effect of CHA0 cell free spent medium on the growth of Sp245, 100 µL of Nfb-Fe-NO_3^- cell
114 filtered media were added to 100 µL of fresh medium containing 5.10^4 Sp245 cells.mL^{-1}. The
115 opposite combination was also tested. When necessary, different combinations of bacterial
116 densities were tested varying the initial number of Sp245 cells: 10^4, 10^5 or 10^6 but keeping 10^5
117 cells of CHA0. Plates were statically incubated for 2 days at 30ºC. For total cell growth
118 quantification, biofilms developed in the wells were vigorously pipetted with a sterile tip for
119 mechanical disaggregation and mixing with planktonic cells. OD_{600} and fluorescence (ABI 7500
120 in fluorimeter mode: Ex: 538 nm, Em: 605 nm for CHA0/pME7134 and Ex: 460, Em: 525nm
121 for Sp245/pMP2444) were registered. Cells in the biofilm were quantified in the same way after
122 discarding planktonic cells of paralleled wells. Percentage of biofilm-associated cells was
123 calculated as Biofilm-associated cells/ Total cells × 100. Biofilms formed in polystyrene
124 paralleled wells were also quantified by crystal violet dye staining (OD_{550}) and

ScholarOne Support 1-434/964-4100

1
2
3 125 normalized to total bacterial growth ($OD_{600)}$ as previously described (Arruebarrena Di Palma *et*
4
5 126 *al*. 2013).
6
7 127 The experimental design consisted of three to nine replicated plates depending on the measured
8
9 128 variable, in which three independent starter cultures were each sown in triplicate wells. Results
10
11 129 were statistically analyzed by ANOVA and Tukey test. Correspondence between the
12
13 130 fluorescence values and the $CFU.mL^{-1}$ of bacterial suspensions was determined by linear
14
15 131 regression for each strain (Sp245/pMP2444 R square: 0.9869 and CHA0/pME7134 R square:
16
17 132 0.9855; Graphpad Prism 5.03 software). Then, $CFU.mL^{-1}$ values were indirectly estimated by
18
19 133 measuring fluorescence and interpolating values from the curve (Fig S3). In order to ensure the
20
21 134 correspondence of fluorescent measurements to $CFU.mL^{-1}$, CFU counts in total and biofilm
22
23 135 fractions were analyzed by the drop method on Nfb-Fe-NO_3^- medium supplemented with 25µg
24
25 136 mL^{-1} Gm or 125 µg mL^{-1} Tc for Sp245/pMP2444 or CHA0/pME7134, respectively. Controls to
26
27 137 evaluate the level of fluorophore crosstalk were done by registering values of fluorescence (red:
28
29 138 Ex: 538 nm Em: 605 nm and green: Ex: 460nm Em: 525nm) for different concentrations of
30
31 139 CHA0/pME7134. A regression curve between green and red fluorescence (R^2: 0.9905) was
32
33 140 done. Correction of crosstalk was made by subtracting the value of green fluorescence obtained
34
35 141 from the curve to the fluorescence measured in co-cultures.
36
37 142
38
39 143 **Colony morphology experiments**
40
41 144 Suspensions of Sp245 and CHA0 obtained from overnight cultures in LB with agitation (100
42
43 145 rpm) were adjusted to 10^8 $cells.mL^{-1}$ and 10^9 $cells.mL^{-1}$, respectively. Serial dilutions of each
44
45 146 strain were spotted onto Nfb-Fe-NO_3^- agar plates that had been previously inoculated with 1 mL
46
47 147 of either CHA0 or Sp245 lawn, or 1 mL of CHA0 or Sp245 cell-free culture filtrate (spent
48
49 148 medium).
50
51
52
53
54
55
56
57
58
59
60

For the drop assay, 1000 CFU 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 five 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 30ºC. 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 *A. brasilense* and *P. protegens* were evaluated in single and mixed-species static cultures of Sp245 and CHA0 strains carrying the plasmid pMP2444 (Gm^r, P_{lac}-*egfp*) or pME7134 (Tc^r, P_{lac}-*dsRed*), respectively. After two days of cultivation, overall bacterial growth was greater when both species grew together (Fig. 1A). Moreover, fluorescence measurements revealed that Sp245 and CHA0 cell number exhibited a 3 and 2 fold increase, respectively, when co-cultured (Fig. 1B and 1C). 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. S1). 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). 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, 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, 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

199 density and homogeneity on the layers closer to the slide (not shown) and a sponge-like
200 structure with channels in the upper layers (Fig. 3C). Surprisingly, CHA0 single species biofilm
201 was much thicker than the mixed-species biofilm, reaching a depth of about 600 μm (25 μm for
202 Sp245 and 200 μm for Sp245+CHA0). As it was previously demonstrated, quantification
203 performed on microtiter plates revealed higher levels of bacterial biofilm in mixed than in
204 single-species cultures. This apparent contradiction might be explained by the fact that Sp245
205 biofilm formed all over the microplate well (surface, walls and bottom) during static co-culture
206 with CHA0, which would account for the observed increase in total biofilm mass (Fig. 2C).
207 Mixed biofilms were multilayered and complex in structure and showed a particular strain-
208 specific distribution within the biofilm. Sp245 cells were mainly placed towards the slide
209 forming the bottom layers of the biofilm, whereas CHA0 cells were located towards the biofilm
210 surface, in contact with the liquid medium (Fig. 3A, B). Nevertheless, a small fraction of Sp245
211 cells was also located close to the surface (Fig. 3A, B).
212
213 **Sp245 colony diameter is reduced by CHA0**
214 Sp245 colonies were noticeably smaller nearby a CHA0 spot, whereas there was no difference
215 in Sp245 colony diameter close to the control spot (Fig. 4). The diameter of Sp245 colonies was
216 restored at distances higher than 7.5 mm away from the CHA0 spot. This result suggests that a
217 diffusible metabolite produced by CHA0 cells is responsible for the observed phenotype. When
218 plates were cultivated for longer periods (23 d), the small colony phenotype of Sp245 in the
219 proximity of CHA0 spot remained unaltered (data not shown). Small colonies picked from this
220 region of the plate were re-streaked onto fresh medium and they displayed a normal sized
221 phenotype (Fig 4C). To determine whether the reduction of Sp245 colony size requires the
222 presence of CHA0 cells, serial dilutions of both strains were placed over a CHA0 or Sp245
223 strains lawn (1 mL of a 10^6 CFU.mL^{-1} suspension), or over their overnight spent cell-free media,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

