RESEARCH ARTICLE

Tryptophan, thiamine and indole-3-acetic acid exchange between *Chlorella sorokiniana* and the plant growth-promoting bacterium *Azospirillum brasilense*

Oskar A. Palacios¹, Gracia Gomez-Anduro², Yoav Bashan¹,³,⁴ and Luz E. de-Bashan¹,³,⁴,*

¹Environmental Microbiology Group, The Northwestern Center for Biological Research (CIBNOR), Av. IPN 195, La Paz 23096, Mexico, ²Molecular Biology of Plants Group, Northwestern Center for Biological Research (CIBNOR), Av. IPN 195, La Paz, B.C.S. 23096, Mexico, ³The Bashan Institute of Science, 1730 Post Oak Ct., AL 36830, USA and ⁴Department of Entomology and Plant Pathology, Auburn University, 301 Funchess Hall, Auburn, AL 36849, USA

*Corresponding author: Environmental Microbiology Group, The Northwestern Center for Biological Research CIBNOR, AV IPN 195, La Paz 23096, Mexico. Tel: +52 612 1238484 ext 3419; E-mail: legonzal04@cibnor.mx

One sentence summary: Signaling in synthetic mutualism between microalgae and bacteria.

Editor: Angela Sessitsch

ABSTRACT

During synthetic mutualistic interactions between the microalga *Chlorella sorokiniana* and the plant growth-promoting bacterium (PGPB) *Azospirillum brasilense*, mutual exchange of resources involved in producing and releasing the phytohormone indole-3-acetic acid (IAA) by the bacterium, using tryptophan and thiamine released by the microalga, were measured. Although increased activities of tryptophan synthase in *C. sorokiniana* and indole pyruvate decarboxylase (IPDC) in *A. brasilense* were observed, we could not detect tryptophan or IAA in the culture medium when both organisms were co-immobilized. This indicates that no extra tryptophan or IAA is produced, apart from the quantities required to sustain the interaction. Over-expression of the *ipdC* gene occurs at different incubation times: after 48 h, when *A. brasilense* was immobilized alone and grown in exudates of *C. sorokiniana* and at 96 h, when *A. brasilense* was co-immobilized with the microalga. When *A. brasilense* was cultured in exudates of *C. sorokiniana* and at 96 h, when *A. brasilense* was co-immobilized with the microalga. When *A. brasilense* was cultured in exudates of *C. sorokiniana*, increased expression of the *ipdC* gene, corresponding increase in activity of IPDC encoded by the *ipdC* gene, and increase in IAA production were measured during the first 48 h of incubation. IAA production and release by *A. brasilense* was found only when tryptophan and thiamine were present in a synthetic growth medium (SGM). The absence of thiamine in SGM yielded no detectable IAA. In summary, this study demonstrates that *C. sorokiniana* can exude sufficient tryptophan and thiamine to allow IAA production by a PGPB during their interaction. Thiamine is essential for IAA production by *A. brasilense* and these three metabolites are part of a communication between the two microorganisms.

Keywords: *Azospirillum*; *Chlorella*; indole-3-acetic acid; mutualism; signaling; tryptophan
INTRODUCTION

Natural microalgae—bacteria mutualisms were proposed over four decades ago (Bell and Mitchell 1972). Only in the last decade has research been intensified, mainly in marine environments (Azam and Malfatti 2007; Amin, Parker and Armbrust 2012). Synthetic mutualism is a relatively new field of study (Momori et al. 2011), serving as a part of the synthetic ecology approach. Synthetic mutualism was artificially created between the fresh water microalgae Chlorella sorokiniana and the microalgae/plant growth-promoting bacterium (MGFP/PGPB) Azospirillum brasilense, when both were co-immobilized in alginate beads to enforce initial association between the non-motile microalgae and the highly motile bacterium (Gonzalez and Bashan 2000; de-Bashan and Bashan 2008; de-Bashan, Hernandez and Bashan 2015). Both microorganisms have completely different life histories and in nature, do not share the same habitat. Azospirillum spp. are rhizosphere-dwelling, free living, aerobic and microaerophilic root-associated bacteria (Bashan, Holguín and de-Bashan 2004) that are highly motile in soil (Bashan and Levanony 1987) and efficiently colonize roots of many plant species (Levanony et al. 1989). For decades, numerous studies have demonstrated that these bacteria induce various beneficial effects in crops, mainly on their yield, and positively affect the microalgae Chlorella spp. (Bashan, Holguín and de-Bashan 2004). There is no single mechanism involved in promoting plant growth with Azospirillum, but rather a combination of a few to many mechanisms in each specific case of inoculation. These mechanisms work together or in tandem and the phenomenon is known as ‘Multiple Mechanisms Theory’ (Bashan and de-Bashan 2010). Several studies claimed that the main mechanism by which Azospirillum affects growth and metabolism of microalgae and higher plants is hormonal, mainly, but not solely, indole-3-acetic acid (IAA) that most strains produce in abundance (Crozier et al. 1988; Bottini et al. 1989; Dobbelare et al. 1999; Martinez-Morales et al. 2003; Cohen, Bottini and Piccoli 2008; de-Bashan et al. 2008). Azospirillum has three metabolic pathways to produce IAA, using tryptophan as a precursor: (i) the indole pyruvate route; main pathway (Speepo et al. 2007), (ii) the indole-3-acetamide route and (iii) the indole-3-acetonitrile route (Carreño-Lopez et al. 2000). Thiamine was proposed as a cofactor in IAA production (Koga, Adachi and Hidaka 1992).

