Changes in the metabolism of the microalga *Chlorella vulgaris* when coimmobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum*

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Abstract: In an agroindustrial wastewater pond, a naturally occurring unicellular microalga, *Chlorella vulgaris*, was closely associated with the terrestrial plant-associative N_2-fixing bacterium *Phyllobacterium myrsinacearum*. When the two microorganisms were artificially coimmobilized in alginate beads, they shared the same internal bead cavities, and the production of five microalgal pigments increased, but there were no effects on the number of the cells or the biomass of the microalga. The association, however, reduces the ability of *C. vulgaris* to remove ammonium ions and phosphorus from water. The bacterium produced nitrate from ammonium in synthetic wastewater with or without the presence of the microalga, and fixed nitrogen in two culture media. Our results suggest that interactions between microalgae and associative bacteria should be considered when cultivating microalgae for wastewater treatment.

Key words: alginate, bacterial immobilization, microalgae, nitrogen fixation, *Phyllobacterium*, wastewater treatment.

Resume : Dans les bassins d'eau usée d'origine agroindustrielle, on retrouve naturellement l'algue microscopique *Chlorella vulgaris* et celle-ci est directement associée à la bactérie du sol *Phyllobacterium myrsinacearum* impliquée dans la fixation de N_2 chez les plantes. Lorsque ces deux microorganismes étaient artificiellement co-immobilisés dans des billes d'alginate, ils se retrouvaient aux mêmes endroits à l'intérieur des billes et on observait une augmentation de la production de cinq pigments par cette algue microscopique, mais il n'y a pas eu de changement au niveau du nombre et de la biomasse des algues. Par contre cette association entraînait une diminution de la capacité de *C. vulgaris* à éiminer le phosphore et les ions ammonium de l'eau usée. Cette bactérie produisait des nitrates à partir d'eaux usées synthétiques contenant on non cette algue microscopique et elle fixait 1'azote dans ces deux milieux de culture. Nos résultats indiquent qu'il serait important de prendre en consideration les interactions algues-bactéries lors de la culture d'algues microscopiques destinées au traitement des eaux usées.

Mots clés : alginate, immobilisation de bactéries, algues microscopiques, fixation d'azote, *Phyllobacterium*, traitement d'eau usée.

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Introduction

The freshwater unicellular microalga *Chlorella vulgaris is* used in tertiary wastewater treatment for removal of nitrogen and phosphorus compounds as well as heavy metals (Oh-Hama and Miyachi 1992; Aksu et al. 1992; Tam et al. 1994, 1998). In Colombia, it has been used experimentally for removal of ammonium ion and phosphorus from agroindustrial wastewater in nonaxenic bioreactors (Gonzalez et al. 1997). It is also used for several industrial processes such as formation of keto acids from amino acids (Kayano et al. 1981; Wikstrom et al. 1982). Because the wastewater used is nonsterile, and incubation periods are commonly long, contamination or natural association of the microalga culture by indigenous bacteria is inevitable. Most wastewater treatment studies using microalgae largely ignored the possibility that such associations might significantly affect the performance of the wastewater treatment agent, the microalgae (Cañizares et al. 1994; Chevalier and de la Noüe 1985; Proulx and de la Noüe 1988; Talbot and de la Nobe 1993), although wastewater treatment by bacteria is common (Daubaras and Chakrabarty 1992). A single study indicated that *Pseudomonas diminuta* and *P. vesicularis*, two obligate aerobes isolated from laboratory algal cultures, stimulated the

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growth of the green microalgae *Scenedesmus bicellularis* and *Chlorella* sp., without releasing any growth promoting substances (Mouget et al. 1995).

Preliminary microscopic observations indicated that bacterial cells might be tightly associated with the wastewater treatment agent *C. vulgaris* (VK. Lebsky, L.E. Gonzalez, and Y Bashan, submitted) isolated from a secondary effluent of a wastewater-treatment stabilization pond near Santafe de Bogota, Colombia. The aims of this study were to isolate and identify *C. vulgaris*-associative bacterium and to partially characterize their relationship with the microalga when coimmobilized in alginate beads, a common way of using microorganisms for environmental applications (Cassidy et al. 1996; Trevors et al. 1993).

