Distribution and Capacity for Utilization of Lower Fatty Acids of Phototrophic Purple Nonsulfur Bacteria in Wastewater Environments

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Populations of phototrophic purple nonsulfur bacteria (PNSB) of up to 10⁴–10⁷ CFU ml⁻¹ were found in photosynthetic sludge and activated sludge plants treating wastewater having different biological oxygen demand (BOD) levels and containing acetate as a main BOD source. There was a positive correlation between the population density of PNSB and the strength of BOD in the aeration tanks. All of the PNSB strains isolated were identified as species of the genera Rhodobacter and Rhodopseudomonas by 16S rRNA gene sequencing. The Rhodobacter species were abundant in high BOD-loaded wastewater environments, while the Rhodopseudomonas species increased at lower BOD levels. Therefore, the PNSB population structure appeared to be greatly affected by the concentration of lower fatty acids as major BOD sources, varying over time and space. The utilization of lower fatty acids with different carbon numbers (C2 to C6) by aerobically or semi-aerobically grown cells of authentic and isolated PNSB strains was evaluated by monitoring substrate-dependent oxygen uptake. The oxidation of the fatty acids by the Rhodobacter strains depended upon the number of carbons in the substrate, while the Rhodopseudomonas strains utilized all substrates equally. A low carbon number was much preferred by the Rhodobacter strains. The affinity for acetate of the Rhodobacter and Rhodopseudomonas strains ranged from 0.14 to 3.0 mM and 0.032 to 0.096 mM, respectively. These results suggest that the concentration and kind of lower fatty acids as major BOD sources are important factors affecting not only the population level but also the species composition of PNSB in wastewater environments.

Key words: phototrophic purple non-sulfur bacteria, lower fatty acids, oxygen uptake, photosynthetic sludge, wastewater treatment

Anoxicogenic phototrophic bacteria are regarded as suitable for treating organic wastewater благодаря these bacteria, especially purple nonsulfur bacteria (PNSB), are able to grow not only by anaerobic photosynthesis but also by aerobic respiration at full atmospheric oxygen tension, utilizing a wide variety of simple organic compounds as sources of carbon and energy. In addition, some PNSB are capable of growing via anaerobic respiration in darkness. Actually, phototrophic bacteria are detected at all purification stages in standard activated sludge plants at levels in the magnitude of 10³ to 10⁴ CFU ml⁻¹, although the population is much smaller than the co-existing chemotrophic population.

Thus, the treatment of wastewater using PNSB, termed the photosynthetic sludge process, has been studied in particular applied to the purification of highly concentrated organic wastewater. PNSB strains have been isolated from various wastewater environments including photosynthetic sludge plants and classified as species of the genera Rhodobacter, Rhodocyclus, Rhodocista, Rhodopseudomonas and Rubrivivax. Towards the application of PNSB to wastewater treatment, several investigators have studied optimal environmental conditions for their growth using pure and mixed cultures. There have been several postulates on how wastewater environments affect phototrophic bacterial behavior. For instance, environmental factors such as light intensity, dissolved oxygen (DO) tension and nutrient strength...
have been considered to be responsible for changes in phototrophic bacterial populations. The operation of laboratory-scale wastewater treatment reactors using a PNSB species has conventionally required sufficient illumination to generate energy\textsuperscript{36-40}. Oxygen-depleted and low oxidation-reduction potential (ORP) conditions yielded a maximum population density of PNSB in photobioreactors\textsuperscript{31}. Obviously, the limited availability of light or the presence of too much oxygen, as is the case under standard operating conditions in activated sludge plants, reduces bacterial photosynthesis\textsuperscript{31}. Due to this suppression, the phototrophic bacteria are not capable of competing with co-existing chemotrophic bacteria for organic nutrients under standard conditions for aerobic wastewater treatment.