The second partners in this synthetic mutualism are Chlorella spp. They are green, unicellular microalgae (Krienitz, Huss and Bock 2015) that produce and exude diverse compounds, such as carbohydrates, proteins, amino acids and vitamins to the culture medium (Aaronson, Dhawale and Patni 1977; Nishijima, Shiozaki and Hata 1979; Watanabe et al. 2006). This microalgae—bacteria association was proposed as a model to study the interaction between plant cells and prokaryotic cells and as a candidate for several biotechnological applications (de-Bashan and Bashan 2008; de-Bashan, Hernandez and Bashan 2015; Perez-Garcia and Bashan 2015). During mutualistic interaction of Chlorella spp. and A. brasilense, enhancement of population growth and significant physiological, morphological and biochemical pathways occur in the microalgae (Lebsky, Gonzalez-Bashan and Bashan 2001; de-Bashan et al. 2002, 2008; Choix, de-Bashan and Bashan 2012a,b; Choix et al. 2014; Leyva et al. 2014; Leyva, Bashan and de-Bashan 2015; Meza et al. 2015; Meza, de-Bashan and Bashan 2015). The interaction between C. sorokiniana and A. brasilense also enhances thiamine release to the culture medium (Palacios et al. 2016a).

In nature, the composition of exudates produced by an organism can indicate the type of microorganisms that are interacting in a specific environment. Exudates from plants are a key determinant of the microbial community structure in the rhizosphere (Nannipieri et al. 2008; Hartmann et al. 2009; Cesco et al. 2012; Barea et al. 2013). Algae-bacteria mutualisms in natural assemblages, specifically in marine environment, involving metabolite signaling were described (Karp-Boss, Boss and Jumars 1996; Amin, Parker and Armbrust 2012; Amin et al. 2015). Recently, the mutual benefit to C. sorokiniana and A. brasilense and demonstration of creation of a true mutualism during this interaction was shown by active exchange of C and N molecules between single cells of interacting microalga and bacteria. This exchange was beneficial to both the microorganisms and enhanced the growth of both (de-Bashan et al. 2016). To demonstrate further that A. brasilense has the ability to use exudates produced by Chlorella spp. to produce IAA that is then used by Chlorella spp. for promotion of growth, it is essential to understand what kind of compounds are involved during this mutualistic interaction. Therefore, our hypothesis was that, during this synthetic mutualism, A. brasilense can use tryptophan and thiamine released in exudates of C. sorokiniana to produce IAA.

To test this hypothesis, our objectives were to measure: (i) production of IAA and consumption of tryptophan and the effect of added thiamine in the medium when C. sorokiniana and A. brasilense are co-immobilized in alginate beads and (ii) the effect of the mutualistic interaction on the activity of IPDC and tryptophan synthase and on the expression of the ipdC gene in A. brasilense that codifies for IPDC in the IAA production pathway.

MATERIALS AND METHODS

Microorganisms and initial growth conditions in prior experiments

The unicellular microalga C. sorokiniana (UTEX 2714, University of Texas, Austin, TX) (formerly designated as C. vulgaris) (see Bashan et al. 2016) and the bacterium Azospirillum brasilense Cd (DSM 1843, Leibniz-Institut DMSZ, Braunschweig, Germany) were used. To produce the inoculum, 10 mL of axenic culture of microalgae were inoculated into 90 mL sterile mineral medium (C30; Gonzalez, Canizares and Baena 1997). The culture was incubated for 7 days at 27 °C ± 2 °C at 140 rpm under light intensity of 60 μmol photon m−2 s−1. The bacterium was cultivated in BTB-2 medium (Bashan, Trejo and de-Bashan 2011). The culture was incubated at 32 °C ± 2 °C at 120 rpm for 16 h.

