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EFFECTS OF THE HERBICIDE, BUTACHLOR, ON NITROGEN FIXATION IN PHOTOTROPHIC NONSULFUR BACTERIA

Kyung Mi Lee, Jaisoo Kim[1], and Hyun Soon Lee[†]
Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Korea
[1]Department of Life Science, Kyonggi University, Suwon 443-760, Korea
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Abstract : In an effort to identify possible microbes for seeking bioagents for remediation of herbicide-contaminated soils, seven species of phototrophic nonsulfur bacteria (*Rhodobacter capsulatus and sphaeroides, Rhodospirillum rubrum, Rhodopseudomonas acidophila, blastica and viridis, Rhodomicrobium vannielii*) were grown in the presence of the herbicide, butachlor, and bacterial growth rates and nitrogen fixation were measured with different carbon sources. Under general conditions, all species showed 17-53% reductions in growth rate following butachlor treatment. Under nitrogen-fixing conditions, *Rb. capsulatus* and *Rs. rubrum* showed 1-4% increases in the growth rates and 2-10% increases in nitrogen-fixing abilities, while the other 5 species showed decreases of 17-47% and 17-85%, respectively. The finding that *Rp. acidophila, Rp. blastica, Rp. viridis* and *Rm. vannielii* showed stronger inhibitions of nitrogenase activity seems to indicate that species in genera *Rhodobacter* and *Rhodospirillum* are less influenced by butachlor than those in *Rhodopseudomonas* and *Rhodomicrobium* in terms of nitrogen-fixing ability. Overall, nitrogenase activity was closely correlated with both growth rate and glutamine synthetase activity (representing nitrogen metabolism). When the carbon sources were compared, pyruvate (three carbons) was best for all species in terms of growth rate and nitrogen fixation, with malate (four carbons) showing intermediate values and ribose (five carbons) showing the lowest; these trends did not change in response to butachlor treatment. We verified that each of the 7 species had a plasmid (12.2~23.5 Kb). We found that all 7 species could use butachlor as a sole carbon source and 3 species were controlled by plasmid-born genes, but it is doubtful whether plasmid-born genes were responsible to nitrogen fixation.

Key Words : Butachlor, Herbicide, Nitrogen fixation, Plasmid, Purple nonsulfur bacteria

INTRODUCTION

Nitrogen fixation by phototrophic nonsulfur bacteria was first demonstrated through the study of hydrogen production by *Rhodospirillum rubrum*,[1)] and subsequently shown in other phototrophic bacteria. However, although many phototrophic bacteria are able to fix nitrogen, the associated nitrogenases have been studied in only a few species.[2-6)] The effects of herbicides on nitrogen fixation in cyanobacteria are of interest to researchers because this process is vital to the fertility of the rice fields.[7-10)] However, only a few studies have examined the effect of herbicides on nitrogen fixing phototrophic nonsulfur bacteria.[11)]

Butachlor (2-chloro-2', 6'-diethyl-N (butoxymethyl) acetanilide; commercial name Machete), alachlor and propachlor belong to the chloroacetanilide chemical family and were used extensively in the 1980s and 1990s for killing weeds in rice-fields, especially in Korea, Japan and other Southeast Asian countries. There have

[†] Corresponding author
E-mail: jkimtamu@kyonggi.ac.kr
Tel: +82-31-249-9648, Fax: +82-31-249-9139

been reports of the negative effects of butachlor on cyanobacteria, Rhizobium and Nostoc; these effects include mutagenesis, as well as inhibition of growth, photosynthesis and nitrogenase activity.[12] In contrast, the growth and aerobic nitrogen fixing ability of butachlor-treated *Gloeocapsa* species (oxygen-utilizing diazotrophs) were shown to be possible due to plasmid-born herbicide resistance genes.[12,13] Nitrogen fixation concurrent with oxygen or ammonia treatment was studied using phototrophic nonsulfur bacteria and diazotrophic bacteria,[13] whereas Habte and Alexander[14] showed that herbicide-treated *Chromatium* and *Thiospirilum* showed increased nitrogen fixation ability, while cyanobacteria showed the opposite in lowland rice culture.

Butachlor is generally removed from the soil through volatilization and decomposition by light[15] or soil microorganisms. The herbicide, chloroacetanilide, was degraded 50 times faster by microorganisms than by light or volatilization, indicating that some microorganisms could use herbicide as a carbon source.[7,8,10,16,17] Likewise, microorganisms capable of using alachlor or propachlor as a carbon source were found in sewage and lake water. Madigan et al.[18] and Serebrayakova et al.[19] examined the nitrogenase activities of nonsulfur photosynthetic bacteria, and the studies have examined the relationships among nitrogen fixation, plasmids and genes.[5,20-22]

This study examined the influence of butachlor on the nitrogen-fixing ability and growth rates of phototrophic nonsulfur bacteria, and then further assessed the utilization of butachlor as a carbon source, and the plasmids involved in butachlor degradation.

MATERIALS AND METHODS

Microorganisms

All strains were purchased from American Type Culture Collection (ATCC, USA) and Deutsche Sammulung für Mikroorgnismen (DSM, Germany), except *Rhodospirillum rubrum* KS-301, which was isolated in our laboratory from lake water using an anaerobic enrichment culture method. Those are *Rhodobacter capsulatus* DSM 1710, *Rhodobacter sphaeroides* ATCC 17023, *Rhodospirillum rubrum* KS 301, *Rhodopseudomonas acidophila* ATCC 25092, *Rhodopseudomonas blastica* ATCC 33485, *Rhodopseudomonas viridis* DSM 133, *Rhodomicrobium vannielii* DSM 162.

Chemical

Butachlor (2-chloro-2', 6'-diethyl-N (butoxymethyl) acetanilide; commercial name, Machete) with purity of 98.7% was purchased from the Korean Agriculture Chemical Company (Seoul, Korea).

Media and Growth Conditions

All strains were grown in Ormerod medium[4] supplemented with 0.3% of the indicated carbon source at pH 7.0, except that *Rhodopseudomonas* (*Rp.*) *acidophila* and *Rhodomicrobium* (*Rm.*) *vannielii* were kept at pH 5.6, and the media for *Rp. blastica* was supplemented with 0.4% NaCl. For experiments testing growth in dinitrogen gas, the $(NH_4)_2SO_4$ and yeast extract in the Ormerod medium (basal medium) was replaced with biotin (8 μg/mL) and a 50 mL/min flow of nitrogen gas.

To study the utilization of butachlor as a sole carbon source, 10^{-2} to 10^{-5} M of butachlor was added to the basal medium without yeast extract. Bacteria were cultured in screw cap tubes (10 mL) or glass bottles (30 or 50 mL) under 2000 Lux illumination at 28 ± 2°C. For growth under nitrogen gas, a suction flask was filled with 50 ml of medium and nitrogen gas was passed through a high temperature copper tube for complete elimination of oxygen. Growth of the microorganisms was determined by measuring optical density (OD) at 660 nm and calculating the dry weight according to Hilmer and Gest.[23] The specific growth rate (μ, unit = h^{-1}) was calculated from the equation of Schlegel.[24] For protein estimation, the Lowry method[25] was used with bovine serum albumin employed as the standard. The cells were disrupted by a sonicator.