Contrary to the aforementioned arguments, our previous study focusing on aerobic photosynthetic sludge plants revealed that population densities of PNSB as high as 10\textsuperscript{7} CFU ml\textsuperscript{-1} were obtained when wastewater contained much acetate as a major BOD source\textsuperscript{18}. The plants studied were constantly aerated, like standard activated sludge plants, and were not equipped with any illuminating apparatus, daylight being the sole light source. This observation led to our assumption that the organic nutrient gradient is an important determinant of the proliferation of PNSB in the wastewater treatment process\textsuperscript{15,18}. Similarly, Siefert et al. 41 have implied from their studies on PNSB in activated sludge that the presence of purple phototrophic bacteria is particularly dependent upon the degree to which water is polluted by organic matter. However, information about the relationships between organic nutrient gradients and the population structures or activity of PNSB in wastewater environments is still lacking.

The major objective of this study was to elucidate what triggers the PNSB population change in wastewater environments. Common organic compounds utilized by PNSB are acetate and some lower fatty acids (LFAs), which are produced by the breakdown of organic matter in the pretreatment stage of the photosynthetic sludge process\textsuperscript{8,24}. Therefore, we focused on differences in the capacity for utilization of selected LFAs among different phylogenetic groups of PNSB isolated from photosynthetic sludge and activated sludge plants.

Materials and Methods

Sludge samples

Sludge samples were taken from main treatment tanks of photosynthetic sludge plants in Shiogama, Miyagi Prefecture, and Nishiakashi, Hyogo Prefecture, Japan, as described previously\textsuperscript{15,19}. These plants were being operated for the treatment of soybean curd (tofu) wastewater with BOD levels of 1,800–7,000 mg l\textsuperscript{-1}. Sludge samples were also collected from activated sludge plants in Toyohashi, Japan, where municipal sewage or swine wastewater was being treated. All samples were collected from the main purification tanks which were constantly aerated, taken into polyethylene bottles, and subjected to examination immediately upon return to the laboratory.

Analytical methods

The organic pollution level as BOD was measured according to the standard method for wastewater\textsuperscript{5}. Total organic carbon in samples was measured with a Shimadzu model 5000A TOC analyzer (Shimadzu Corp., Kyoto, Japan). LFAs (C2 to C6 fatty acids) in wastewater were directly separated and identified by ion-pair HPLC with external standards. The analytical conditions were as follows: pump, Shimadzu LC-10AD (Shimadzu Corp., Kyoto, Japan); column, Waters Organic Acid Column (7.8 mm i.d.\times300 mm; Japan Waters, Tokyo, Japan); column temperature, 40°C with a CO-8020 column oven (TOSO, Tokyo, Japan); mobile phase, 0.1% phosphoric acid buffer; flow rate, 1 ml min\textsuperscript{-1}. Eluates were monitored with a TOSO model UV-8020 spectrophotometric detector at 210 nm. Fatty acids were identified according to HPLC retention times, and their concentrations were determined by comparing peak areas with those of pure agents.

Direct cell counting

Sludge samples were harvested by centrifugation at 1,100 \times 10 for 10 min, resuspended in the same volume of filter-sterilized phosphate-buffered saline (PBS), and then sonicated for 90 sec (20 kHz; output power, 100 W) to disperse the sludge flocs. The dispersed samples were decimally diluted with PBS, and appropriate dilutions were used for direct cell counting by epifluorescence microscopy with ethidium bromide or SYBR Green staining. Detailed information on the cell-counting method has been described previously\textsuperscript{29,45}.

