Original article

Growth promotion of the freshwater microalga Chlorella vulgaris by the nitrogen-fixing, plant growth-promoting bacterium Bacillus pumilus from arid zone soils

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A B S T R A C T
Immobilization of Bacillus pumilus ES4 from arid land soils, a plant growth-promoting bacterium and the freshwater, green microalga Chlorella vulgaris enhanced microalgal growth only in the absence of combined nitrogen in synthetic growth medium (SGM), but not in medium with combined nitrogen. B. pumilus was able to fix nitrogen in N-free SGM and its growth yielded an accumulation of ammonium in the medium. On its own, B. pumilus is a poor agent for removing nitrogen and phosphorus from wastewater, while C. vulgaris is a capable microorganism. By jointly immobilizing the two microorganisms, the capacity to remove nitrogen and phosphorus from the medium by the microalgal culture was not enhanced, but, at the cell level, removal of these nutrients was significantly enhanced. It appears that growth promotion induced by B. pumilus on C. vulgaris is related to nitrogen fixation.

E U R O P E A N  J O U R N A L  O F  S O I L  B I O L O G Y  4 5  ( 2 0 0 9 )  8 8 – 9 3

1. Introduction
Microalgae are a very large group of microscopic algae, primary producers on a global scale, and involved in all marine and freshwater ecosystems, wastewater treatment, and some soil processes. Growth promotion of microalgae by microalgal growth-promoting bacteria has been reported for a few strains of two species of the microalga Chlorella vulgaris and C. sorokiniana and several strains of terrestrial Azospirillum spp. [13,15,21], as well as for few aquatic bacteria and microalgae, mainly phytoplankton [1,20,32,38,42,43]. Consequently, it has not been established whether growth promotion of Chlorella is a unique characteristic of species of Azospirillum or if this is a wider phenomenon.

Growth promotion of agricultural and wild plants by plant growth-promoting bacteria (PGPB) [3] is commonplace,
involving different plant-bacteria mechanisms in which the end product of these numerous associations is a better plant feature, usually depending on the usefulness of the plant for human consumption [2]. Promotion of aquatic microalgae by bacteria, although revealed initially decades ago [44], is an emerging field in which almost all studies have been conducted in recent years [14,15,21,45]. The main interest in this artificial association and in joint associations of microalgae and bacteria in general, so far, has been because the community associations were better at removing pollutants from wastewater [11,12,25,33] than microalgae alone [8,9,16] or the microalgae grew better when they were used in aquaculture [20].

The hypotheses of this exploratory study were that: (1) there are other PGPB than Azospirillum, a common PGPB for crop plants [5], capable of promoting the growth of the microalga and these do not necessarily originate from the aquatic natural habitat of the microalgae; (2) the interaction of microalgae and PGPB are not specific; this study employed a nitrogen-fixing PGPB, Bacillus pumilus ES4, originally isolated from the rhizoplane of an arid land cactus; and (3) the mechanism by which this operates relates to its nitrogen-fixing ability.

2. Material and methods

2.1. Microorganisms and initial growth conditions

Prior to immobilization in beads, 10 ml of axenic Chlorella vulgaris Beijerinck UTEX 2714 were inoculated into 100 ml of sterile mineral medium C30 and incubated at 27 ± 2 °C and stirred at 140 rpm under light intensity of 60 μmol photon m⁻² s⁻¹ for 7 days [22].

Bacillus pumilus ES4 [35] (FJ032017, NBCR) was used in these experiments. The bacteria were stored in liquid nitrogen and, for daily use, were kept on tryptic soy slants (Sigma, St. Louis, MO). Two days before immobilization, a loop of B. pumilus was transferred to 25 ml of liquid tryptic soy broth (Sigma) and incubated overnight at 30 ± 1 °C and agitated at 120 rpm. The day before immobilization, 3–4 ml of pre-inoculum were introduced into 50 ml of fresh tryptic soy broth and incubated at 30 ± 1 °C for 18 h at 120 rpm. Cells were harvested by centrifugation at 1000 × g for 20 min. The pellet was suspended in 0.85% saline solution to a final concentration of 10⁵ colony-forming units (cfu) ml⁻¹.

2.2. Immobilization of C. vulgaris and B. pumilus in alginate beads

Microorganisms were immobilized according to the method described by de-Bashan et al. [12]. Briefly, axenic cultures (either C. vulgaris or the PGPB B. pumilus) were mixed with 2% alginate solution. The solution was dripped from a sterile syringe into 2% CaCl₂ solution, with periodic mixing of the solution. For joint immobilization of the two microorganisms in the same bead, after washing the cultures, each of them was re-suspended in 10 ml of 0.85% saline solution and then mixed together in the alginate before forming the beads. Because immobilization normally reduces the number of B. pumilus cells in the beads, a second, overnight incubation in diluted nutrient broth was necessary.