224 and incubated for 3d at 30ºC. Sp245 developed small colonies in the presence of a CHA0 lawn
225 (Fig. 5A, D), while CHA0 colonies remained unaffected when grown over Sp245 lawn (Fig.
226 5A, inset). However, Sp245 colonies grown in plates with CHA0 cell-free media remained
227 unaffected (Fig. 5A, E). This indicates that the metabolite responsible for the reduction in Sp245
228 colony size in co-culture with CHA0 in agar plates (Fig. 4A, B), was either absent or in
229 inadequate concentration in this experimental setup. A CHA0 cell-free filtrate from overgrown
230 cultures (3 days, DO_{600} = 6) was used to test this possibility; however, Sp245 colonies again
231 exhibited a normal size, discarding a dilution effect (data not shown). Taken together, the results
232 indicate that the development of Sp245 small colonies requires the presence of CHA0 cells in
233 their proximity, or alternatively, a metabolite that is only secreted in cells growing in solid
234 medium and that is induced by the presence of Sp245.
235

236 **DISCUSSION**

237 PGPR have been studied for their ability to stimulate the growth and health of economically
238 important crops (Lugtenberg & Kamilova, 2009, Hayat*, et al.*, 2010, Beneduzi*, et al.*, 2012,
239 Bhattacharyya & Jha, 2012, Drogue*, et al.*, 2013). However, cell-to-cell communication
240 between PGPR and other rhizosphere-inhabiting microorganisms has received much less
241 attention. Static co-cultivation of strains Sp245 and CHA0 results in a clear interspecific
242 cooperation, as overall growth was enhanced for both species (Fig. 1 and S4). Moreover, cell-
243 free stationary-phase supernatants from CHA0 and Sp245 cultures were sufficient to exert
244 interspecies growth promotion (Fig. 1C). Bacteria are found to interact synergistically, by either
245 providing nutrients, removing some inhibitory products, or stimulating each other through
246 physical or biochemical mechanisms (Burmølle *et al*., 2006). Growth enhancement upon CHA0
247 and Sp245 interaction can be explained by several mechanisms like pH changes,
248 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 1 B 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 & Cao, 2012, Powers*, et al.*, 2015). In this regard, sub-inhibitory 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 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, 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

1
2
3 274 tolerance to desiccation, heat, and osmotic shock, at different degrees depending on the identity
4
5 275 of the species involved (Joe, et al., 2009). Recently, Ren, et al. (2015) showed the prevalence of
6
7 276 synergistic effects in biofilm formation among isolates from different soils when co-cultured in
8
9 277 combinations of four species. Moreover, absolute individual strain cell numbers were
10
11 278 significantly enhanced when compared with those of single-species biofilms, indicating that all
12
13 279 the individual strains benefit from inclusion in the multispecies community (Ren et al., 2015).
14
15 280 The fact that the *A. brasilense* biofilm is thicker (Fig. 2A) and denser (Fig. 3) when co-cultured
16
17 281 with *P. protegens* suggests that this particular interspecific relationship could be an adaptation
18
19 282 to complex environments where microbial communities co-exist. This response may result in an
20
21 283 increased ability of *A. brasilense* to colonize roots in the rhizosphere when both microorganisms
22
23 284 are present. On the contrary, the outcome of the interaction depends on the species partner, as it
24
25 285 has been reported that biofilms of *Bacillus subtilis* are inhibited by *P. protegens* and *P. putida*
26
27 286 (Powers*, et al.*, 2015). To our knowledge, our work is the first to demonstrate a true
28
29 287 collaboration between statically co-cultured *A. brasilense* and *P. protegens* that is reflected not
30
31 288 only in an increase in the total cell numbers, but also in a distribution of niche occupancy within
32
33 289 an interspecies biofilm.
34
35 290 On the other hand, interaction on agar plates revealed that Sp245 colonies are non-pigmented
36
37 291 and noticeably smaller nearby a CHA0 spot, but they are unaffected when spread onto agar
38
39 292 plates containing a cell-free CHA0-spent-medium (Fig. 5A, D and E). The smaller Sp245
40
41 293 colonies around a CHA0 spot cannot be interpreted as slowly-growing colonies since their size
42
43 294 remained unaltered even after 23 days of culture (data not shown). An inoculum of Sp245
44
45 295 produced ca. 10^8 CFU. mL^{-1} when plated either on fresh media or on a lawn of CHA0 (Fig. 5B).
46
47 296 The fact that the number of CFU.mL^{-1} of the same Sp245 inoculum is not diminished, or even
48
49 297 showed a statistically non-significant tendency to augment, revealed that CHA0 did not affect
50
51 298 Sp245 viability but only its colony phenotype. A nutrient depletion effect cannot be discarded in
52
53
54
55
56
57
58
59
60