Materials and methods

Microorganisms and axenic growth conditions

Chlorella vulgaris Beijerinck (UTEX 2714, Austin, Tex.) was isolated from secondary effluent of a wastewater-treatment stabilization pond near Santafe de Bogota (Gonzalez et al. 1997). Before immobilization into the beads, purified (see below for protocol) *C. vulgaris* was cultivated in a sterile mineral medium (C30) as previously described (Gonzalez et al. 1997) for 5 days. *Phyllobacterium myrsinacearum* was grown in liquid nutrient broth (Difco, Detroit, Mich.) at 30°C ± 2°C for 48 h in a rotary shaker.

Isolation of *Phyllobacterium myrsinacearum* from *Chlorella vulgaris* culture and purification of the microalga from associative bacteria

Aliquots (100 µL) from the original nonaxenic microalgabacterium culture were spread on nutrient agar (NA, Difco) plates supplemented with microalgae C30 medium and incubated at 30°C ± 2°C for 48 h. Bacterial colonies were picked and streaked several successive times on similar plates until separate colonies were obtained. The only contaminant of the microalga culture was a single morphotype later identified as *Phyllobacterium myrsinacearum*. No attempt was made to quantify the level of microalga-bacterial association by *Phyllobacterium myrsinacearum* in the wastewater pond (described in the Results section). The purified strain was stored in liquid nitrogen. For daily use it was stored on nutrient agar slants.

The *C. vulgaris* culture was purified from its associative bacteria as follows: 1 mL of the culture was centrifuged at 10 300 x g for 2 min. The pellet was suspended in 1 mL C30 medium supplemented with a mixture of 300 µg/mL for each antibiotic compound (ampicillin, penicillin, and tetracycline, Sigma, St. Louis, Mo.), incubated in the dark for 72 h, after which possible bacterial growth in the suspension was evaluated under light microscopy every 24 h. This procedure was repeated several times until no bacterial growth was observed in association with the microalga. The antibiotic treatment did not inhibit microalgal growth capacity, and the microalgal population increased during the purification process (data not shown). Microalgal colonies were then picked after washing the cells twice in sterile C30 medium. The microalga was incubated as described by Gonzalez et al. (1997) for 7 days until normal growth was observed. The elimination of bacteria from the microalgal culture was verified after aliquots (100 µL) of the purified microalga culture were transferred into an antibiotic-free nutrient agar medium and incubated as above.

Immobilization of microalgae and bacteria into alginate beads and bead solubilization

Microorganisms were immobilized using the method described by Bashan (1986). Briefly, 20 mL of axenically grown cultures of *C. vulgaris* containing 6 x 10^6 cells/mL were mixed with 80 mL of sterile, 6000 centipoise (cps), 2% alginate solution (a solution made of 14 000 and 3 500 cps alginate, Sigma) and stirred for 15 min. The solution was dripped from a sterile syringe into 2% $CaCl_2$ solution (Bettman and Rehm 1984) under slow stirring. The beads formed were left for 1 h at 22°C ± 2°C for curing and were then washed in sterile saline solution (0.85% NaCl). *Phyllobacterium myrsinacearum* cultures (approximately 10^9 cfu/mL) or untreated *C. vulgaris* cultures (containing naturally occurring *P. myrsinacearum*) were immobilized similarly. Because immobilization normally reduces the number of microorganisms in the beads (Bashan 1986), a second incubation step was necessary; this was done overnight in nutrient broth medium for beads containing *P. myrsinacearum*, and in 6.5 mM phosphate buffer, pH 7.0, for beads containing *C. vulgaris* at illumination of 60 µmol m^{-2} s^{-1}. The beads containing *P. myrsinacearum* were rinsed 5 times in saline solution (0.85% NaCl) to remove traces of the nutrient broth. The low concentra tion of phosphate in the medium was insufficient to dissolve the beads. Where artificially made cocultures of *P. myrsinacearum* and the microalga were used, the same concentration of each microorganism used in pure cultures was mixed prior to incorporation with alginate and bead formation, but the volume of each microbial culture was reduced to 10 mL (making a total of 20 mL) before add ing the alginate solution.