Ammonium Estimation

For estimation of ammonium production, 10

mL of each cell culture was digested with 0.1 mL of digestion solution (conc. H_2SO_4, 1 L: potassium sulfate, 100 g; selenium, 1 g) at 300°C for 1 hour until the mixture became transparent. After centrifugation for 10 min, 50 μL of supernatant was used for measuring the absorbance at 625 nm according to the method of Chaney and March.[26] NH_4Cl was dried for 24 hours at 100°C was used as the standard.

Enzyme Assays

Nitrogenase activity was determined by measurement of acetylene reduction, as described.[18,27] Briefly, cell cultures were centrifuged, and each pellet was washed with nitrogen-free medium and then suspended in Ormerod medium without a nitrogen source. Two ml of this cell suspension was inoculated into a tube, sealed with suba seal (No. 17), treated with argon gas for 10 min and then treated with acetylene gas (calcium carbide + H_2O) up to 10% (v/v). The cells were cultured under 10,000 Lux at 30°C for 60 hours and the production of ethylene gas (0.5-1 mL) was measured by gas chromatography (Varian 3700, CA, USA).

In order to assay the activity of glutamine synthetase, γ-glutamylhydroxamate formation was determined as described by Shapiro and Stadtman[28] and Bender et al.[29]

Preparation of Plasmids

Plasmids from seven strains of phototrophic nonsulfur bacteria were prepared by the rapid method described by Birnboim and Doly[30] and Ish-Horowitz and Burke,[31] which allows isolation of undenatured ccc-plasmid DNA between pH 12-12.5 in SDS alkaline solution. For comparison and validation of the rapid method, plasmid DNA was also isolated by the large-scale preparation method described by Maniatis et al.[32]

Curing Test of Plasmids

To determine whether butachlor degradation depended on the presence of the plasmids, a curing test was performed as described by Rheinwald et al.[33] Approximately 500 cells were inoculated into 5 ml of LB medium including Mitomycin C (0-20 μg/mL), and the inoculums were cultured at 30°C for 48 hours. A second generation was cultured as above and then inoculated to LB medium including butachlor, the plasmids were isolated from cured cells, and resolved by agarose gel electrophoresis.

Agarose Gel Electrophoresis

Electrophoresis was carried out on 0.8% agarose gels at 100 V for 2.5 hours using TEA buffer.[32] Hind III DNA fragments were used as molecular size markers. After electrophoresis, the agarose gels were stained with Ethidium Bromide (1 mg/mL) for 40 min, washed with distilled water, and photographed with Polaroid film Type 667.

Analysis of Results

Each experiment was performed in triplicate and repeated two times. The standard deviation was less than 10 % of the mean. All comparisons were statistically compared using a t-test with $P < 0.05$.

RESULTS

Effects of Butachlor on Bacterial Growth

We first identified the effective concentration of butachlor required to build a proper experimental design. Protein concentration (mg/mL) at stationary phase was used as a representation of *Rs. rubrum* KS-301 growth in basal medium containing pyruvate, malate or ribose as a carbon source, supplemented with concentrations of butachlor ranging from 10^{-1} to 10^{-6} M (Fig. 1). Our results revealed that the bacterial growth decreased with increasing concentrations of butachlor up to 10^{-3} M, and almost no growth was seen at butachlor concentrations of 10^{-2} M or higher. Interestingly, cultures grown in the presence of 10^{-3} M butachlor using ribose as the carbon source showed poor growth.

We then studied the effects of $(NH_4)_2SO_4$, dinitrogen gas, organic carbon (pyruvate and malate),

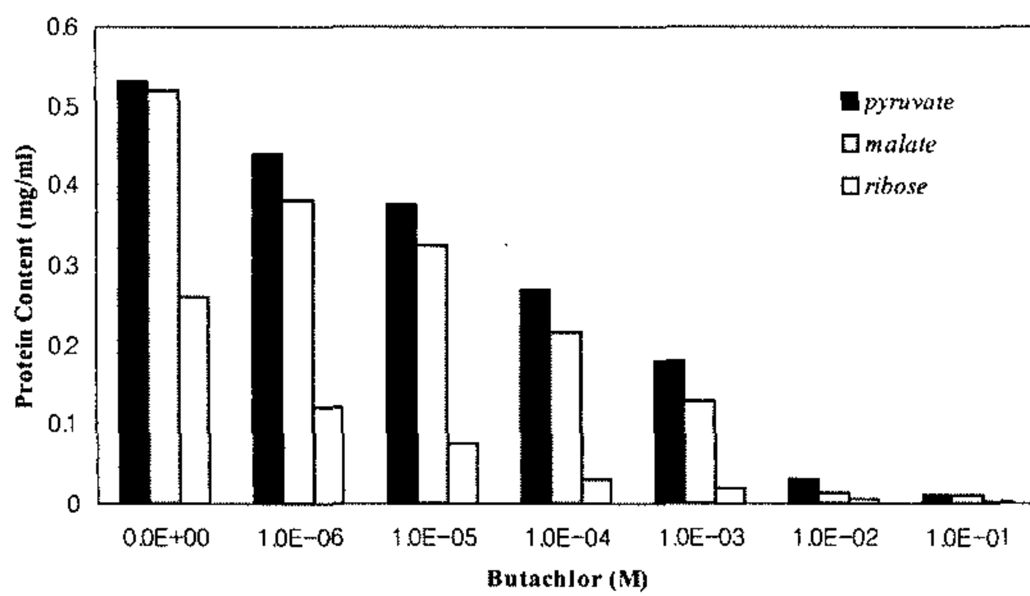

Figure 1. The effect of butachlor concentration versus substrate on growth of *Rs. rubrum* KS-301.