Enumeration and isolation of PNSB

Sludge samples were harvested by centrifugation, resuspended in the same volume of 20 mM phosphate buffer (pH 7.0) containing 0.1% peptone and sodium tripolyphosphate (50 mg l\textsuperscript{-1}), and then sonicated as noted above. The dispersed sludge samples were serially diluted with this buffer, and appropriate dilutions were plated by the pour-plating method with SAYS agar medium (pH 6.8), which consisted
of mineral base RM2\textsuperscript{14}, 5 mM succinate, 5 mM acetate, 0.1% yeast extract (Difco Laboratories, Detroit, MI, USA), 10 µg of vitamin B\textsubscript{12} and 1.5% agar. Inoculated plates were incubated anaerobically with the AnaeroPak system (Mitsubishi Gas Chemical Co., Niigata, Japan) under incandescent light (ca. 2,000 lx). After 10 days of incubation, red to red-brown colonies recovered onto the agar medium were counted as CFU of PNSB. Single red colonies recovered on the enumeration medium were picked up randomly and subjected to a standard purification procedure by repeated streaking of medium. Microscopic observation with a phase-contrast microscope was also performed to ensure cultural purity. Purified isolates were preserved in MYS\textsuperscript{(4)} stub cultures. PNSB counts were also obtained by the most-probable-number (MPN) method using SAYS medium. Aliquots (1 ml) of each dilution of the homogenate were transferred into 20-ml screw-capped test tubes containing 10 ml of the growth medium, which were then completely filled with a filter-sterilized 0.1% ascorbate solution. The enrichment tubes were set up in triplicate for each step of dilution and incubated for 10 days before the final recording. The tubes showing a brown to red color were regarded as positive for the growth of PNSB. All culture media were incubated at 30\textdegree C under incandescent light (ca. 2,000 lx).

16S rRNA gene sequencing and phylogenetic analysis

Crude cell lysate was prepared as the source of genomic DNA according to a protocol described previously\textsuperscript{(2)}. The 16S rRNA genes from the cell lysate were amplified by PCR with the bacterial universal primers fD1 (27\textsuperscript{f}) and rP1 (1492\textsuperscript{r})\textsuperscript{(43)}. PCR products were purified by the polyethylene glycol precipitation method\textsuperscript{(47)}, sequenced with a SequiTherm Long Read Cycle Sequencing kit (Epicentre Technologies, Madison, USA), and analyzed with an Amersham-Pharmacia model ALFexpress automated DNA sequencer. The newly determined sequences were compiled using the GENETYX-MAC program (Software Developing Co., Tokyo, Japan) and compared with the sequences deposited in the database using the BLAST search program\textsuperscript{(2)}. The multiple alignment of sequence data, calculation of the nucleotide substitution rate using Kimura’s two-parameter model\textsuperscript{(2)}, and construction of a phylogenetic tree by the neighbor-joining method\textsuperscript{(35)} were conducted using the CLUSTAL W program\textsuperscript{(42)}. The bootstrap analysis of the tree\textsuperscript{(3)} was performed using CLUSTAL W with 1,000 bootstrapping trials.

The 16S rRNA gene sequences determined in this study have been deposited under DDBJ accession numbers AB196354 to AB196356.

Measurement of oxygen uptake

Representative strains of Rhodobacter and Rhodopseudomonas species isolated from the photosynthetic sludge and activated sludge were used to measure the uptake of oxygen (see Table 2). For comparison, Rhodobacter (Rba.) capsulatus strain DSM 1710\textsuperscript{T}, Rba. sphaeroides strain DSM 158\textsuperscript{1} and Rba. azotoformans strain KA25\textsuperscript{T}\textsuperscript{(15)} were used. ACY medium containing 1% sodium acetate, 0.05% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2% casamino acid and 3% yeast extract (pH 7.0) was used for culturing. For Rba. azotoformans strain KA25\textsuperscript{T}, half of the acetate in the medium was replaced with glucose on a weight basis to prevent any viscous excretion during the growth\textsuperscript{(16)}. Precultures were made semi-aerobically in 10 ml of the medium in 20-ml screw-capped tubes under incandescent light (ca. 3,000 lx). Precultures at the exponential phase of growth were then transferred to fresh medium (1% inoculum) and incubated either semiaerobically in light similar to the preculturing or aerobically in the dark with reciprocal shaking. Incubation was at 30\textdegree C in all cases. Cells at the early to mid exponential phases of growth were harvested by centrifugation at 11,000xg for 10 min at 4\textdegree C, washed twice with 50 mM phosphate buffer (pH 7.0), and resuspended in this buffer to give an optical density at 660 nm (OD\textsubscript{660}) of 2.0–2.5. The cell suspensions were immediately used for measuring oxygen uptake. Since cooling of cells with ice before the measurement resulted in a decrease in oxygen uptake in all cases, the suspensions were kept at room temperature until used. Oxygen uptake was measured at 25\textdegree C using an Iijima model B-505 DO analyzer with a reaction chamber (2 ml capacity) and a polarograph type of oxygen macro electrode. As the substrate for oxygen consumption, neutralized forms (2 mM each) of acetate, propionate, butyrate, valerate and caproate were used. The cell suspension was introduced into the reaction chamber and stirred for a few minutes until the endogenous substrate-dependent oxygen uptake became constant. Then, the reaction was triggered by adding one of the substrates noted above. When the cells grown in the dark under aerobic conditions were used for measurements, the chamber was shaded to minimize the oxygen sparing effect (decreased oxygen uptake under illumination) of natural light or room lighting\textsuperscript{(44)}. All experiments were performed in triplicate.