Beads were solubilized for cell counts by immersing 5 of them (1 bead/mL) in a solution of 0. 25 M saline phosphate buffer (PBS) for 1 h at 30°C ± 2°C. *Phyllobacterium myrsinacearum* was counted by plating a dilution series (in PBS) on nutrient agar plates (Difco, Detroit, Mich.), and *C. vulgaris* was counted using a Neubauer haemocytometer. Counts of up to 100 cells were done in each observation field.

Culture conditions for coimmobilized microorganisms

Coimmobilized microorganisms were grown in batch cultures in the mineral salts of residual water medium (RWM) (Gonzalez et al. 1997) containing the following (mg/L): NaCl, 7; $CaC1_2$, 4; $MgSO_4.7H2O$, 2; K_2HPO_4, 21.7; KH_2PO_4, 8.5; Na_2HPO_4, 33.4; NH_4Cl, 10. The level of phosphate in the medium was insufficient to dissolve the constructed beads. Cultures (500 mL) were incubated in Erlenmeyer flasks at 22°C ± 2°C, 150 rpm, and with a light intensity of 60 µmol $m^{-2}.s^{-1}$ for 7 days. Samples for analysis were taken aseptically.

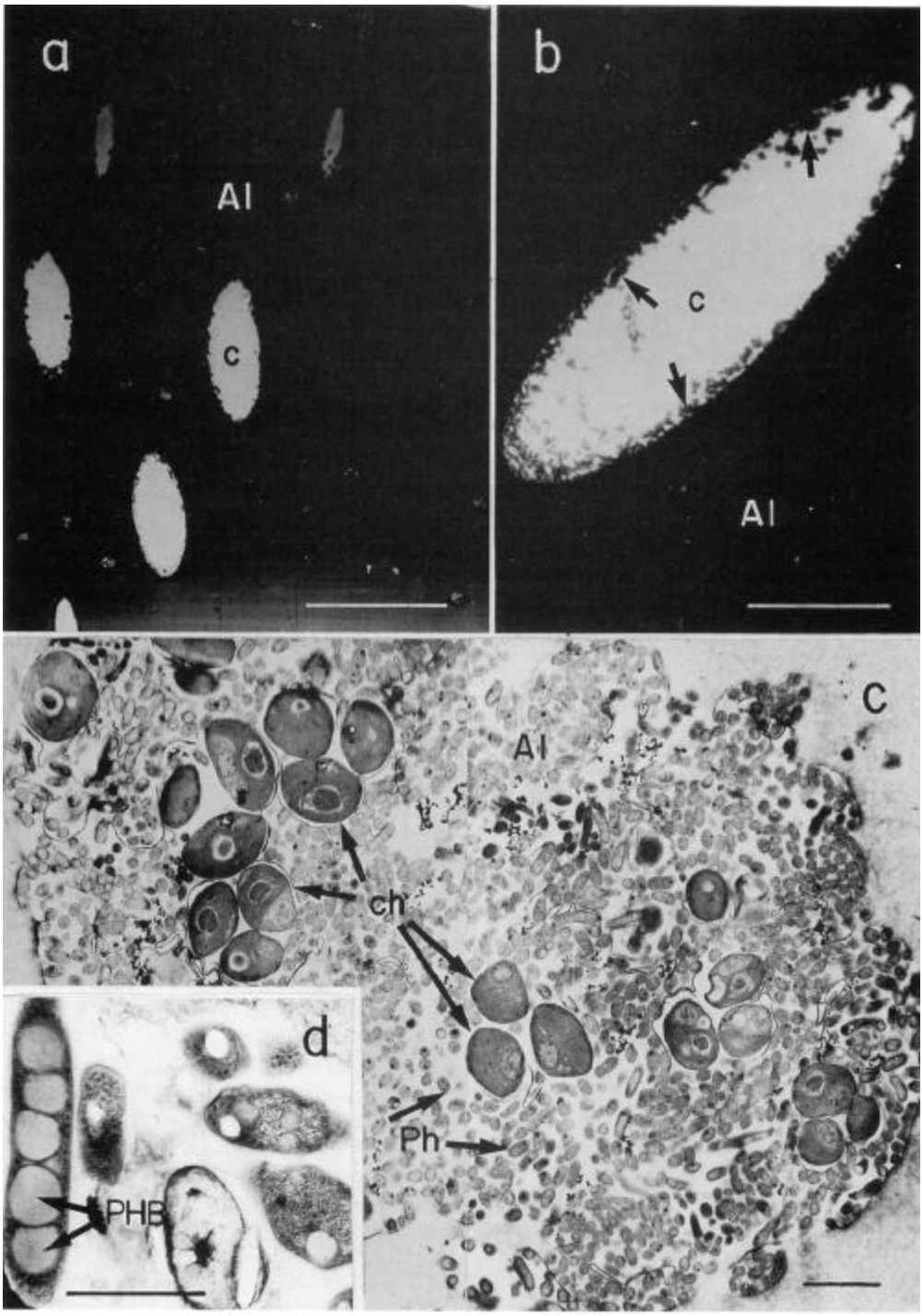
Light and transmission electron microscopy (TEM)