1
2
3 299 this experimental approach in which a lawn of CHA0 coexists with colonies of Sp245. Although
4
5 300 Nfb-Fe-NO_3^- is a complete rich medium supplemented with nitrate to favor biofilm formation
6
7 301 (Arruebarrena Di Palma, *et al.*, 2013) and with iron to limit siderophores production that can
8
9 302 impair Sp245 growth, a different experimental set up in which CHA0 was spotted in the center
10
11 303 of the plate showed a radial negative effect on Sp245 colony development (Fig. 4A, B). This
12
13 304 undoubtedly accounts for one or more soluble factors that diffuse and affect the size of Sp245
14
15 305 colony. The small non-pigmented colony phenotype is usually observed in pathogenic bacteria
16
17 306 under stress conditions. Such variants are called *small colony variants* (SCV) and have been
18
19 307 extensively studied in several genera including *Pseudomonas* (Häußler, *et al.*, 1999). SCV
20
21 308 constitute a subpopulation of non-pigmented colonies that are ca. 10 times smaller than their
22
23 309 counterparts on agar plates and less susceptible to antibiotics (Proctor, *et al.*, 2006). The
24
25 310 observed effect of CHA0 on Sp245 colonies might be a result of exposition to a stressful
26
27 311 metabolite that induces a SCV-like phenotypic response. The fact that Sp245 small colonies
28
29 312 restore to a normal colony phenotype upon being streaked in fresh medium (Fig 4C), implies
30
31 313 that the probability of plasmid loss or genetic rearrangement is negligible in the SCV.
32
33 314 Moreover, the radial and dose-dependent SCV phenotype observed in our experiments (Fig 4A,
34
35 315 B) is neither consistent with genetic rearrangements. Antibiotic production by *P. protegens*
36
37 316 CHA0 is tightly regulated by the post-transcriptional cascade Gac/Rsm. This signal transduction
38
39 317 pathway regulates the production of important secondary metabolites for the biocontrol of root
40
41 318 pathogens (Lapouge, *et al.*, 2008). Since the SCV-like phenotypic response of Sp245 might be
42
43 319 the result of exposition to stressful metabolites such as those released by CHA0, future studies
44
45 320 using CHA0 Gac/Rsm mutants, which are impaired in antibiotic production, could be useful to
46
47 321 elucidate the participation of metabolites secreted by CHA0 in the development of Sp245 SCV.
48
49 322 In conclusion, the observations presented here suggest that interspecies co-culture forming
50
51 323 mixed biofilms is a favorable way of cooperation, in which the growth of interacting strains is
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

324 enhanced, and the development of spatially structured biofilms may be a cooperative solution
325 for better exploitation of available resources. The two species used in this work are major
326 inhabitants of rhizosphere and their mutually cooperative interaction could probably prevail in
327 this habitat. There is a need for integrative studies in soil microbiology, and a holistic
328 consideration of the interactions between the various species at play in the rhizosphere would
329 undoubtedly improve management of the rhizosphere microbiota and PGPR performance.
330

331 **FUNDINGS**

332 This work was supported by 'Secretaría de Ciencia y Técnica', UNMdP, Argentina (AGR
333 411/15). LP and CC are researchers from UNMdP, Argentina. CK is researcher from University
334 of Lausanne, Switzerland. GM, LP and CV are researchers from CONICET, Argentina. FS is
335 doctoral fellow from CONICET, Argentina.
336

337 **ACKNOWLEDEMENTS**

338 We thank Dr. M. Pechy-Tarr and Dr. E. Baehler (Département de Microbiologie Fondamentale,
339 Université de Lausanne, Switzerland) for help with the construction of pME7134 plasmid, Dr. J.
340 Carella (Departamento de Ciencia e Ingeniería de Polímeros, FI, UNMdP) for valuable
341 assistance with polystyrene slides and S. Larraburu for technical assistance.
342

343 **REFERENCES**

344

345 Arruebarrena Di Palma A, M. Pereyra C, Moreno Ramirez L, *et al.* (2013) Denitrification-
346 derived nitric oxide modulates biofilm formation in Azospirillum brasilense. *FEMS*
347 *Microbiology Letters* **338**: 77-85.

348 Beneduzi A, Ambrosini A & Passaglia LMP (2012) Plant growth-promoting rhizobacteria
349 (PGPR): their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology*
350 **35**: 1044-1051.
351 Bhattacharyya PN & Jha DK (2012) Plant growth-promoting rhizobacteria (PGPR): emergence
352 in agriculture. *World Journal of Microbiology and Biotechnology* **28**: 1327-1350.
353 Burdman S, Jurkevitch E, Schwartsburd B, Hampel M & Okon Y (1998) Aggregation in
354 Azospirillum brasilense: effects of chemical and physical factors and involvement of
355 extracellular components. *Microbiology* **144**: 1989-1999.
356 Burmølle M, Webb JS, Rao D, Hansen LH, Sørensen SJ & Kjelleberg S (2006) Enhanced
357 Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion
358 Are Caused by Synergistic Interactions in Multispecies Biofilms. *Applied and Environmental*
359 *Microbiology* **72**: 3916-3923.
360 Burmølle M, Hansen L & Sørensen S (2007) Establishment and Early Succession of a
361 Multispecies Biofilm Composed of Soil Bacteria. *Microbial Ecology* **54**: 352-362.
362 Combes-Meynet E, Pothier JF, Moënne-Loccoz Y & Prigent-Combaret C (2010) The
363 Pseudomonas Secondary Metabolite 2,4-Diacetylphloroglucinol Is a Signal Inducing
364 Rhizoplane Expression of Azospirillum Genes Involved in Plant-Growth Promotion. *Molecular*
365 *Plant-Microbe Interactions* **24**: 271-284.
366 Couillerot O, Combes-Meynet E, Pothier JF*, et al.* (2011) The role of the antimicrobial
367 compound 2,4-diacetylphloroglucinol in the impact of biocontrol Pseudomonas fluorescens
368 F113 on Azospirillum brasilense phytostimulators. *Microbiology* **157**: 1694-1705.
369 Creus CM, Graziano M, Casanovas EM, *et al*. (2005) Nitric Oxide is involved in the
370 *Azospirillum brasilense*-induced lateral root formation in tomato. Planta **221**: 297-303.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