Immoblization of microorganisms

Microorganisms were immobilized individually and co-immobilized in alginate beads as described by de-Bashan et al. (2004). Using an automatic bead maker, beads of 2 mm diameter were formed (de-Bashan and Bashan 2010) and initial populations for experiments were adjusted according to de-Bashan et al. (2004). It is well established that immobilization of these microorganisms in alginate beads prevent their release from the beads, thus reducing error in counting data (Covarrubias et al. 2012). Close confinement forces interaction with each other (de-Bashan and Bashan 2008; de-Bashan, Hernandez and Bashan 2015).

Experimental culture conditions

After formulation and establishing initial populations of each microorganism in the bead for 24 h, the beads were washed three times in sterile saline solution to remove remnants of
the medium. For experiments, 20 g of beads with microorganisms either immobilized alone or co-immobilized, were inoculated in 150 mL synthetic growth medium (SGM) described in de-Bashan et al. (2011). For treatments with A. brasilense cultivated alone, the SGM was supplemented either with 1.5 μg mL⁻¹ synthetic L-tryptophan (#93659, Sigma-Aldrich, this concentration corresponds to the amount present in C. sorokiniana exudates; determination described later) or a mixture of 1.5 μg mL⁻¹ tryptophan and 70 ng mL⁻¹ thiamine diphosphate (C8754, Sigma-Aldrich) (Palacios et al. 2016a). In another treatment, immobilized bacteria were grown on 150 mL of the exudates produced by C. sorokiniana. To obtain the exudates, C. sorokiniana was cultured for 3 days under the conditions described earlier. The culture was centrifuged at 6000 × g for 5 min and then filtered through a 0.22 μm membrane (GSWP02500, EMD Millipore, Billerica, MA). At each step, the pellet was discarded. All experiments were stirred at 140 rpm at 27 °C ± 2 °C for 120 h.

Counting microorganisms in beads

For each treatment, three beads from each 250 mL flask were selected. Each bead was dissolved by immersion for ~30 min at ~28 °C in 1 mL citrate buffer containing (in mM): sodium citrate (55), EDTA anhydride (30) and NaCl (150), and adjusted to pH 8 with NaOH. Azospirillum brasilense cells were counted by the fluorescein diacetate method described by Chrzanowski et al. (1984) under a fluorescent microscope (BX41, Olympus, Tokyo). Chlorella sorokiniana was counted under a light microscope, using a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD).

Analytical methods

Determination of tryptophan in exudates of C. sorokiniana

To quantify tryptophan release by C. sorokiniana, the microalgae was cultured in SGM under autotrophic conditions (60 μmol photon m⁻² s⁻¹, stirred at 120 rpm) for 5 days. A total of 5 mL of culture medium (in triplicates) were sampled every 24 h and centrifuged at 6000 × g for 5 min. The pellet was discarded and the supernatant was filtered through a 0.22 μm membrane (GSWP02500, EMD Millipore) and analyzed by HPLC.

Determination of IAA and tryptophan with microorganisms immobilized alone and co-immobilized

Samples of 1 mL culture media from each treatment (per replicate and per treatment, n = 27) were taken every 24 h. These were filtered through a 0.22 μm membrane (GSWP02500, EMD Millipore) and analyzed by HPLC. Analyses of tryptophan and IAA were performed (Zakharova et al. 1999). Chromatograms were analyzed and recorded with HPCHEM integrating software (G2170BA; Hewlett-Packard, Wilmington, DE).

The HPLC system (Agilent 1100; Agilent Technologies) was equipped with a reversed phase column (TSKgel ODS-120A, 5 μm particle size, 150 × 46 mm; Supelco, Bellefonte, PA) and was run isocratically (all analyses used the same ratio of solvents), using the ratio of methanol:water:acetic acid (36:64:1 v/v) as the mobile phase. Injection volume was 100 μL; flow rate was 0.5 mL min⁻¹. The wave length for detection was 290 nm. The standards were: L-tryptophan (PHR1176, Sigma-Aldrich) and IAA (5148, Sigma-Aldrich). The limit of IAA detection by the HPLC method used was 195 ng mL⁻¹.