Random samples of beads were transversely cut and immediately mounted on a glass slide at ambient temperature (23°C ± 2°C) and viewed by light microscopy using a conventional light microscope (Zeiss, Germany). For transmission electron microscopy, intact beads were first fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 20°C ± 2°C, rinsed in the same buffer, and postfixed in 1% osmium-tetroxide for 1 h at the same temperature. The beads were then rinsed with distilled water and dehydrated in a series of increasing ethanol concentrations (30%-100%) for 20 min each, and finally with 100% acetone. The dehydrated beads were embedded in araldite resin (Sigma). Ultrathin sections were cut using a Reichert-Young Ultracut ultramicrotome (Vienna, Austria), stained with uranyl acetate and lead citrate, and viewed using a Hitachi H-300 transmission electron microscope (Japan) at 70 kV.

Pigment analysis

The effect of coculturing *C. vulgaris* with *P. myrsinacearum* on the quantity of some pigments of *C. vulgaris* was determined after 7 days of incubation by HPLC (Series 1100, Hewlett Packard), using a column C8 MOS Hypersil, and detection at wavelengths of 440 and 667 nm (Vidussi et al. 1997). The elution buffer was 1 M

Fig. 1. Light and transmission electron microscopy (TEM) of alginate beads coimmobilized with *C. vulgaris* (UTEX 2714) and *P. myrsinacearum* (BOG-1-98). (a) Light microscopy of cross sections of alginate beads showing various size cavities created during solidification of the beads. (b) Magnification of one cavity where both microorganisms were coimmobilized. Arrows indicate concentration of microorganisms. (c) TEM of cross section of one cavity in an alginate bead coimmobilized with *C. vulgaris* and *P. myrsinacearum*. (d) Insertion shows an enlargement of a few *P. myrsinacearum* cells. Bars represent 0.25 mm (a), 0.05 mm (b), and 1 µm (c,d). Abbreviations: Al, solidified alginate; c, internal cavity in the bead; ch, *C. vulgaris*; Ph, *P. myrsinacearum*; PHB, possibly poly-β-hydroxybutyrate.



ammonium acetate at a flow rate of 1 mL/min and at ambient temperature using an injection volume of 100 µL. Standards used were chlorophyll *a* and *b*, violoxantin, lutein, and (3-carotene (VKI Water Quality Institute, Denmark).

Biomass determination

Ten grams of beads containing the coimmobilized microalga and bacteria were dissolved in 100 mL PBS as described above. The suspension was then filtered through a 3.0-µm plankton net leaving a pellet of mostly microalgae on the filter. This pellet was resus-

Table 1. Residual concentration of ammonium ion and phosphorus from water with *C. vulgaris* UTEX 2714 and *C. vulgaris* coimmobilized with *P. myrsinacearum* BOG-1-98.