371 Dimitriu T, Lotton C, Bénard-Capelle J, Misevic D, Brown SP, Lindner AB & Taddei F (2014)
372 Genetic information transfer promotes cooperation in bacteria. *Proceedings of the National*
373 *Academy of Sciences* **111**: 11103-11108.
374 Döbereiner J & Day J (1976) Associative symbioses in tropical grasses: characterization of
375 microorganisms and dinitrogen-fixing sites. 518-538. Washington State University Press
376 Pullman.
377 Drogue B, Combes-Meynet E, Moënne-Loccoz Y, Wisniewski-Dyé F & Prigent-Combaret C
378 (2013) Control of the Cooperation Between Plant Growth-Promoting Rhizobacteria and Crops
379 by Rhizosphere Signals. *Molecular Microbial Ecology of the Rhizosphere,* pp. 279-293. John
380 Wiley & Sons, Inc.
381 Häußler S, Tümmler B, Weißbrodt H, Rohde M & Steinmetz I (1999) Small-colony variants of
382 Pseudomonas aeruginosa in cystic fibrosis. *Clinical infectious diseases* **29**: 621-625.
383 Hayat R, Ali S, Amara U, Khalid R & Ahmed I (2010) Soil beneficial bacteria and their role in
384 plant growth promotion: a review. *Annals of Microbiology* **60**: 579-598.
385 Heeb S, Itoh Y, Nishijyo T, Schnider U, Keel C, Wade J, Walsh U, O'Gara F, Haas D (2000)
386 Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative,
387 plant-associated bacteria. *Molecular Plant Microbe Interaction* **13**:232-7.
388 Joe MM, Jaleel CA, Sivakumar PK, Zhao C-X & Karthikeyan B (2009) Co-aggregation in
389 Azospirillum brasilensense MTCC-125 with other PGPR strains: Effect of physical and
390 chemical factors and stress endurance ability. *Journal of the Taiwan Institute of Chemical*
391 *Engineers* **40**: 491-499.
392 Karatan E & Watnick P (2009) Signals, Regulatory Networks, and Materials That Build and
393 Break Bacterial Biofilms. *Microbiology and Molecular Biology Reviews* **73**: 310-347.

Karunakaran E, Mukherjee J, Ramalingam B & Biggs C (2011) “Biofilmology”: a multidisciplinary review of the study of microbial biofilms. *Applied Microbiology and Biotechnology* **90**: 1869-1881.

Keel C, Wirthner P, Oberhänsli T, Voisard C, Burger, Haas D & Défago G (1990) Pseudomonads as antagonists of plant pathogens in the rhizosphere: role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. *Symbiosis* **9**: 327-341.

Kloepper J, Leong J, Teintze M & Schroth M (1980) Pseudomonas siderophores: A mechanism explaining disease-suppressive soils. *Current Microbiology* **4**: 317-320.

Lapouge K, Schubert M, Allain FHT & Haas D (2008) Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour. *Molecular Microbiology* **67**: 241-253.

López D, Vlamakis H & Kolter R (2010) Biofilms. *Cold Spring Harbor Perspectives in Biology* **2**.

Lugtenberg B & Kamilova F (2009) Plant-Growth-Promoting Rhizobacteria. *Annual Review of Microbiology* **63**: 541-556.

Molina-Favero C, Creus CM, Simontacchi M, Puntarulo S & Lamattina L (2008) Aerobic Nitric Oxide Production by Azospirillum brasilense Sp245 and Its Influence on Root Architecture in Tomato. *Molecular Plant-Microbe Interactions* **21**: 1001-1009.

Notz R, Maurhofer M, Schnider-Keel U, Duffy B, Haas D & Défago G (2001) Biotic factors affecting expression of the 2, 4-diacetylphloroglucinol biosynthesis gene phlA in Pseudomonas fluorescens biocontrol strain CHA0 in the rhizosphere. *Phytopathology* **91**: 873-881.

Ona O, Van Impe J, Prinsen E & Vanderleyden J (2005) Growth and indole-3-acetic acid biosynthesis of Azospirillum brasilense Sp245 is environmentally controlled. *FEMS Microbiology Letters* **246**: 125-132.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

418 Philippot L, Raaijmakers JM, Lemanceau P & van der Putten WH (2013) Going back to the
419 roots: the microbial ecology of the rhizosphere. *Nat Rev Micro* **11**: 789-799.
420 Powers MJ, Sanabria-Valentín E, Bowers AA & Shank EA (2015) Inhibition of Cell
421 Differentiation in Bacillus subtilis by Pseudomonas protegens. *Journal of Bacteriology* JB.
422 02535-02514.
423 Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M & Peters G (2006)
424 Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent
425 infections. *Nat Rev Micro* **4**: 295-305.
426 Ren D, Madsen JS, Sorensen SJ & Burmolle M (2015) High prevalence of biofilm synergy
427 among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. *ISME J*
428 **9**: 81-89.
429 Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular cloning*. Cold spring harbor laboratory
430 press New York.
431 Sörensen M, Lippuner C, Kaiser T, Misslitz A, Aebischer T, Bumann D (2003) Rapidly
432 maturing red fluorescent protein variants with strongly enhanced brightness in bacteria.
433 *FEBS Letters* **552**:110-4.
434 Tien TM, Gaskins MH & Hubbell DH (1979) Plant Growth Substances Produced by
435 Azospirillum brasilense and Their Effect on the Growth of Pearl Millet (Pennisetum
436 americanum L.). *Applied and Environmental Microbiology* **37**: 1016-1024.
437 Wenner N, Maes A, Cotado-Sampayo M, Lapouge K (2014) NrsZ: a novel, processed, nitrogen-
438 dependent, small non-coding RNA that regulates Pseudomonas aeruginosa PAO1 virulence.
439 *Environmental Microbiology* **16**:1053-68.
440 Yang F & Cao Y (2012) Biosynthesis of phloroglucinol compounds in microorganisms—
441 review. *Applied Microbiology and Biotechnology* **93**: 487-495.
442