Enzymatic activity of indole pyruvate decarboxylase and tryptophan synthase β-subunit

To determine enzymatic activity of indole pyruvate decarboxylase (IPDC) (E.C.4.1.1.74) and tryptophan synthase (E.C.4.2.1.20), 6 g of alginate beads (1 g = 48 beads) from each treatment were sampled every 24 h. The beads were dissolved in 30 mL of citrate buffer and centrifuged at 2000 × g for 6 min. The supernatant was discarded and the pellet containing the cells was washed three times with sterile 0.85% saline solution. IPDC activity was determined according to Minamisawa et al. (1996): the cells were suspended in 10 mL potassium phosphate buffer (pH 8.2), containing 5 mM MgCl₂ (M8266, Sigma-Aldrich) and 1 mM thiamine diphosphate. Liquid nitrogen was added to the homogenates and the cells were disrupted with a pestle and mortar. The extract was centrifuged at 10 500 × g for 10 min. The reaction mixture, in a final volume of 500 μL, containing cell-free extract and 0.2 mM indole-3-pyruvic acid, was incubated for 30 min at 30 °C under shaking (120 rpm). The reaction was stopped with 0.1 M HCl with an equal volume of reaction mixture. The supernatant was analyzed by HPLC, as described above.

Tryptophan synthase β-subunit activity was determined according to Last et al. (1991): The cells were suspended in 2 mL 0.1 M potassium phosphate buffer at pH 8.2, containing 600 mg polyvinylpolypyrrolidone (#77627, Sigma-Aldrich). Liquid nitrogen was added to the homogenates and cells were disrupted with a pestle and mortar. The extract was centrifuged at 10 500 × g for 10 min. The reaction mixture, in a final volume of 1 mL, contained 400 μL of cell-free extract and 600 μL reaction solution (60 μmol L-serine [#84959, Sigma-Aldrich], 0.2 μmol indole-3-pyridoxal phosphate (#92955, Sigma-Aldrich) dissolved in 600 μL potassium phosphate buffer) and incubated at 30 °C with shaking of 120 rpm for 90 min. The reaction was stopped by adding 0.1 mL 0.2 M NaOH. The remaining indole was extracted into 4 mL toluene (#244511, Sigma-Aldrich) by gentle vortexing. After centrifugation for 15 min at 1500 × g, 0.5 mL of the toluene layer was added to 2 mL ethanol (#459844, Sigma-Aldrich) and 1 mL Ehrlich’s Reagent (#159477, Sigma-Aldrich). The color developed for 30 min at room temperature and the product was measured spectrophotometrically at 540 nm.

RNA isolation, cDNA synthesis and quantitative Real Time PCR

To determine the expression of the ipdC gene, 18 g of alginate beads from each treatment were sampled. The beads were dissolved in 100 mL citrate buffer and centrifuged at 2000 × g for 6 min. The supernatant was discarded and the pellet containing the cells was washed two times with citrate buffer to eliminate remnant alginate. The pellet was then washed three times with sterile saline solution. The pellet was homogenized with TRIzol (#94242- Sigma-Aldrich) and kept at −80 °C for 20 min. The frozen sample was mixed vigorously by vortexing for 30 sec to extract the RNA. Purified RNA was quantified with a ND-1000 NanoDrop UV Spectrophotometer (Thermo Scientific, Waltham, MA). Total RNA (1 μg) was treated with DNase I (Invitrogen, Carlsbad, CA) and cDNA synthesis was done in a final volume of 20 μL containing 1.5 mM MgCl₂, 1X reaction buffer, 500 ng random primers, 500 μM dNTP mix, 20 U recombinant RNasin Ribonuclease and 1 μL ImProm-II Reverse Transcription System (Promega, Madison, WI) and stored at −20 °C. Primers (Table 1) were designed based on ipdC GenBank sequence (NC0156417.1); two potential reference genes (recA and gylA; McMillan and Pereg 2014) were included. In the absence of a complete genome sequence of C. sorokiniana (Krienitz, Huss and Bock 2013), primers for tryptophan synthase are not yet available. For each standard curve,
Table 1. Sequences of primers of target and constitutive genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ipdC</td>
<td>Forward: 5′-AGTCGTCCAGGTCGTTGAAG-3′</td>
<td>95.9</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-ACCCCATCGTGATCCTG-3′</td>
<td></td>
</tr>
<tr>
<td>recA (McMillan and Pereg 2014)</td>
<td>Forward: 5′-GTCGAACTGCCTGGTGAT-3′</td>
<td>97.2</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GACGGAGGCGTAGAACTT-3′</td>
<td></td>
</tr>
<tr>
<td>glyA (McMillan and Pereg 2014)</td>
<td>Forward: 5′-GGAGATCGCCAAGAAGAT-3′</td>
<td>96.6</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GCTCTTGGCGTAGGTCTT-3′</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Tryptophan production (filled circle) and growth (open circle) of Chlorella sorokiniana growing alone in synthetic growth medium. Values denoted by letters for each curve separately differ significantly, using one-way ANOVA and LSD post-hoc analysis at $P < 0.05$. Whisker lines represent SE.