	$N\text{-}NH_4$ (mg/L)			$P\text{-}PO_4$ (mg/L)		
Microorganisms	0 h	48 h	144 h	0 h	48 h	144 h
C. vulgaris	3.09±0.1aA	3.35±0.6aA	0 bA	3.86 aA	3.0±0.28 abA	2.46±0.33 bA
C. vulgaris + *P. myrsinacearum*	3.09±0.1aA	4.38±0.1bAB	1.66±0.04 cB	3.86 aA	4.4±0.08 bB	3.13±0.14 cA
P. myrsinacearum	3.09±0.1aA	5.0±0.37bBC	7.3±0.81cC	3.86 aA	5.3±0.32 bC	3.6±0.58 aA

Note: Numbers followed by a different lower case letter in each row, for ammonium and phosphorus respectively, differ significantly at $P \leq 0.05$ in one-way ANOVA. Numbers followed by a different capital letter in each column differ significantly at $P \leq 0.05$ in one-way ANOVA. Results are presented ± SE.

pended in 100 mL PBS. Ten-millilitre aliquots were centrifuged for 3 min at 1400 x g in tubes containing filter paper of uniform size and weight (Jecaber 5098, U.S.A.) at the bottom. The supernatant containing the bacteria was discarded. The dry mass of the microalga was measured after removing and drying the filter paper containing the microalga pellet at 105°C for 1 h, which was sufficient to dry the sample completely.

Ammonium, nitrate, and phosphate ion determination, and acetylene-reduction assay (ARA)

Ammonium, nitrate, and phosphate ions were determined in 75-mL samples of the various culture growth media, sampled at 24 hoax intervals, by using water determination standard methods (APHA et al. 1992). All analyses were colorimetric. Ammonium was determined by the Nessler method as having a detection range between 0-3.5 mg/L $N\text{-}NH_4^+$. For calibration, we used an ammonium nitrogen standard solution (Hach Co., Loveland, Colo.). Nitrates were measured by Hach NitraVer 5 reagent (Hach), having a detection range of 0-30 mg/L $N\text{-}NO_3$. Phosphorus was measured by the Molibdovanadate method, having a detection range of 0-45 mg/L $P\text{-}PO_4^{-3}$. The standard for calibration was phosphate standard solution (Kitson and Mellon 1944 as modified by Hach Co.). pH was measured using a pH meter (pHx1495 EBRO, Germany), and oxygen level by oximeter (Orion 820, Boston, Mass.). ARA was performed as previously described using gas chromatography (Holguin et al. 1992) after the bacterium was grown in microaerophylic OAB medium (Bashan et al. 1993), or OAB medium supplemented with (g/L): citric acid, 1.25; glucose, 2; manitol, 2; and myo-inositol, 1; for 24 h; at which point a population of 3.6×10^4 cfu/mL was reached.

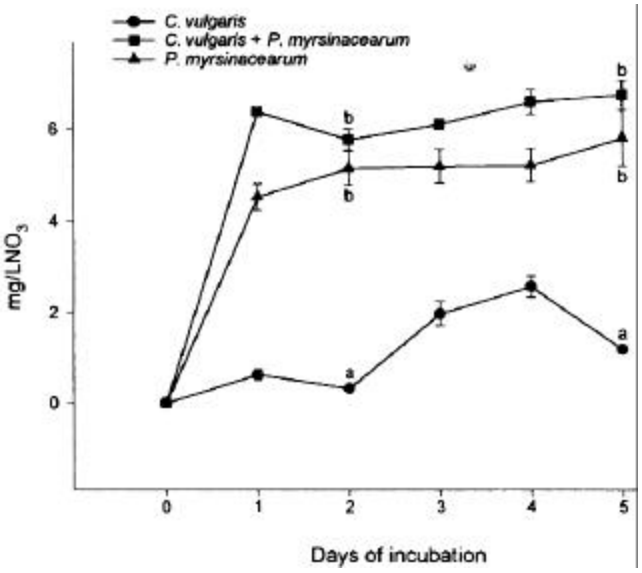
Bacterial identification

Apart from several morphological tests (described in the Results section), bacterial identification was done by gas chromatography of methyl esters of cell fatty acids (FAME analysis) and by comparing 16S rRNA profiles of our strain to known *P. myrsinacearum* strains. These analyses were done as a commercial service by the Department of Plant Pathology, Auburn University, Alabama, and by Acculab (Newark, Del.).