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig 1

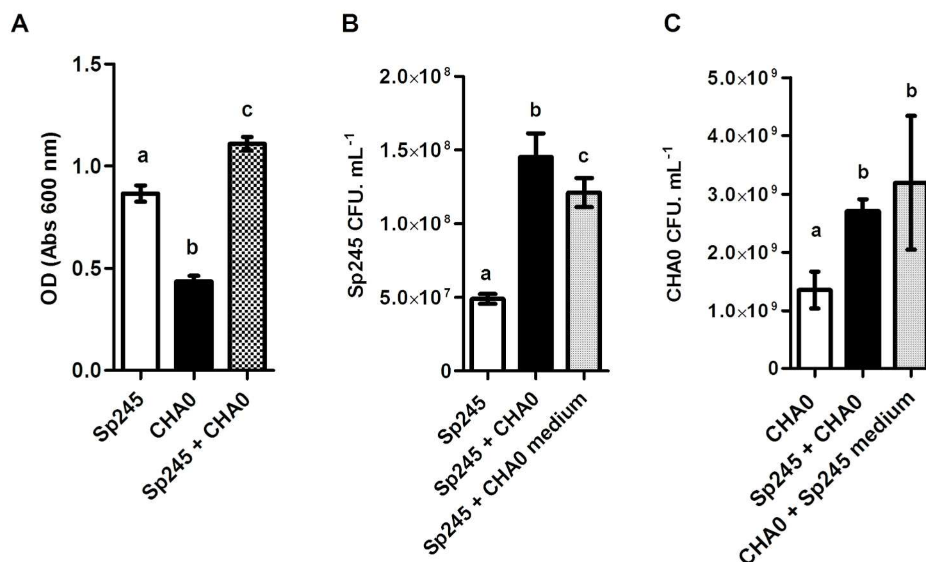
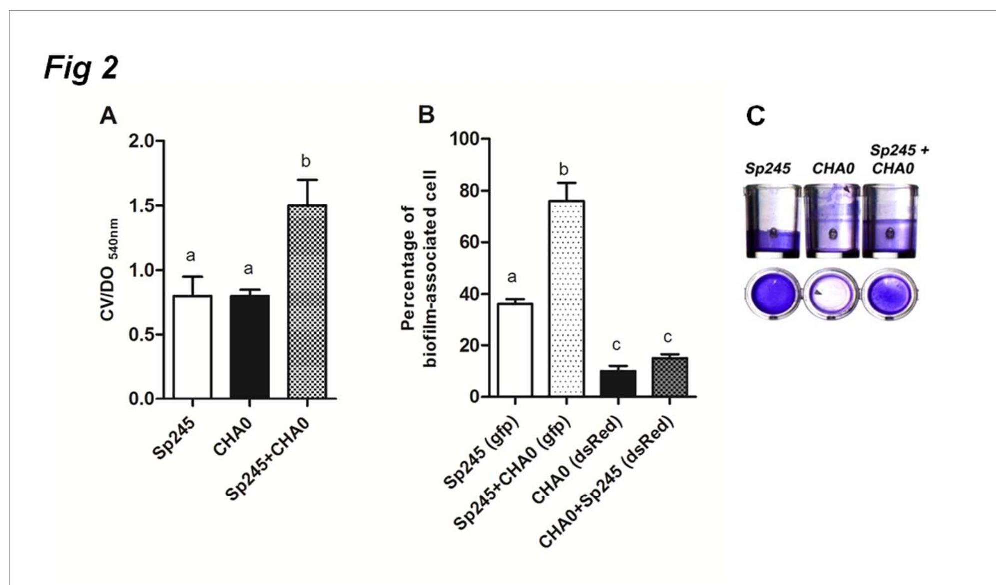


Fig. 1. Static co-cultivation enhances growth of *A. brasilense* Sp245 and *P. protegens* CHA0 strains. Total growth (planktonic plus attached cells after disaggregation) of *A. brasilense* Sp245 and *P. protegens* CHA0 strains was analyzed after 2 days of static culture in Nfb-Fe-NO_3^- at 30ºC in 96 wells plates. Single cultures were initiated with 10^5 cells and mixed cultures with 5.10^4 cells of each strain. Optical density at 600nm (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 S3). 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).

Fig 1

191x129mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26



27 Co-cultivation enhances *A. brasilense* Sp245 biofilm. Biofilm of *A. brasilense* Sp245 and *P. protegens* CHA0
28 strains was monitored after 2 days of static culture in Nfb-Fe-NO_3^- at 30ºC in 96 wells plates. Crystal violet
29 staining of the biofilm was quantified and normalized by the total cell growth (OD550nm/OD600nm, A).
30 Fluorescence of disaggregated cells was also determined and normalized as percentage of biofilm-associated
31 cells (B). Biofilm phenotypes are shown for each condition (C). Values are means ± SE of three to nine
32 independent experiments, with wells replicates each. Results were statistically analyzed by ANOVA and
33 Tukey's Multiple Comparison posttests. Different letters indicate significant differences (P<0.05).
34 Fig 2
35 196x114mm (150 x 150 DPI)