the efficiency was estimated using the equation: $E = (10^{-1/slope}) - 1$ (Bustin et al. 2009). Standard curves were obtained from serial dilutions (dilution factor 1:2) of cDNA pool. The quantitative Real Time PCR were conducted in triplicate for all genes, using SsoFast Evagreen Supermix (#1725200, Bio-Rad Laboratories, Hercules, CA) on a Rotor-Gene 6000 real-time qPCR machine (Qiagen, Hilden, Germany): 95 °C for 1 min; 95 °C for 10 s; 60 °C for 15 s; 72 °C for 20 s (40 cycles); 72 °C for 5 min. At the end, a dissociation step from 75 °C to 99 °C (stepped at 1 °C s$^{-1}$) was performed to ensure the absence of artifacts and primer dimmers and verify the specificity of the PCR product. Results were analyzed using Rotor-Gene 6000 1.7 software. For determining changes in relative gene expression, data were analyzed with the $2^{-\Delta\DeltaCT}$ algorithm (Livak and Schmittgen 2001).

Experimeantal design and statistical analysis

Each experiment was independently repeated twice. The setup of each experiment was in batch culture. Each individual experiment was performed in triplicates, where each 500 mL Erlenmeyer flask, containing 300 mL SGM served as a replicate. Each setup contained six treatments: (i) beads containing C. sorokiniana and A. brasilense, (ii) beads containing only C. sorokiniana, (iii) beads containing only A. brasilense, (iv) beads containing A. brasilense in SGM supplemented with tryptophan, (v) beads containing A. brasilense in SGM supplemented with tryptophan and thiamine and (vi) beads containing A. brasilense containing exudates of C. sorokiniana. The last five treatments served as controls. The following variables were analyzed: tryptophan production by C. sorokiniana, tryptophan consumption and IAA production in the media by A. brasilense, activity of tryptophan synthase and IPDC and expression of the ipdC gene. Data from each treatment from the two independent experiments ($n = 30$) were combined for statistical analysis, first by one-way ANOVA and then by LSD post-hoc analysis. Significance was set at $P < 0.05$, using Statistica 6.0 software (StatSoft, Tulsa, OK).

RESULTS

Release of tryptophan by free cultures of C. sorokiniana

Cultures of C. sorokiniana produced tryptophan during the first 72 h of growth, with the highest release ($1.5 \mu g \text{mL}^{-1}$) at this time (Fig. 1). This concentration was added later to the treatments of A. brasilense growing in SGM enriched with either synthetic tryptophan, or synthetic tryptophan and thiamine.

Tryptophan consumption and IAA production by A. brasilense

As expected, we could not detect IAA in the SGM when A. brasilense was grown alone in the absence of tryptophan (data not shown). Under these conditions, A. brasilense showed higher growth rates after 72 h and then decreasing growth rates after 120 h (Fig. 2C). When the SGM was supplemented with tryptophan, A. brasilense consumed it in full until 120 h, but no release of IAA was observed (Fig. 2A).
When thiamine was added along with tryptophan to the SGM, all tryptophan was consumed by *A. brasilense* after 72 h, and IAA release was observed (0.448 ± 0.165 μg mL⁻¹) after 96 h (Fig. 2A). Growth of *A. brasilense* in SGM, enriched with tryptophan and thiamine, reached its stationary growth phase after 72 h (Fig. 2C). When *A. brasilense* was grown in exudates of *C. sorokiniana*, tryptophan was rapidly consumed in the first 48 h (Fig. 2A). Yet, a higher release was measured at 72 h (0.557 ± 0.319 μg mL⁻¹) (Fig. 2A). Under these conditions, *A. brasilense* reached the stationary phase growth at 48 h (Fig. 2C). When *A. brasilense* is co-immobilized with *C. sorokiniana*, no tryptophan or IAA was detected in the SGM; therefore, no data is presented in Fig. 2A. Nevertheless, when both are co-immobilized, we observed an increase in the population of *C. sorokiniana* during the first 48 h (Fig. 2B).