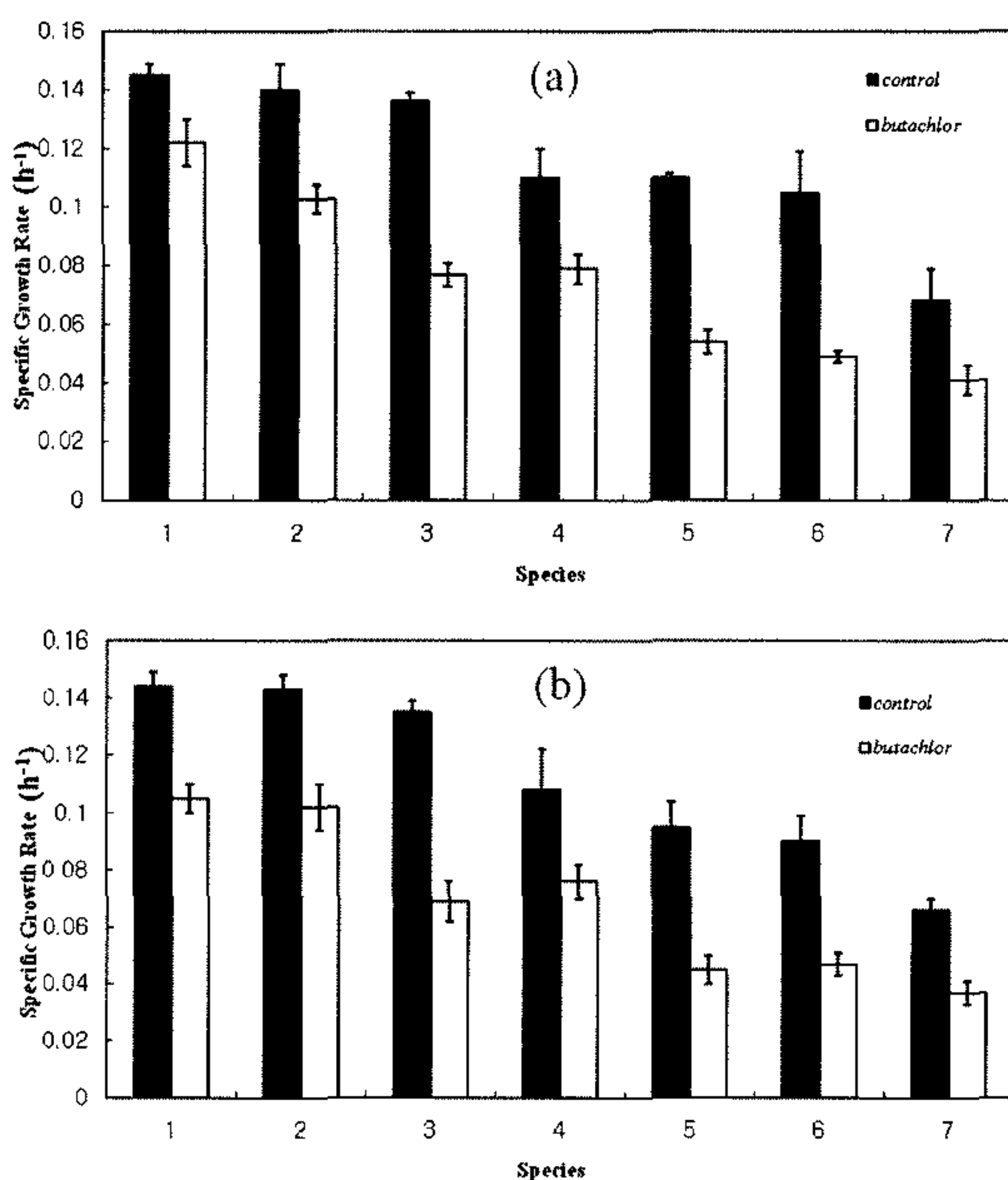

Figure 2. The effect of butachlor (10^{-3} M) on the growth rate of phototrophic nonsulfur bacteria using $(NH_4)_2SO_4$ as nitrogen source. (a),. pyruvate as the carbon source; (b), malate as the carbon source. 1, *Rb. Capsulatus*; 2, *Rs. Rubrum*; 3, *Rb. Sphaeroides*; 4, *Rp. Viridis*; 5, *Rp. Acidophila*; 6, *Rm. Vannielii*; 7, *Rp. Blastica.*

and the pH value on the influence of butachlor on seven species of phototrophic nonsulfur bacteria, as measured in terms of the specific growth rate (μ). In medium containing $(NH_4)_2SO_4$ as the nitrogen source, untreated cells grew 1.2-2.1 times faster than those treated with butachlor ($P < 0.05$) (Fig. 2). *Rb. capsulatus* showed the highest growth rate regardless of butachlor treatment, while *Rhodopseudomonas* (*Rp.*) *blastica* showed the lowest growth rate among the seven tested species. The growth rates of *Rb. capsulatus* were about 2 times higher than those of *Rp. blastica* in the absence of butachlor and about 3 times higher in the presence of butachlor, regardless of whether the carbon source was pyruvate or malate. The growth rates of *Rs. rubrum* were second in both cases, regardless of butachlor treatment. The growth rate of *Rp. viridis* was slightly (within the error range) higher than that of *Rb. sphaeroides* in the presence of butachlor, while the opposite was true in the absence of butachlor. Butachlor treatment decreased the growth rates of *Rb. sphaeroides, Rp. acidophila* and *Rm. vannielii* by around 50%, regardless of the carbon source (Fig. 2).

Effects of Butachlor on Nitrogen-Fixing Ability

The cell growth rates were generally lower when using N_2 gas as a nitrogen source versus $(NH_4)_2SO_4$ (Fig. 3), but these rates were not significantly different in the presence or absence of butachlor, especially for *Rb. capsulatus* and *Rs. rubrum* ($P > 0.05$). *Rb. capsulatus* showed a 4-fold higher growth rate than *Rp. blastica* in the presence of butachlor, regardless of the carbon source (pyruvate or malate). *Rs. rubrum* showed relatively higher growth rates as *Rb. capsulatus*, regardless of butachlor treatment. *Rp. viridis* showed higher growth rates than *Rb. sphaeroides* in the presence of butachlor, but the opposite was true in the absence of butachlor.

To determine the ability of nitrogen fixation, we cultured *Rs. rubrum* cells for seven days with pyruvate, malate or ribose as the carbon source, and measured ammonium production (Fig. 4). Among the three carbon sources, ammonium production was the highest (87 μg/mL) in pyruvate-containing cultures treated with 10^{-5} M butachlor for 5 days, with levels declining by day 7. Malate-containing cultures showed a similar pattern, but yielded lower levels of ammonium production, while ribose-containing cultures showed the lowest levels of ammonium production. In