Experimental design and statistical analysis

Cultures were prepared in triplicate (a single flask was one replicate), and each experiment was repeated 5 times. Controls were prepared similarly, but without microorganisms in the beads. Five beads were taken randomly (from each culture) and dissolved for counting the total number of cells. Dry mass of the microalga was measured in triplicate (10 grams of beads dissolved in 100 mL PBS was a single replicate). Pigment content and acetylene reduction assays were also analyzed in triplicate (level of pigments in 5 dissolved beads was one replicate, and one bacterial culture was a replicate for ARA). Results of all repetitions were combined and analyzed by one-way analysis of variance (ANOVA) ($P \leq 0.05$).

Fig. 2. Nitrate formation from ammonium ion by *C. vulgaris* (UTEX 2714), *P. myrsinacearum* (BOG-1-98), and *C. vulgaris* coimmobilized with *P. myrsinacearum* in alginate beads. Points denoted by a different letter at each incubation time differ significantly at $P \leq 0.05$ using one-way ANOVA. Bars represent SE. The absence of SE means that the value is smaller than the point.



Results

Observation of Chlorella vulgaris bacterium associations

Light microscopy observations of fresh mounted *C. vulgaris* colonies indicated the presence of a straight rodshaped, motile, Gram-negative bacterium closely associated with, and sometimes attached to, the microalga cells. All other bacterial cells found in abundance in the wastewater pond could be completely removed by successive washing of the microalga cells. Because of their close proximity and attachment to the microalga cell, it was impossible to quantify the number of the naturally associated bacterial cells. Nevertheless, the unidentified strain was easily grown on nutrient agar medium, and purified. *Chlorella vulgaris* could be freed of its associative bacterium by several consecutive treatments of its culture with antibiotics. Both FAME and 16S rRNA analyses identified the bacterium as *Phyllobacterium myrsinacearum* (ex Knösel 1962) nom. rev. *(Phyllobacterium myrsinacearum* Knösel 1962, 1996) and designated as strain BOG-1-98. Bacterial cell measurements done on TEM photomicrographs matched the published dimensions for the *Phyllobacterium* genus (Knösel 1962, 1984). Colonies on nutrient

Table 2. Cell pigment content by the microalga *C. vulgaris* (UTEX 2714) after coimmobilization with *P. myrsinacearum* (BOG-1-98) in alginate beads and incubated under batch culture for 7 days.

Pigment	*C. vulgaris* (µg/g microalgae cells)	*C. vulgaris* + *P. myrsinacearum* (µg/g cells)
Chlorophyll *a*	473.62±1.58 a	502.06±0.89 a
Chlorophyll *b*	71.92±0.83 a	130.97±3.3 b
β-carotene	7.1±0.76 a	12.55±3.9 a
Lutein	52.02±2.75 a	105.66±0.67 b
Violoxantin	10.41±0.53 a	22.75±0.27 b

Note: Numbers denoted by a different letter in each pigment differ significantly at $P \leq 0.05$ using Student's *t*-test. Results are presented ± SE.

agar medium were nonpigmented to beige, opaque in the center, slimy, and circular. This strain fixed atmospheric nitrogen at a level of 2.85 pmol - 3.6 x 10^4 cfu^{-1} Ml^{-1} (regardless of the culture media used), using the acetylenereduction assay. No other bacterial morphotype was found associated with *C. vulgaris*. Transmission electron microscopy of immobilized *C. vulgaris* and its associative bacterium in alginate beads revealed close association between the two microorganisms. Bacterium and microalga sometimes shared the same cavities inside the bead (Fig. 1). Because only the two microorganisms coexist in the culture at the time of coimmobilization, there was no need to immunolabel *P. myrsinacearum* cells for identification in the photomicrographs.