**Tryptophan synthase activity**

Tryptophan synthase activity in the treatments of *Chlorella*, whether immobilized alone or co-immobilized with *A. brasilense*, was cyclic, with periods of low and high activity. However, the time at which each treatment had higher enzymatic activity was different. Generally, activity of tryptophan synthase in the microalgae co-immobilized with *A. brasilense* was higher in the first 24 h of interaction (34.29 ± 6.467 U), which is interpreted as tryptophan production in the beginning of the interaction, and
Figure 3. Tryptophan synthase activity in Chlorella sorokiniana immobilized alone (filled circle) and co-immobilized with Azospirillum brasilense Cd (filled triangle) and in A. brasilense alone (filled square). Values denoted by different capital letters differ significantly at various sampling times, where values at each sampling time that is denoted by different lower case letters differ significantly, using one-way ANOVA and LSD post-hoc analysis at $P < 0.05$. Whisker lines represent SE. Absence of a line indicates negligible SE.

Expression of the ipdC gene

The most stable reference gene for normalization during quantitative analysis of ipdC expression was recA. This gene was used to establish differences in relative expression between treatments. Relative transcript levels of ipdC mRNA were significantly overexpressed ($P > 0.05$) at 48 h in A. brasilense grown in exudates of C. sorokiniana (Fig. 5A). When both microorganisms were co-immobilized, ipdC mRNA was over-expressed at 96 h (Fig. 5B). Nevertheless, in all other treatments at both times, the expression of ipdC was significantly repressed ($P > 0.05$).

DISCUSSION

Production of IAA by the PGPB Azospirillum brasilense is a main mechanism for promoting growth in plants and microalgae (Dobbelare et al. 1999; de-Bashan et al. 2008; Bashan and de-Bashan 2010). Although some bacteria can degrade IAA, the importance of this metabolism in bacterial cell is unclear (Spaepen and Vanderleyden 2011); yet, it is widespread among PGPB (Duca, Lorv and Patten 2014). Therefore, production and extrusion of IAA by A. brasilense indicate potential manipulation by bacteria of the metabolism of responsive microalgae. Studies indicate that during mutualistic interaction between plants or microalgae with a PGPB, there is a cascade of events, where the precursor tryptophan needed for IAA production is produced and released by the plant or the microalgae (Zakharova et al. 1999; Carreno-Lopez et al. 2000). This allows production of IAA in the PGPB. Then, the IAA is released and promotes plant growth and production of more tryptophan. This feedback declined after 72 h of incubation (Fig. 3). A second production of tryptophan occurred after 96 h of interaction, reaching a peak of tryptophan synthase activity at 34.82 ± 13.51 U, comparable to the first 24 h of interaction. When the microalgae was immobilized alone, the tryptophan synthase activity showed highest activity at 48 h of incubation (34.17 ± 1.503 U), which corresponds with tryptophan production (Fig. 1), and then decreased, but increased again at 120 h of incubation (Fig. 3). Enzymatic activity by A. brasilense was negligible (Fig. 3).

IPDC activity

The highest activity of IPDC occurred when A. brasilense was co-immobilized with C. sorokiniana at 72 h (2.581 ± 0.19 µg IAA mg protein$^{-1}$), decreased in the next 24 h, but increased again at 120 h (Fig. 4, capital letter analysis). Similarly, when A. brasilense was immobilized alone and grown in exudates of C. sorokiniana, the highest activity of IPDC was recorded at 72 h (1.35 ± 0.12 µg IAA mg protein$^{-1}$), and then declined over time. Growing in SGM without tryptophan and thiamine, A. brasilense immobilized alone showed an increase in IPDC activity, also at 72 h (0.992 ± 0.023 µg IAA mg protein$^{-1}$) and then declined (Fig. 4). Yet, the IPDC activity was significantly lower than the activity of IPDC shown by A. brasilense co-immobilized with C. sorokiniana or immobilized alone and growing in the exudates (Fig. 4, lower case analysis). In SGM enriched only with tryptophan (without supplementation of thiamine), the activity of IPDC in A. brasilense was low at all intervals during incubation. However, when thiamine was added to SGM with tryptophan, the activity of IPDC in A. brasilense increased over time and reached their highest level at 120 h (0.587 ± 0.01 µg IAA mg protein$^{-1}$) (Fig. 4).

The microalgae C. sorokiniana immobilized alone also showed IPDC activity, peaking at 120 h (0.402 ± 0.035 µg IAA mg protein$^{-1}$). Yet, the amount of IPDC activity showed by C. sorokiniana at all other sampling intervals were lower than the activity by A. brasilense grown with this microalga or when this bacterium was grown alone in exudates of C. sorokiniana.
mechanism progresses in a close loop during the interaction of the two organisms (Kamilova et al. 2006; Idris et al. 2007). IAA affects several metabolic pathways in this microalgae (de-Bashan et al. 2008; Meza et al. 2015; Meza, de-Bashan and Bashan 2015). Here, we demonstrated that tryptophan and thiamine in exudates produced by *C. sorokiniana* (Palacios et al. 2016a) are precursors for producing and releasing IAA during their mutualistic interaction.