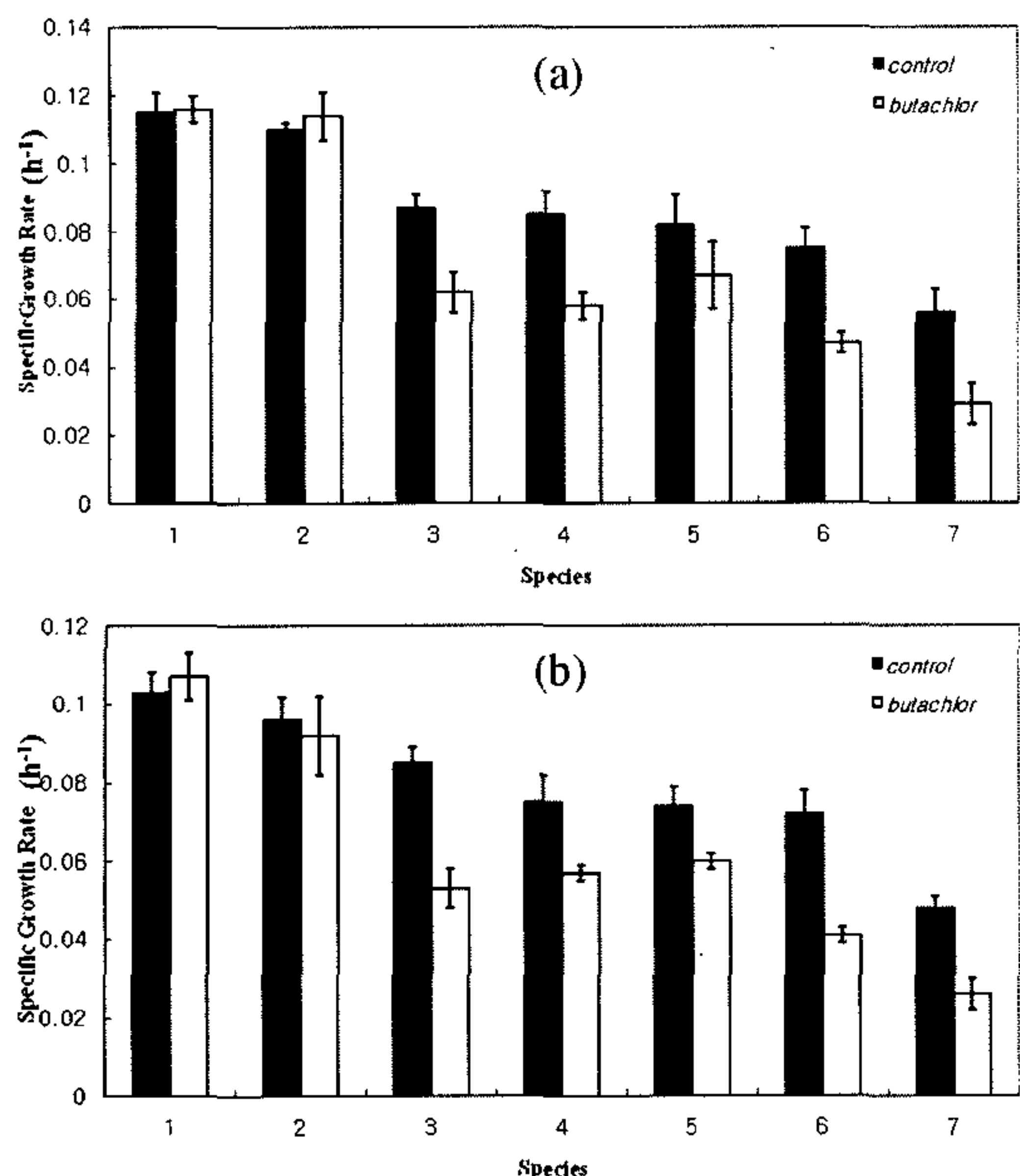

Figure 3. The effect of butachlor (10^{-3} M) on the growth rate of phototrophic nonsulfur bacteria using N_2 gas as the nitrogen source. (a), pyruvate as the carbon source; (b), malate as the carbon source. 1, *Rb. Capsulatus*; 2, *Rs. Rubrum*; 3, *Rb. Sphaeroides*; 4, *Rp. Viridis*; 5, *Rp. Acidophila*; 6, *Rm. Vannielii*; 7, *Rp. Blastica*.

general, ammonium formation decreased with increasing concentrations of butachlor ranging from 10^{-5} to 10^{-2} M.

As shown in Figure 5(a), butachlor-treated *Rb.*

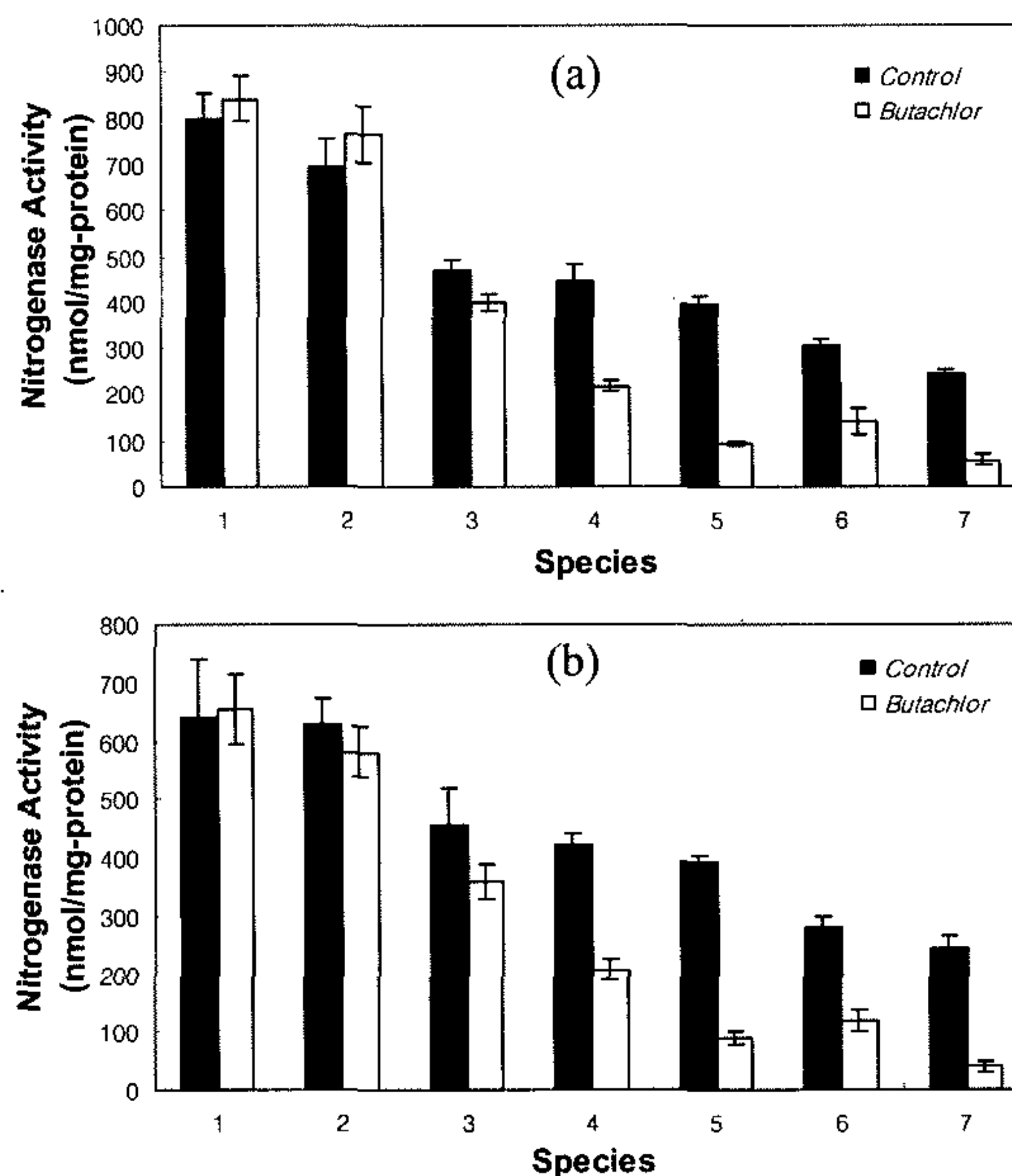

Figure 5. Comparison of nitrogenase activity in nonsulfur photosynthetic bacteria. (a), pyruvate as the carbon source; (b), malate as the carbon source. 1, *Rb. Capsulatus*; 2, *Rs. Rubrum*; 3, *Rb. Sphaeroides*; 4, *Rp. Viridis*; 5, *Rp. Acidophila*; 6, *Rm. Vannielii*; 7, *Rp. Blastica*.