36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig 3

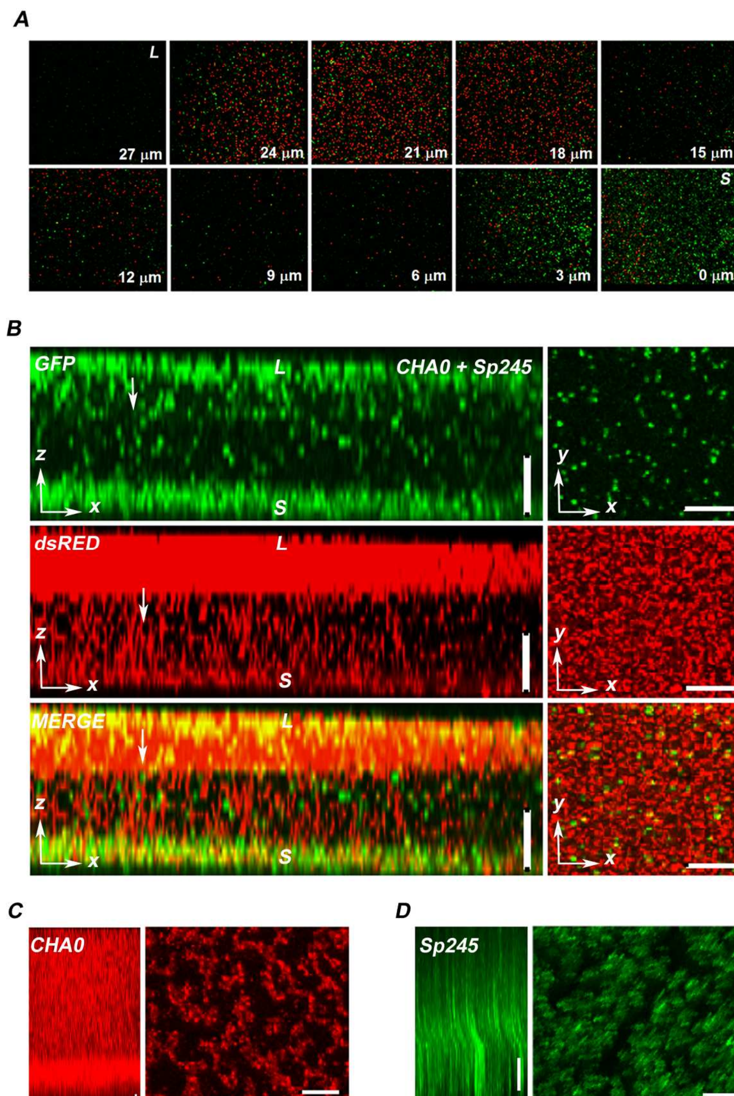


Fig. 3. Confocal laser scanning microscopy showing strain distribution within the mixed biofilm located on the air-liquid interface zone. Polystyrene covered slides were placed in 3 mL Nfb-Fe-NO_3^- with 10^5 Sp245 cells, 10^5 CHA0 cells or a combination of both strains and statically cultured for 2 days at 30ºC. Fluorescence across the biofilm formed over the slides on the air-liquid interface zone was monitored, reconstruction was done with 3µm 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-projection were reconstructed with 3 µm-interspace between images. L: in contact with culture media; S: in contact with slide. Bar scale: 20 µm.
Fig 3
500x688mm (96 x 96 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

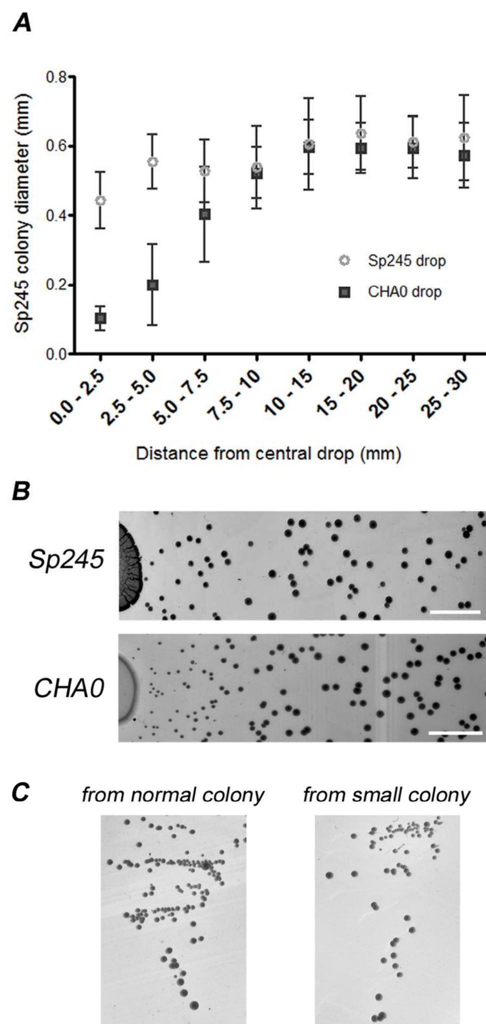


Fig. 4. Sp245 colony diameter is reduced by CHA0. 1000 CFU of Sp245 were spread onto Nfb-Fe-NO_3^- agar plate, and 10 µL of a suspension of CHA0 (10^9 cells. mL^{-1}) or Sp245 (10^8 cells. mL^{-1}), was spotted at the center of the plate and incubated for five days at 30°C. Diameters of Sp245 colonies 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.
Fig 4
80x157mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