Although bacteria produce small amount of endogenous tryptophan, generally, cellular tryptophan levels in bacteria are at a concentration of ~0.004 ng·mL⁻¹ (Patten, Blakney and Coulson 2012), to produce large quantities of IAA, different bacteria require varying amounts of exogenous tryptophan supplementation (Duca, Lorv and Patten 2014). This likely corresponds to each bacterium’s inherent ability to synthesize tryptophan endogenously. Endogenous tryptophan reserves in bacteria are first used for protein synthesis, while the production of secondary metabolites, such as IAA, requires an additional nitrogen source (Duca, Lorv and Patten 2014). Addition of tryptophan to the bacterial growth medium yielded 6–8-fold more IAA by *A. brasilense* Sp7 than was produced by a culture grown without tryptophan supplementation (Zimmer and Bothe 1988). In our study, *A. brasilense* produced IAA only when growing on either exudates of *C. sorokiniana* (which contain tryptophan) or on SGM medium enriched with tryptophan and thiamine. In one study, L-tryptophan present in exudates of duckweed, *Lemna minor*, is a precursor of IAA. This suggests a close symbiotic relationship between the macrophyte and the colonizing *Bacillus amyloliquefaciens* (Idris et al. 2007).

Thiamine is known as a cofactor with the main enzyme in the biosynthesis of IAA (Koga, Adachi and Hidaka 1992; Jurgenson, Begley and Ealick 2009) and for numerous other enzymes of central cell metabolism. Minamisawa et al. (1996) showed that, in the PGPR *Bradyrhizobium elkanii*, adding thiamine and Mg²⁺ provided a 20-fold increase in the concentration of indole-3-acetaldehyde, a precursor in IAA biosynthesis. In our study, supplementing thiamine to a medium containing tryptophan allows IAA production by *A. brasilense* and also enhanced IPDC activity and tryptophan uptake from the medium. Thiamine production for the microalgae as a mere response to general enhancement in metabolic activity occurring during this synthetic mutualism is unlikely because, in this interaction, more thiamine is produced under conditions of stress (Palacios et al. 2016a).

During development of synthetic mutualism, the interaction got stronger over time (de-Bashan et al. 2011). The paradigm of mutualism is that during interaction, both microorganisms produce compounds that are used by the other, as is the case of tryptophan and IAA in this synthetic mutualism. The absence of tryptophan or IAA in the culture medium, when both microorganisms were co-immobilized, can be explained: no extra tryptophan or IAA is produced, apart from the exact quantities needed to sustain the interaction. In such a scenario, tryptophan is consumed by *A. brasilense* and converted to IAA. IAA is directly transferred to the cells of *C. sorokiniana* and does not leak to the medium. Thus, analysis of the medium showed no free IAA. Such intimate transfer was recently shown for unidentified C and N molecules in this interaction (de-Bashan et al. 2016).

This explanation is additionally supported by the fact that we measured an increase in IPDC activity in the co-immobilized treatment after 72 h, together with increased tryptophan synthase activity. The latter is typical for tryptophan production by *C. sorokiniana* at 72 h onward. Finally, as supportive evidence for IAA production, we found that when *A. brasilense* was cultured in...
exudates of C. sorokiniana, but in the absence of the microalgae, it released abundant IAA to the medium.

Under natural conditions, tryptophan extruded by C. sorokiniana can attract a large variety of bacterial species, including those living in its phycosphere (Gonzalez-Bashan et al. 2000). Yet, only bacteria capable of converting tryptophan to IAA, such as A. brasilense in this study, can create a positive feedback loop between microalgal-tryptophan and bacterial IAA. In our study, the absence of competing bacteria in this synthetic mutualistic assemblage ensured maximum, mutual signal exchange between the two microorganisms.

The tryptophan synthase β-subunit catalyzes the last step in the tryptophan synthesis. The tryptophan inhibits this enzyme as a feedback-inhibiting effect (Swift and Stewart 1991). In our study, the activity of the β-chain in tryptophan synthase in C. sorokiniana, when grown alone, is highest at 48 h. Decline in tryptophan synthase activity, corresponding with the highest amount of tryptophan in exudates of this microalga, indicates feedback inhibition. Synthetic mutualism between C. sorokiniana and A. brasilense led to a change in the microalga’s metabolism, specifically in AGPase (Choix et al. 2014) and amylase (Palacios et al. 2016b) after 24 h. Our study shows that this interaction also affects tryptophan production by C. sorokiniana.