capsulatus and *Rs. rubrum* showed higher levels of nitrogenase activity than untreated cells although statistically not different ($P > 0.05$), while the other five strains showed 1.2~6 times higher nitrogenase activity in the absence of butachlor ($P < 0.05$). Cells cultured in malate showed a

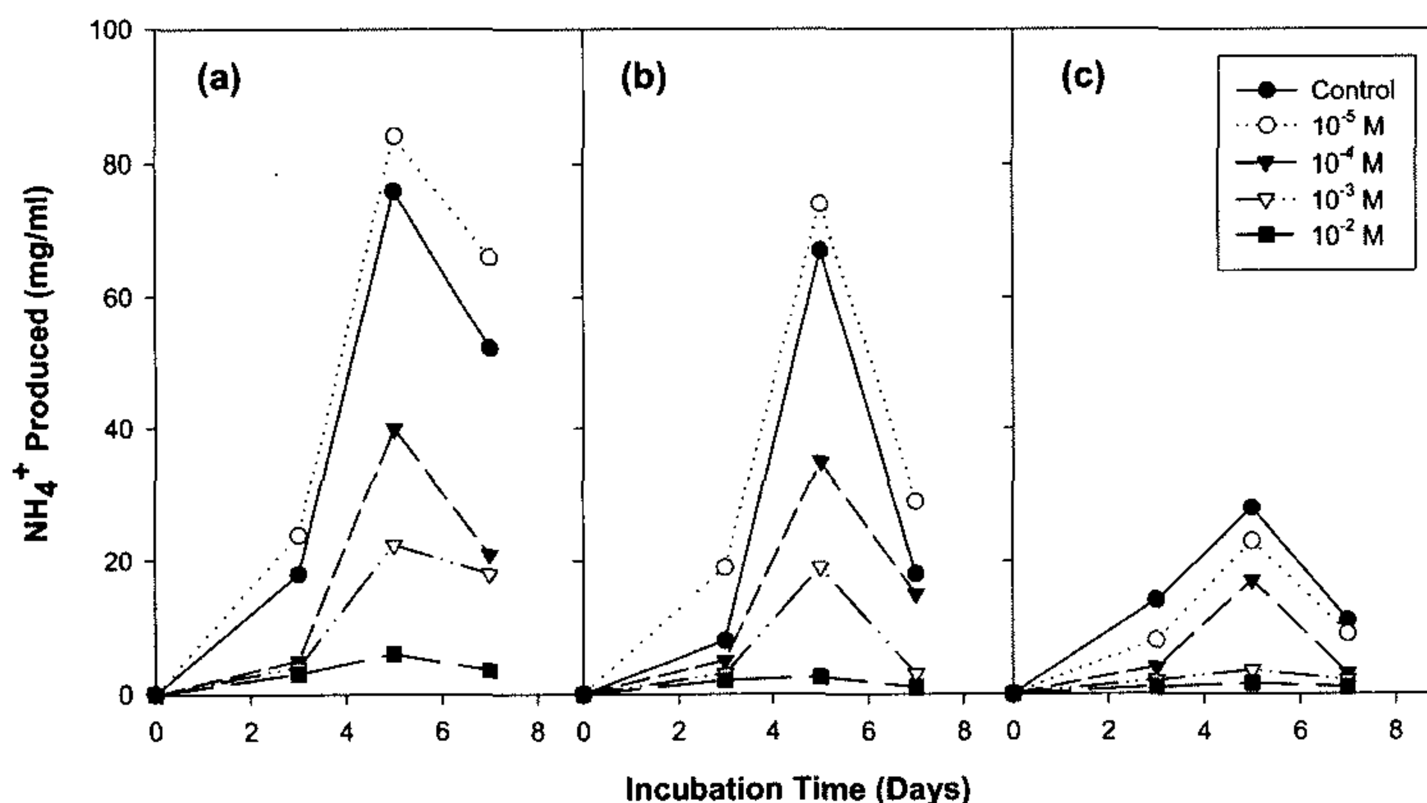

Figure 4. The effect of various concentration of butachlor on changes in NH_4^+ production by *Rs. rubrum* KS-301 using pyruvate, malate and ribose as the carbon sources under nitrogen fixing conditions. (a), pyruvate; (b), malate; (c), ribose.

similar pattern as pyruvate but 1~1.3 times lower nitrogenase activity than those cultured in pyruvate in all seven strains, regardless of butachlor treatment. Of the untreated cultures, *Rb. capsulatus* cultured in pyruvate showed the highest nitrogenase activity (798 nM/mg protein), with *Rs. rubrum* showing the second highest activity (693 nM/mg protein). *Rp. acidophila* and *Rm. vannielii,* which grew well in acidic media, had comparatively low nitrogenase activities (about 38-60% that of *Rb. capsulatus*). *Rp. blastica* showed the lowest nitrogenase activity at 246 nM/mg protein, which was 31-38% that of *Rb. capsulatus*. Of the butachlor-treated cultures, *Rb. capsulatus* grown in pyruvate-containing cultures showed the highest nitrogenase activity of 842.7 nM/mg protein, with *Rs. rubrum* showing the next highest nitrogenase activity of 766.8 nM/mg protein. The activities of these two species were little higher than those of their untreated counterparts, and 15~16 times higher than the lowest activity observed (*Rp. Blastica*). The nitrogenase activities of *Rb. sphaeroides* were lower than those of *Rs. rubrum*, regardless of carbon source or butachlor treatment. *Rb. sphaeroides* cultured in pyruvate without butachlor showed 1.5~1.9 fold higher activity than *Rm. vannielii* and *Rp. blastica*. In *Rp. acidophila* and *Rp. blastica*, butachlor treatment decreased the nitrogenase activities to 16~24% of control levels, with a larger decrease seen in cultures using malate as the carbon source versus those using pyruvate.