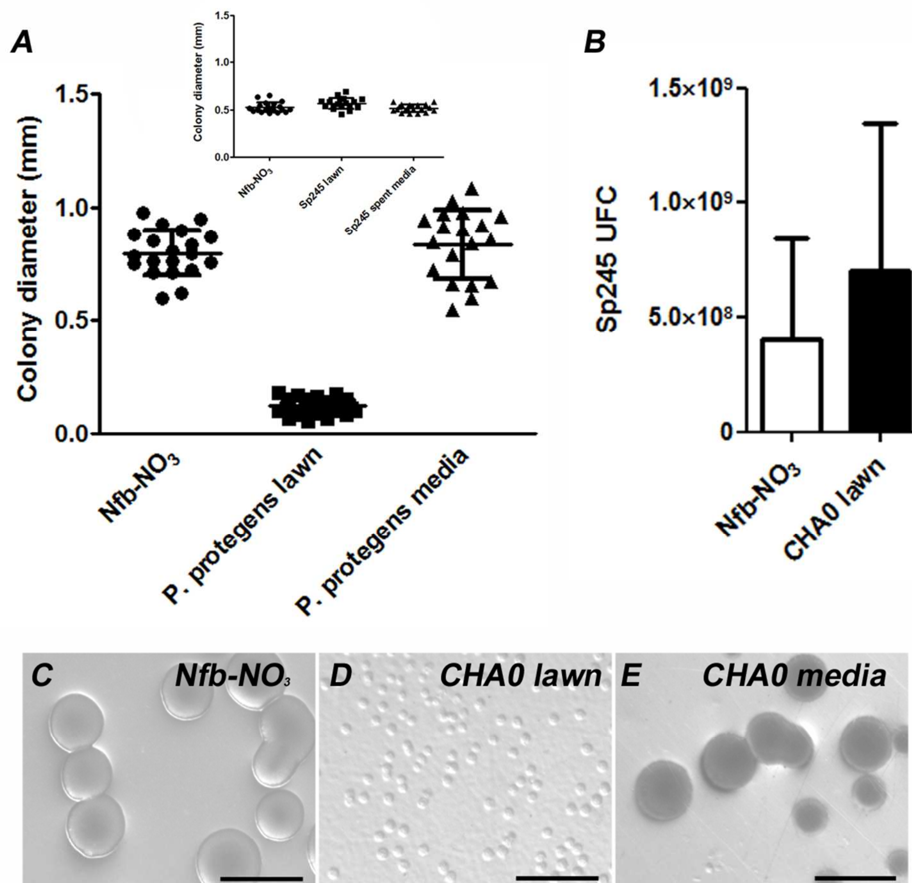
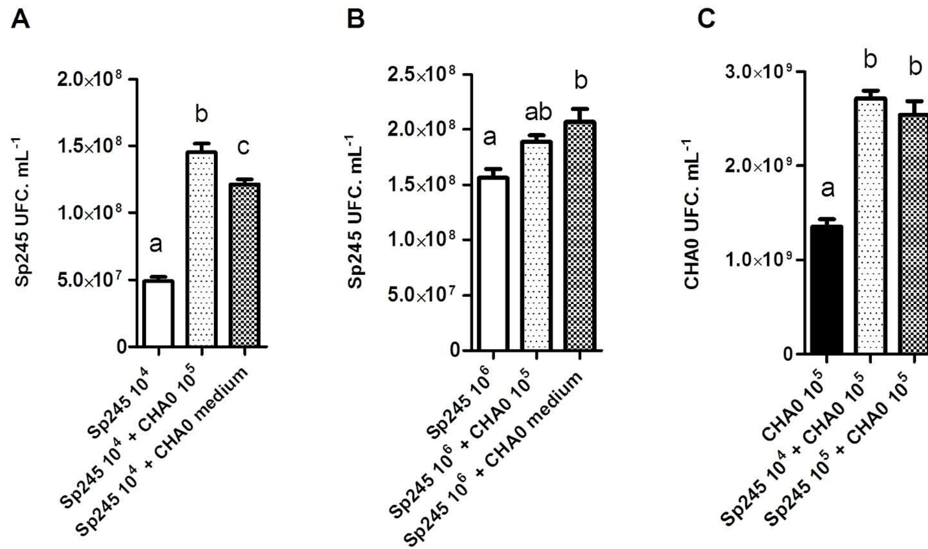


Fig. 5. Sp245 colony diameter is not affected by CHA0 stationary-phase supernatants. Serial dilutions of each strain were spotted onto Nfb-Fe-NO_3^- agar plates, over a CHA0 or Sp245 lawn or over cell-free culture filtrates. Sp245 (A) or CHA0 (A, inset) colonies diameter were measured, Sp245 CFU.mL^{-1}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

Fig 5

109x111mm (300 x 300 DPI)

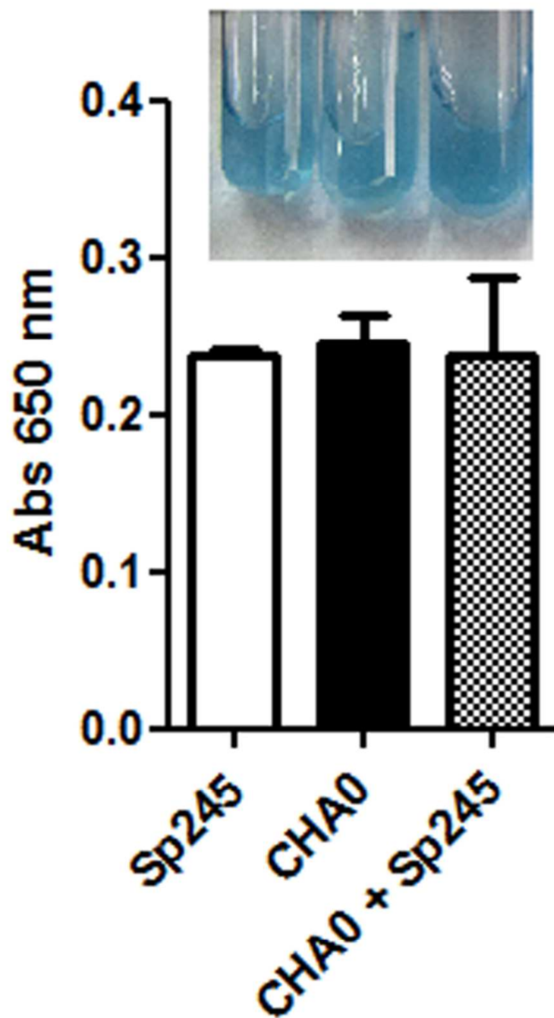
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



178x118mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig S2

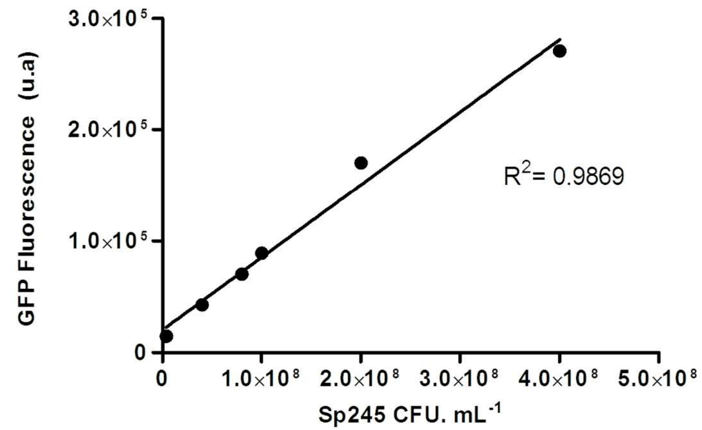


80x147mm (300 x 300 DPI)