2.3. Culturing conditions for joint immobilization of microorganisms, solubilization of beads, and cell counts

Initial concentration of ammonium was 10 mg l⁻¹ NH₄Cl; initial concentration of phosphorus was 35.5 mg l⁻¹ PO₄³⁻. Experiments were carried out in SGM [21] with and without dissolved nitrogen. The medium did not contain tryptophan. After secondary multiplication of the microorganisms inside the beads, the beads were washed twice with saline solution (0.85% NaCl) and beads weighing 40 g were added to 200 ml of SGM. Batch cultures were incubated for 5 days in Erlenmeyer flasks at 28 °C with continuous stirring at 140 rpm under light intensity of 60 μmol m⁻² s⁻¹. Cells were released from the beads and counted, using five beads solubilized by immersion in 5 ml of 4% sodium bicarbonate for 30 min at room temperature (24–26 °C). B. pumilus was counted using fluorescein diacetate (FDA) stain [27]. The slides were...
observed and counted with an episcopic fluorescent microscope (Leitz Laborlux-S, Wetzlar, Germany) and C. vulgaris was counted with a Neubauer hemocytometer.

2.4. Ammonium and phosphorus analyses

Removal and accumulation of ammonium ions were measured by the salicylate method and removal of phosphorus by the molybdenum blue method, both standard water analysis techniques using special kits (Hach, Loveland, CO, USA) and spectrophotometer (Hach, Model DR 4000).

2.5. Nitrogen-fixing measurement

To measure the nitrogen-fixing activity of bacteria, 10% acetylene was injected into flasks showing bacterial growth. Acetylene reduction assay was done by gas chromatography as described by Holguin et al. [26]. Results are expressed as nmol ethylene (10^4 cfu)^−1 h^−1.

2.6. Experimental design and statistical analysis

All experiments were carried out in batch culture in Erlenmeyer flasks using SGM with and without nitrogen for 5 days under the cultivation conditions described in Section 2.3. Each treatment was done in triplicate using one Erlenmeyer flask as a replicate. Each experiment was repeated three or four times in full. Beads without microorganisms were prepared similarly to serve as controls. Results of all experiments, showing normal distribution, were analyzed by one-way ANOVA and then by Tukey's HSD post-hoc analysis at the P ≤ 0.05 significance level or by Student's t-test at P ≤ 0.05. STATISTICA software was used (Statsoft, Tulsa, OK, USA).

3. Results

When B. pumilus was immobilized in alginate beads, it fixed nitrogen at an intermediate level of 58 ± 0.1 nmol ethylene flask^−1 h^−1. When immobilized with C. vulgaris, the level of detected nitrogen fixation in the medium was reduced. In the absence of combined nitrogen in the medium, B. pumilus did not fix any nitrogen from the air (Fig. 1A). The level of ammonium in microalgae-free medium (B. pumilus growing alone) accumulated with time. C. vulgaris, by itself, did not contribute ammonium to the medium. Immobilizing the two microorganisms together slightly increased ammonium accumulation, but at very low levels (Fig. 1B).

In the presence of combined nitrogen in the medium, B. pumilus did not induce any growth promotion in C. vulgaris and both systems (microorganisms immobilized alone and jointly immobilized) developed similar high populations at levels of about 7 × 10^6 after incubation for 96 h (Fig. 2A). In the absence of combined nitrogen in the medium, C. vulgaris grew slowly. Joint immobilization of B. pumilus with the microalga enhanced the growth of the microalga after 48 h and afterwards (Fig. 2B). B. pumilus also grew in this medium (Fig. 2C).

C. vulgaris was capable of removing ammonium and phosphorus from the medium, but B. pumilus was a poor remover of both nutrients. Joint immobilization of C. vulgaris with B. pumilus did not enhance removal of combined nitrogen and phosphorus by the microalgal cultures (Fig. 3A, B). However, by calculating removal of ammonium and phosphorus on a per-cell basis of the microalgae, removal of these two nutrients significantly increased during joint immobilization with the PGPB (Tables 1 and 2).

4. Discussion

The mechanisms by which PGPB (nonbiocontrol-PGPB) [3] affect plant growth varied greatly. PGPB directly affect the metabolism of plants by providing substances that are usually in short supply. These bacteria are capable of fixing atmospheric nitrogen, solubilizing phosphorus and iron, and producing plant hormones, such as auxins, gibberelins, cytokinins, and ethylene and nitrite and nitric oxide. Additionally,
they improve a plant’s tolerance to stress, such as drought, high salinity, metal toxicity, and pesticide load. One or more of these mechanisms may contribute to the increases in plant growth and development that are higher than normal for plants grown under conditions of standard cultivation [2,5,30,40]. Most PGPB Bacillus spp. operate via control of soilborne diseases [28], but some bacilli promote plant well-being directly in the absence of a disease [4,6,7,19,24,34,36,37,41]. The possible interactions of Bacillus spp. with microalgae are unknown.