To test whether butachlor affected nitrogen metabolism, we examined glutamine synthetase activity in treated and untreated bacteria. In order to maintain the activity of glutamine synthetase, bacteria were pretreated with cethyltrimethylammonium bromide (0.1 mg/mL) in 0.8 M Tris- HCl buffer (pH 7.0) at 37°C and then the glutamine synthetase activity was determined as the amount of γ-glutamylhydroxamate (Fig. 6). The results of the glutamine synthease analysis were comparable to those of the nitrogenase activity assays (Fig. 5). Glutamine synthetase activity showed 1~1.2 times higher activity in cultures using pyruvate as a carbon source versus those using

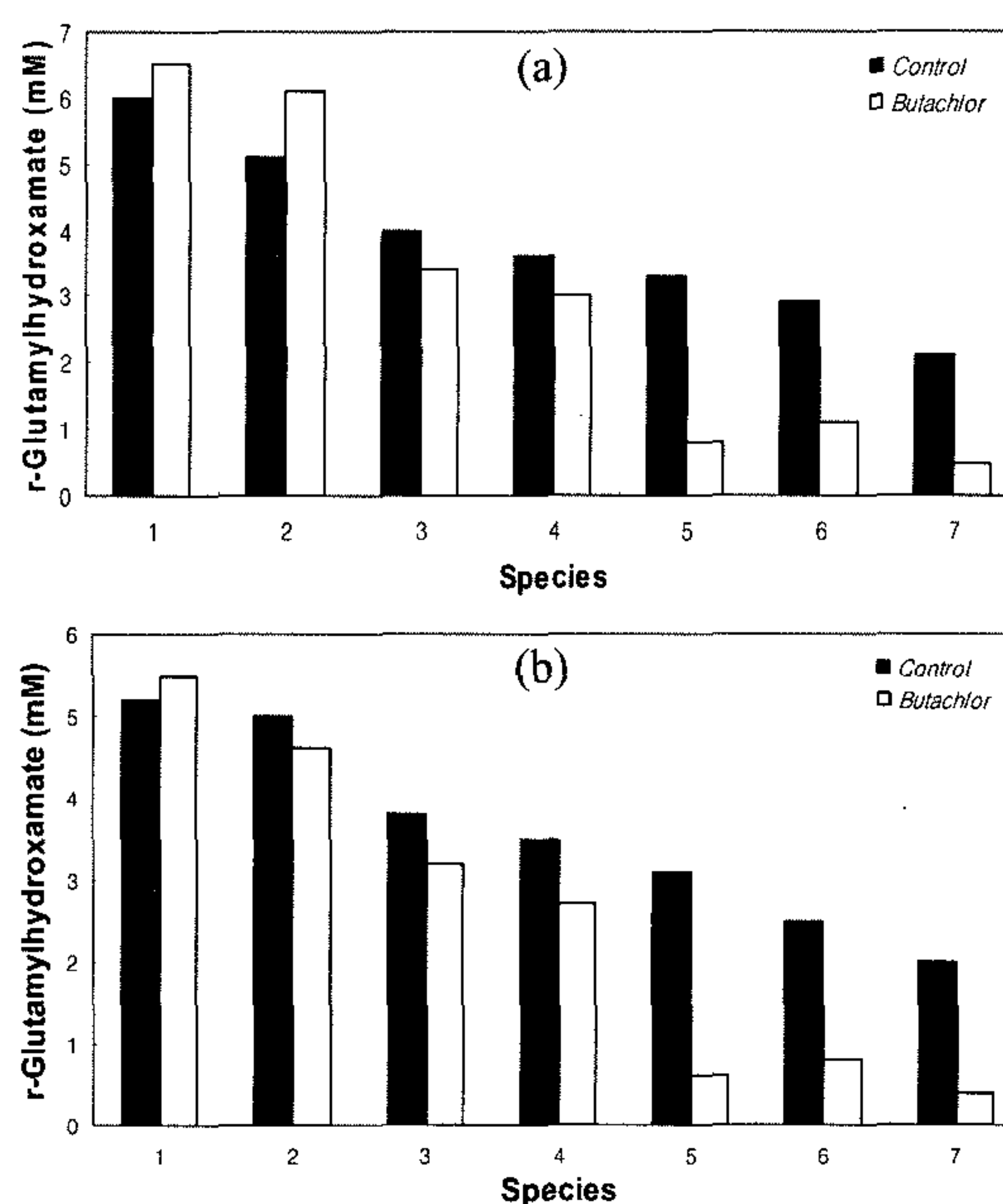

Figure 6. A, pyruvate as carbon source; B, malate as carbon source. and r-glutamylhydroxamate as the glutamine synthetase in N_2-grown phototrophic nonsulfur bacteria. (a), pyruvate as the carbon source; (b), malate as the carbon source. 1, *Rb. Capsulatus*; 2, *Rs. Rubrum*; 3, *Rb. Sphaeroides*; 4, *Rp. Viridis*; 5, *Rp. Acidophila*; 6, *Rm. Vannielii*; 7, *Rp. Blastica*.

malate. In butachlor-treated cultures, *Rb. capsulatus* and *Rs. rubrum* showed 1.1~1.2 times higher glutamine synthetase activity, while the other 5 strains showed lower activities. In the butachlor-treated cultures, the presence of pyruvate was associated with 1.1~1.4 times higher glutamine synthetase activities than those seen in the presence of malate. The glutamine synthetase activities in *Rp. acidophila* cultures treated with butachlor were only 21~24% of the untreated control values.

Use of Butachlor as a Carbon Source

To determine whether the seven species of phototrophic nonsulfur bacteria could utilize butachlor as a sole carbon sourse, the strains were cultured for 7 days in media containing 10^{-5} to 10^{-2} M butachlor. When the growth rates were compared on day 7, all strains showed their highest growth rates at the concentration of 10^{-3}

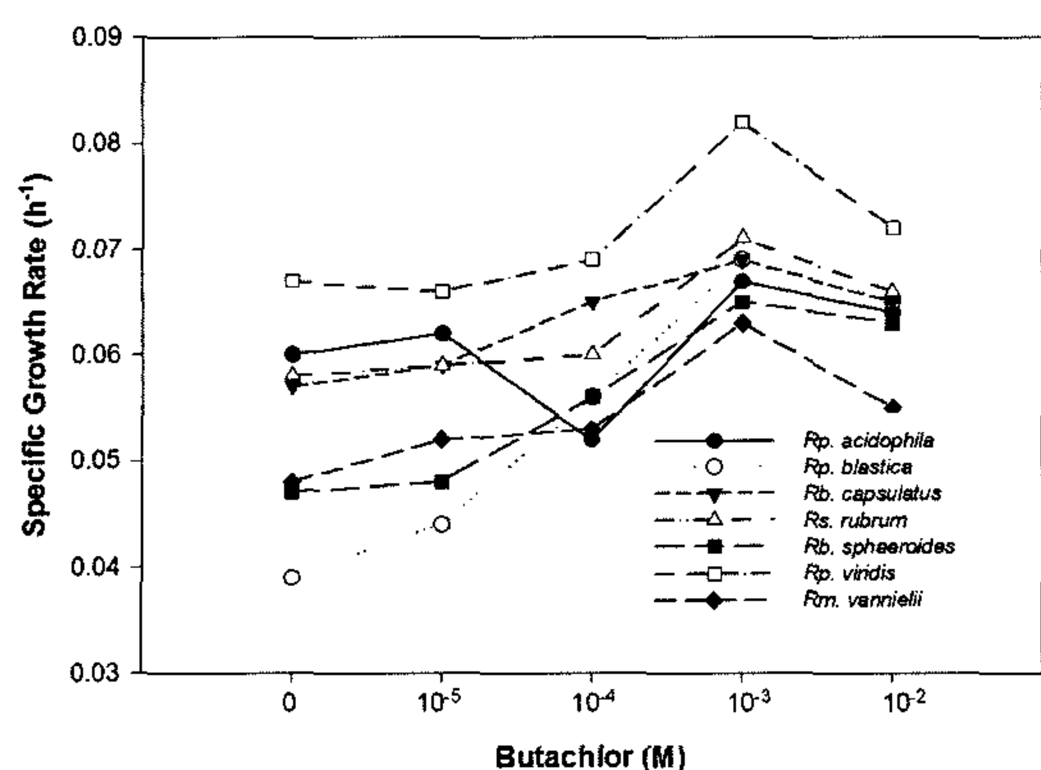

Figure 7. Utilization of butachlor as a sole carbon source in nonsulfur photosynthetic bacteria (unit of growth rate: h^{-1}).