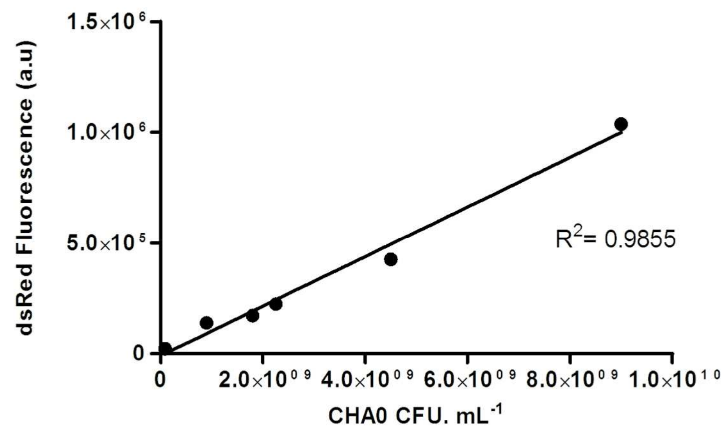
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig S3

A

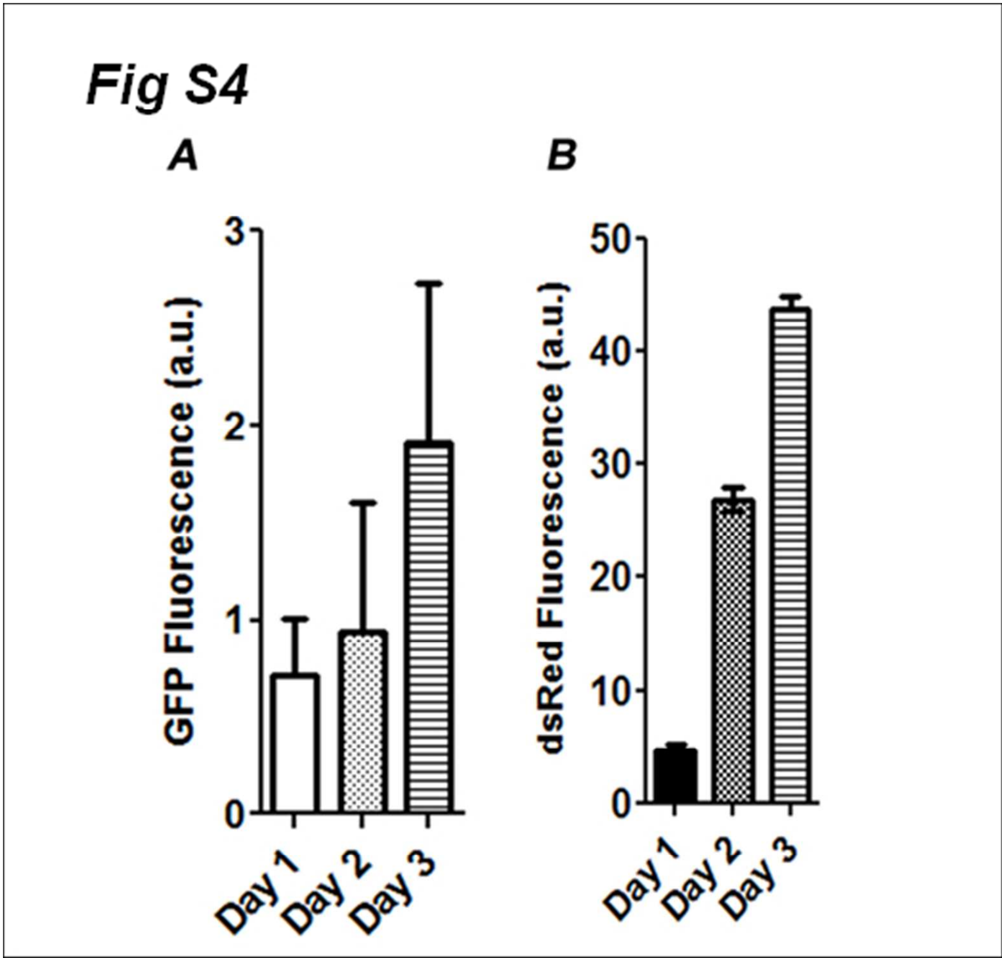


B



122x179mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



88x84mm (150 x 150 DPI)

Legends to Supplementary Figures

Fig. S1. Growth enhancement is dependent from the density of initial inocula. 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 at 30ºC in 96 wells plates. Mixed cultures were initiated with 10^5 CHA0 cells.mL^{-1} and 10^4, 10^6 Sp245 cells. mL^{-1} (A and B) or 10^4 and 10^5 Sp245 cells.mL^{-1} (C). Fluorescence of Sp245 (A and B) and CHA0 (C) were registered. CFU.mL^{-1} was obtained from linear regression with fluorescence values for each strain (Fig S3). Values are means ± SE of three independent experiments, with nine replicates each. Results were statistically analyzed by ANOVA and Tukey's Multiple Comparison posttests. Different letters indicate significant differences ($P<0.05$).

Fig. S2. No pH changes were detected between single and mixed cultures. Media pH was analyzed after 2 days of static culture at 30ºC in 96 wells with Nfb-Fe-NO^{3-} and bromothymol blue. Absorbance at 650nm of Sp245, CHA0 and mixed cultures were registered. Values are means ± SE of three independent experiments, with nine replicates each. Results were statistically analyzed by ANOVA and Tukey's Multiple Comparison posttests.

Fig. S3. Linear regression between fluorescence values and CFU. mL^{-1}. Fluorescence measurements on serial dilutions of *A. brasilense* Sp245 (A) and *P. protegens* (B) were registered and CFU. mL^{-1} of the same samples was determined. Linear regression was calculated between both parameters for each strain and Goodness of fit values were calculated (Graphpad Prism 5.03 software).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig. S4. Total growth (planktonic plus attached cells after disaggregation) of 10^5 initial cells of *A. brasilense* Sp245 (GFP) or *P. protegens* CHA0 (dsRED) strains was analyzed after 1, 2 or 3 days of static culture at 30ºC in 96 wells plates. Fluorescence of Sp245 (A) and CHA0 (B) were registered. (a.u.) arbitrary units of fluorescence.