So far, Azospirillum is the only genus of bacteria known to promote microalgae growth (MGPB) [12,21]. Azospirillum spp. significantly alter the metabolism of microalgae [10] mainly by producing indole-3-acetic acid (IAA) [15] and enhancing enzymes of the nitrogen cycle in the microalgae [16]. Although inoculation of freshwater and marine phytoplankton with bacteria sometimes enhances their productivity [20,43,44], these studies are descriptive and exploratory and no mechanisms for the phenomenon have been demonstrated. Microalgal growth promotion by bacteria notwithstanding, not all interactions are positive; the interaction of C. vulgaris with its bacterium Es4, originally isolated from the rhizoplane of a cactus was tested. This PGPB fixed atmospheric nitrogen [35], produced IAA in vitro in the presence of tryptophan, produced siderophores, and efficiently enhanced growth of cardon cactus seedlings over prolonged periods [36].

The PGPB Bacillus pumilus Es4 proved to be an MGPB capable of promoting the growth of microalgae and enhancing the capacity of individual microalgae cells to absorb nitrogen and phosphorus. However, the jointly-immobilized culture was not useful for the common use of C. vulgaris in wastewater treatment [17]. It did not enhance the capacity of the joint culture to remove pollutants from wastewater as does Azospirillum spp. [11,12,25].

Growth promotion, in the case of B. pumilus, was restricted to the absence of nitrogen, a condition that largely prohibited the growth of the microalgae. Chlorella spp. are capable of growing without combined nitrogen for a limited time, as ammonium can be produced and recycled inside the organism by a variety of metabolic pathways, such as photorepiration, phenylpropanoid metabolism, use of nitrogen transport compounds, and amino acid catabolism [18]. In this regard, the growth of Chlorella in the absence of other microorganisms can be explained by the dissimilatory activity of the enzyme glutamate dehydrogenase. This enzyme serves as a link between carbon and nitrogen metabolism because it is

### Table 1 – Removal of ammonium per cell of Chlorella vulgaris when jointly immobilized with Bacillus pumilus in synthetic wastewater.

<table>
<thead>
<tr>
<th>Incubation period (h)</th>
<th>Removal of NH₄⁻ (µg cell⁻¹)</th>
<th>Alone</th>
<th>Jointly immobilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.87 ± 0.05 Aa</td>
<td>1.01 ± 0.05 Aa</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>3.00 ± 0.37 Ab</td>
<td>3.26 ± 0.43 Bb</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>4.62 ± 0.56 Ac</td>
<td>6.17 ± 0.69 Bc</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>5.05 ± 0.27 Ad</td>
<td>5.48 ± 0.76 Bd</td>
<td></td>
</tr>
</tbody>
</table>

Points for each incubation period denoted by a different capital letter differ significantly by Student’s t-test at P ≤ 0.05. Columns for each treatment, denoted by a different lower case letter, differ significantly by one-way ANOVA and Tukey’s post-hoc analysis at P ≤ 0.05. ± represents standard error.

### Table 2 – Removal of phosphorus per cell of Chlorella vulgaris when jointly immobilized with Bacillus pumilus in synthetic wastewater.

<table>
<thead>
<tr>
<th>Incubation period (h)</th>
<th>Removal of PO₄³⁻ (µg cell⁻¹)</th>
<th>Alone</th>
<th>Jointly immobilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.261 ± 0.393 Aa</td>
<td>2.495 ± 0.423 Ba</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.684 ± 0.169 Ad</td>
<td>0.421 ± 0.190 Bd</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1.882 ± 0.259 Ab</td>
<td>2.065 ± 0.184 Bb</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>1.384 ± 0.145 Ac</td>
<td>1.338 ± 0.168 Ac</td>
<td></td>
</tr>
</tbody>
</table>

Points for each incubation period denoted by a different capital letter differ significantly by Student’s t-test at P ≤ 0.05. Columns for each treatment, denoted by a different lower case letter, differ significantly by one-way ANOVA and Tukey’s post-hoc analysis at P ≤ 0.05. ± represents standard error.
the metabolism of nitrogen in previous studies, we found that this enzyme plays a key role in the metabolism of nitrogen in Chlorella when immobilized with the PGPB Azospirillum brasilense [16]. In our present study, when combined nitrogen was present in the wastewater medium, there was no apparent promotion of growth by the MGPB. This might indicate that the bacteria’s potential for producing IAA was not employed, probably because the synthetic wastewater did not contain tryptophan, the precursor of IAA in this species. However, in the absence of combined nitrogen, this species was capable of accumulating sufficient ammonium in the medium that, in the presence of the microalgae, was consumed, probably translating into enhanced microalgae mass. Therefore, the most likely mechanism by which B. pumilus Es4 promotes the growth of C. vulgaris is nitrogen fixation under conditions of severe nitrogen starvation.

In summary, this study shows that useful MGPB can be found even in the most unlikely habitats. Therefore, this artificial association may be tested for a variety of PGPB and not necessarily only aquatic PGPB. However, promoting the growth of microalgae does not always apply to common biotechnological applications of the microalgae.

Acknowledgments

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References


[21] L.E. Gonzalez, Y. Bashan, Growth promotion of the microalgae Chlorella vulgaris when coimmobilized and

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