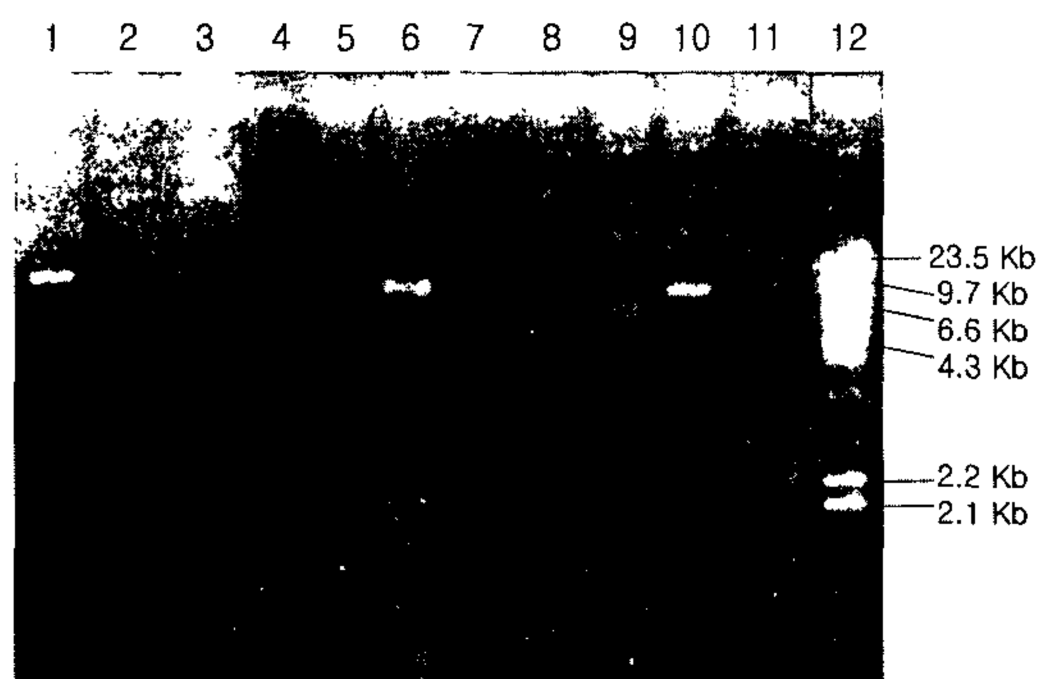

Figure 8. Agarose gel electrophoresis of But^+ and But^- plasmids purified from 3 species of *Rhodospirillaceae*. Lane 1, *Rm. vannielii*, wild type (But^+); lane 2, *Rm. vannielii*, cured cell (But^-) with 0.5 μg/mL Mitomycin C; lane 3, *Rm. vannielii*, cured cell (But^-) with 5 μg/mL Mitomycin C; lane 4, *Rm. vannielii*, cured cell (But^-) with 10 μg/mL Mitomycin C; lane 5, *Rm. vannielii*, cured cell (But^-) with 20 μg/mL Mitomycin C; lane 6, *Rp. blastica*, wild type (But^+); lane 7, *Rp. blastica*, cured cell (But^-) with 5 μg/mL Mitomycin C; lane 8, *Rp. blastica*, cured cell (But^-) with 10 μg/mL Mitomycin C; lane 9, *Rp. blastica*, cured cell (But^-) with 20 μg/mL Mitomycin C; lane 10, *Rs. ruburum*, wild type (But^+); lane 11, *Rs. ruburum*, cured cell (But^-) with 20 μg/mL Mitomycin C; lane 12, Hind III digested λ DNA.

M butachlor (Fig. 7). *Rp. viridis* showed the highest growth rate among the seven, with *Rs. rubrum* and *Rb. capsulatus* and *Rp. blastica* following in descending order. The growth rate of *Rm. vannielii* was the lowest.

To determine whether butachlor could be utilized as a carbon source by plasmid genes, we performed a curing test using Mitomycin C (0.5-20 μg/mL) according to Rheinwald et al.[33] Then, cured cells, which lost the ability to utilize butachlor, were treated for the plasmid isolation. Cured plasmids did not show bands following agarose gel electrophoresis, while uncured plasmids could be visualized as EtBr-stained bands (Fig. 8). The bands were not visible in *Rm. vannielii* cured with 0.5 to 20 μg/mL Mitomycin C, *Rp. blastica* cured with 5-20 μg/mL Mitomycin C, or *Rs. rubrum* cured with 20 μg/mL Mitomycin C (Fig. 8).

DISCUSSION

The toxicity of butachlor to microbes differs among phototrophic nonsulfur bacteria, cyanobacteria and *Rhizobium*. Previous studies revealed that the growth of nitrogen fixing cyanobacteria was inhibited by 2-10 μg/mL butachlor and that *Nostoc muscorum* was mutated by concentrations of 10 μg/mL,[34] while *Anabaena*,[35] *Rhizobium* and *Gloeocapsa*[12,36] could grow in the presence of 100 μg/mL butachlor.

The rice fields in Korea are generally treated with 100-200 μg/mL of butachlor annually. Here, we show that 10^{-3} M (156 μg/mL) butachlor decreased the growth rates of seven species of phototrophic nonsulfur bacteria to 47-83% of control values (Figs. 2 and 3), and 10^{-2} M (1560 μg/ml) butachlor completely inhibited the growth of these organisms (Fig. 1). Our results indicate that these seven species of phototrophic nonsulfur bacteria grew relatively well in the presence of butachlor, and might survive better than other nitrogen-fixing organisms in highly contaminated sites.