IPDC is an α-keto acid decarboxylase with broad substrate specificity that metabolizes phenylpyruvate, pyruvate and benzoylformate and also produces indole-3-acetaldehyde, the IAA precursor. Therefore, it was suggested that the production of the secondary metabolite IAA is not its main role (Duca, Lorv and Patten 2014 and references therein). This nonspecific function of the IPDC can explain high enzymatic activity of IPDC in A. brasilense growing in SGM without adding tryptophan; therefore, yielding no IAA production. Indole acetaldehyde can be used to support the growth of the PGPB Pseudomonas putida in a minimal medium (Leveau and Lindow 2005). In our work, production of indole acetaldehyde could be used as energy source for the growth of A. brasilense.

Analyzing the increase of IPDC activity in the co-immobilized treatment with data on ipdC gene expression, at this point of our knowledge, it can only be suggested that the main contribution of IPDC activity at 72 h is by C. sorokiniana and at 120 h by A. brasilense. Under co-immobilization, the ipdC gene is repressed at 48 h, but over-expressed in A. brasilense growing in exudates. Therefore, the contribution of IPDC activity reflected at 72 h in the co-immobilized treatment cannot be by A. brasilense, but by C. sorokiniana. At 96 h, the ipdC gene is over-expressed in A. brasilense; therefore, the main activity of IPDC obtained at 120 h is from bacterial origin.

Production of IAA by PGPB occurs during the stationary growth phase (Duca, Lorv and Patten 2014). The addition of tryptophan to cultures of A. brasilense increased the length of the logarithmic phase and allows an increase in cell concentration (Ona et al. 2005). This study gives further support to this hypothesis, but from the synthetic mutualism point of view. When A. brasilense reaches its stationary growth phase under three growing conditions: (i) growth on exudates of C. sorokiniana, (ii) in SGM medium enriched with tryptophan and thiamine, or (iii) co-immobilized with C. sorokiniana, either IAA production and/or IPDC activity increases, or over-expression of ipdC gene occurs.

This study provides broader significance than solely explaining a signaling mechanism under specific synthetic mutualism. Tryptophan and its derivatives are common signaling molecules in marine environments (Azam and Malfatti 2007). In a recent study, a similar signaling mechanism (tryptophan-IAA) was found in the globally-distributed diatom Pseudo-nitzschia.
multiseries and a marine bacterium, Sulfitobacter sp. The bacterium promoted cell division in the diatom via secretion of bacterial IAA (Amin et al. 2015). Our study further expanded on the emerging recognition that tightly connected microbial communities, natural or constructed, complex or simple, are controlled through production and exchange of information metabolites.

In summary, this study, combined with our previous studies of IAA in synthetic mutualism, demonstrated that tryptophan and thiamine released by C. sorokiniana are signal molecules that can be used by A. brasilense for synthesizing another signal molecule, IAA, which, when released, facilitates growth of microalgae. Occurrence of signaling compounds, such as tryptophan and thiamine in exudates of C. sorokiniana, support a synthetic mutualistic interaction of C. sorokiniana with the PGPB A. brasilense.

ACKNOWLEDGEMENTS

At CIBNOR, Manuel Moreno and Juan Pablo Hernandez provided technical support, Fernando García Carreño facilitated the use of HPLC and Ira Fogel provided English and editorial suggestions. Alejandro Palacios of the Autonomous University of Baja California Sur provided advice on statistical analysis. This is contribution 2016-012 from Bashan Institute of Science (USA).

FUNDING

This study was supported by Consejo Nacional de Ciencia y Tecnología of Mexico [CONACYT-Basic Science-2009, grant 164548] and time for writing by The Bashan Foundation, USA. O.P. was mainly supported by graduate fellowships from CONACYT (226169) and small periodic grants from The Bashan Foundation.

Conflict of interest. None declared.

REFERENCES

Choix FJ, de-Bashan LE, Bashan Y. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized Chlorella spp. induced by Azospirillum brasilense: II. Heterotrophic conditions. Enzyme Microb Tech 2012b;51:300–9.
de-Bashan LE, Hernandez J-P, Bashan Y. Interaction of Azospirillum spp. with microalgae; a basic eukaryotic-prokaryotic model and its biotechnological applications. In: Cassán FD,


Leyva LA, Bashan Y, de-Bashan LE. Activity of acetyl-CoA carboxylase is not directly linked to accumulation of lipids when Chlorella vulgaris is co-immobilised with Azospirillum brasilense in alginate under autotrophic and heterotrophic conditions. Ann Microbiol 2015;65:339–49.


Palacios OA, Choix FJ, Bashan Y et al. Influence of tryptophan and indole-3-acetic acid on starch accumulation in the