Effect of *Phyllobacterium myrsinacearum* on the growth of the microalga when both microorganisms are artificially coimmobilized in comparison to axenic culture

Coimmobilization of both microorganisms in alginate beads did not enhance the growth of *C. vulgaris* over a period of 6 days, which reached a similar population level (1 x 10^6 cells/ bead) to axenically grown *C. vulgaris*. Dry mass was also unaffected by this association (30 µg/mL). In addition, the solution pH (7.0) and the level of oxygen (4 mg O_2/L) did not change. However, coimmobilization significantly reduced the ability of *C. vulgaris* to remove ammonium ion and phosphorus from the water (Table 1). The ability to remove phosphorus was recovered by the coimmobilized microalga after 144 h of incubation (Table 1). In cultures containing only immobilized *P. myrsinacearum*, the total level of ammonium ion in the medium increased, probably because of the N_2-fixation capacity of the strain, which occurred after the ammonium ion of the medium was depleted during the long incubation periods. Increased total phosphorus in the medium containing immobilized bacteria (Table 1) can be partially explained by the death of some of the immobilized bacterial cells, common during the immobilization process. Although not evaluated in this study, these dead immobilized cells may leak phosphorus to the medium (Bashan 1986). Immobilized *P. myrsinacearum* cells in alginate beads produced nitrate in culture from ammonium ion, whereas the level of nitrate in immobilized *C. vulgaris* culture was minimal and significantly lower. When the bacterium was coimmobilized with *C. vulgaris*, the total level of nitrate ion in the medium was similar to that of the bacterium alone (Fig. 2).

Increase in pigment production in *Chlorella vulgaris* coimmobilized with *Phyllobacterium myrsinacearum*

Microalga pigment production from a mixture of *C. vulgaris* and *P. myrsinacearum* coimmobilized in alginate beads was compared to an axenic culture of *C. vulgaris* immobilized in similar beads under batch-culture conditions. Table 2 shows that production of 3 out of 5 microalgal pigments evaluated, significantly increased as a result of coimmobilization of the two microorganisms in the same bead.

Discussion

The use of several species of microalgae as tertiary water treatment was reviewed more than a decade ago (de la Node and Pauw 1988), and continues to be evaluated today (Tang et al. 1997). The underlying assumption is that the microalgae will transform some of the contaminants to nonhazardous materials so that the water can be reused or safely discharged (Oswald 1992). Although the microbial population in the wastewater is large, most studies have not considered the possibility that microalgae-associative bacteria interaction may alter (positively or negatively) the water bioremediation features of the microalgae. Plant associative bacteria are well recognized and can be defined as saprophytic, pathogenic, or growth promoters (Bashan and Holguin 1998). Bacteria which are exclusively associated with unicellular aquatic plants are not well characterized (Mouget et al. 1995).

Every microalgae-wastewater treatment sample is composed of numerous microorganisms. The relationship, if any, between most of them are largely unknown. This study revealed the natural association between *C. vulgaris* and *P. myrsinacearum* (Knösel 1962, 1984), but the information concerning this bacterial genus is limited. This bacterium is known to occur in leaf nodules of tropical plants (Knösel 1984; Sutrop 1987), in the rhizoplane of sugar beet from Spain and temperate Belgium (Lambert et al. 1990), and as an endophyte in cotton, soybeans, and beans in southern U.S.A. (Hallmann et al. 1997). Tentatively, the genus is within the family *Rhizobiaceae* (Knösel 1984) but it is not known as a nitrogen fixer. Strains of this genus obtained from the rhizosphere of arid tropical marine mangroves in Mexico were N_2-fixers (A. Rojas 1999, unpublished data). The possible relationship of these bacteria, if any, to aquatic plants or microalgae are largely unknown. The microalgae-associative bacterium isolated in this study does not have growth-promoting or pathogenic effects on the microalga, yet it affected the microalgal

metabolism when the two were cultivated as a coculture in alginate beads used for wastewater treatment. Pigment production by the microalga significantly increased, and ammonium ion and phosphate ion removal by the microalga were significantly reduced. However, one can suggest that elimination of these bacteria from the microalga culture used for water bioremediation might improve ammonium ion and phosphorus removal. Yet, its inclusion as a coinoculant for the microalgae in alginate beads, as was done for terrestrial plant-growth-promoting bacteria (Bashan 1998), would promote pigment production when this is the product sought.

This study shows a bacterium-microalga relation when both are confined to cavities inside alginate beads where space is limited, microbial numbers are high, and there is no interference from other microorganisms. It is, however, unknown whether this bacterium retains all of its effects on the microalga in the original habitat of these microorganisms, the wastewater pond. The frequency of *P. myrsinacearum* associated with the microalga relative to other bacteria in the natural consortium in the wastewater pond has not yet been determined.

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