Singh et al.[36] reported that resistance to butachlor appeared to be correlated with evolution, as resistance decreases in the order of *Rhizobium* species > unicellular cyanobacteria > filamentous cyanobacteria > higher plants and *monocotyledons*. This suggests that more primitive organisms have

more resistance to butachlor and that the higher and *monocotyledons* are more sensitive. *Rhizobium leguminosarum* showed 50 times more resistance than cyanobacteria, but we herein show that purple nonsulfur bacteria are more resistant than *Rhizobium*. Thus, purple nonsulfur bacteria may be more primitive organisms than those originally reported by Singh et al.[36)]

Cells cultured on NH_4^+ as a nitrogen source showed 1.3-1.6 times higher growth rates than those grown in nitrogen gas using either pyruvate and malate as carbon sources (Figs. 2 and 3), and butachlor-treated cells also showed 1-1.4 times higher growth rates cultured on NH4, as mentioned by Yoch[37)] who mentioned about the higher growth rates on NH_4^+ than N_2 gas. In terms of growth rates and nitrogen fixation, the seven tested species performed best when using the three-carbon (pyruvate) as a carbon source, followed by the four-carbon (malate) and then the five-carbon (ribose), regardless of butachlor treatment (Fig. 4). This may be due to easier utilization of the smaller molecule, and is consistent with the findings of Schick[38)] and Hillmer and Gest,[23)] who reported that pyruvate was a more relevant carbon source than malate and ribose because it quickly proceeds to the ammonium metabolism that inhibits nitrogenase activity *in vivo*. When the bacteria were cultivated with $(NH_4)_2SO_4$ as a nitrogen source, the initial pH declined to 6.2-6.8 regardless of the absence or presence of butachlor, but under nitrogen fixing conditions, the pH increased to 7.8-8.4 (data not shown), suggesting that NH_4^+ is more readily used for metabolism than nitrogen fixation.[39)]

Previous studies showed the nitrogen-fixing ability was highest in *Rb. capsulatus*, followed by *Rb. sphaeroides* and then *Rs. rubrum*.[18,19)] However, our results revealed that *Rb. capsulatus* had the highest nitrogen-fixing ability, followed by *Rs. rubrum* Ks-301 and then *Rb. sphaeroides* (Fig. 5). Madigan et al.[18)] reported that *Rp. acidophila*, *Rp. viridis* and *Rm. vannielii* had very similar nitrogenase activities, whereas we found that *Rp. blastica* had the lowest nitrogenase activity, i.e. only 31-38% that of *Rb. capsulatus*. Such differences might be due to strain-specific nitrogenase activity or other characteristics.[18,19)] For example, *Rs. rubrum* Ks-301 was previously reported to have higher nitrogenase activity, hydrogen gas production and better immobilization of microorganism beads.[40,41)]

We found that the nitrogenase activity and growth rates of 5 species (excluding *Rb. capsulatus* and *Rs. rubrum* Ks-301) decreased to 17-83% (Fig. 5) and 53-83%, respectively, of the untreated control values following butachlor treatment (Fig. 3) . It appears that butachlor inhibits nitrogenase activity more effectively than growth rate in some species, especially *Rp. acidophila*. In contrast, butachlor treatment increased nitrogenase activity by 2-10% and growth rates by 1-4% in *Rb. capsulatus* and *Rs. rubrum* grown in the presence of pyruvate. As these slight increases are within the error ranges, future work will be required to determine whether butachlor plays an effective role as an energy source in these organisms.

Rb. sphaeroides showed 1-1.6 times more nitrogenase activity than *Rp. viridis, Rp. acidophila, Rm. vannielii* and *Rp. blastica*. The species in genera *Rhodobacter* and *Rhodospirillium* had more nitrogenase activity than those in genera *Rhodopseudomonas* and *Rhodomicrobium*, while the latter were more greatly inhibited by butachlor. So the species in the two genera could be used as the possible bioremediation agents in butachlor-contaminated sites. Consistent with the report of Pandey et al.[9)] that the herbicide, propanil, was more inhibitory against cyanobacteria grown in acidic medium than in alkaline medium, *Rp. acidophila* and *Rm. vannielii* grown in acidic medium were significantly influenced by butachlor treatment.

Nitrogenase activity and growth rates were parallel regardless of butachlor treatment except in the case of *Rp. acidophila*, which grew well in association with lower nitrogenase activity. This seems to indicate that nitrogen is a critical factor for the growth of most species of microbes, even under harsh environmental conditions. Glutamine synthetase behaved in a manner similar to nitrogenase activity (Fig. 6), and was always

proportional to nitrogenase activity in control and butachlor-treated cultures. These findings seem to indicate that nitrogen fixation and nitrogen metabolism take place consecutively in phototrophic nonsulfur bacteria, regardless of butachlor treatment.

Our analysis of butachlor as a sole carbon source seemed to suggest that some phototropic nonsulfur bacteria can utilize 10^{-3} M butachlor to maximize their growth rates (Fig. 7). We further noted a decrease in pH under these conditions, likely indicating that butachlor may be degraded/utilized. However, in the presence of primary substrates (pyruvate, malate and ribose), cell growth was inhibited by 10^{-3} M of butachlor (Fig. 1), implying that the herbicide may act as a competitive inhibitor. Previously, Lee and Lee[42,43] determined the biodegradabilities of butachlor during anaerobic culture of phototropic nonsulfur bacteria (*Rb. capsulatus, Rs. rubrum, Rp. acidophila* and *Rp. viridis*), as assessed by decreased butachlor absorbance measured with a UV-scanning spectrometer. The authors reported that *Rb. capsulatus* and *Rs. rubrum* showed higher removal rates than *Rp. acidophila* and *Rp. viridis*. These findings are consistent with our observation that *Rb. capsulatus* and *Rs. rubrum* showed higher growth rates than *Rp. acidophila* and *Rp. viridis*. Thus, the breakdown and up-take of butachlor under anaerobic conditions are of interest since herbicides are generally degraded under aerobic conditions.

Nitrogen fixation and growth in butachlor-treated cells of genus *Gloeocapsa* has been associated with plasmid-borne genes,[12] as has degradation of herbicides such as 2,4-D and MCPA, as well as toluene, xylene, naphthalene and 2,4,5-trichlorophenoxy acetic acid.[44,45] In this study, we were able to isolate appropriately-sized plasmids from all 7 tested species (data not shown), and we always observed 9.7-23.5 kb plasmid in rapid isolation or ccc-plasmid DNA under all conditions. But it is already known that plasmids do not play any roll about nitrogen fixation and butachlor degradation as cyanobacteria. Klipp et al.[21] noticed that nitrogen fixation in phototrophic bacteria depends on chromosomal DNA not on plasmid. We used a curing test to show that butachlor could be degraded by plasmids in *Rp. blastica, Rm. vannielii* and *Rs. rubrum* (Fig. 8). But a further research is needed to prove whether or not the results are true.

For industrial or bioremedial applications, nonsulfur photosynthetic bacteria have several benefits over other nitrogen-fixing bacteria, such as ubiquitousness, high resistance, degrading ability on various compounds and high productivity. Historically, nonsulfur photosynthetic bacteria have been utilized for bioremediation of water pollution,[46,47] production of industrial hydrogen gas,[48] feeding of livestock, degradation of organic compounds[46] and fertilizing of crops. In addition, phototrophic nonsulfur bacteria have a larger distribution range than cyanobacteria,[14] making them potentially useful candidates for a variety of research areas. As our results indicate that phototrophic nonsulfur bacteria had more resistance to butachlor than other nitrogen-fixing bacteria, we propose that they can be used for bioremediation.

In sum, we herein show for the first time that butachlor treatment of *Rb. capsulatus* and *Rs. rubrum* does not inhibit growth or nitrogen fixation of these organisms, indicating that these two species may be useful candidates for developing nitrogen-fixing strains or effective vectors for use in degrading a variety of persistent contaminants. We recognized a diversity of microorganisms. Each species acts so differently.

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