Kinetic analysis

Kinetic studies were conducted to measure responses to acetate. The concentration of acetate was adjusted from 0.1 mM to 10 mM, as this was considered a range of concentra-
tions that phototrophic bacteria might encounter during wastewater treatment. The apparent maximum velocity ($V_{max}$) and Michaelis-Menten constant ($K_s$) were calculated from a linear Lineweaver-Burk plot. Following the lead of Folsom et al., the term $K_s$ was employed instead of $K_m$, because the activity was measured using intact cells rather than purified enzymes.

Results

Relationships between nutrient strength and PNSB population

The levels of BOD and LFAs in the samples taken from the main treatment tanks of the photosynthetic sludge plants varied remarkably over time, ranging from ca. 120 to 1,100 mg l$^{-1}$ and 110 to 920 mg l$^{-1}$, respectively. The samples from the activated sludge plants had a BOD level of 170 to 3,100 mg ml$^{-1}$ and a total LFA concentration of 150–2,800 mg ml$^{-1}$. In all cases, acetate accounted for more than 90% of the lower fatty acids detected. These data suggested that acetate was the major BOD source in all the wastewater treatment plants studied.

All sludge samples, except one sample with a BOD level of 3,100 mg l$^{-1}$, were subjected to the enumeration of PNSB. The population densities of PNSB detected in the photosynthetic sludge samples fluctuated in the order of magnitude of $10^4$ to $10^7$ CFU or MPN ml$^{-1}$ depending upon sampling time, whereas those in the activated sludge samples ranged from $10^4$ to $10^8$ CFU or MPN ml$^{-1}$. Figure 1 shows the relationship between the population density of PNSB and BOD levels. There were positive correlations between BOD levels and CFU or MPN counts of PNSB. These correlations were statistically significant ($P<0.05$). The results suggested that the growth of PNSB in the wastewater treatment plants was stimulated by increasing concentrations of organic nutrients.

Phylogenetic affiliations of PNSB

A total of 87 PNSB strains were isolated from the photosynthetic sludge and activated sludge samples and phylogenetically identified by 16S rRNA gene sequencing as well as microscopic testing. Of these 87 isolates, 32 were tentatively identified as Rba. azotoformans on the basis of similarity levels of more than 99.5% relative to the type strain of the species, strain KA25$^T$. Using this standard of sequence similarity, 15 isolates could be affiliated to Rps. palustris, 5 isolates to Rba. sphaeroides, and one isolate to Rba. blasticus. The remaining 34 isolates were most similar to Rhodopseudomonas (Rps.) palustris ATCC 17001$^T$ but designated as an unaffiliated Rhodopseudomonas species because of similarity levels of less than 99.5% between the former strains and the latter.

A phylogenetic tree for the PNSB isolates and their relatives based on 16S rRNA gene sequences is shown in Fig. 2. As mentioned above, the isolates were grouped with either Rba. azotoformans, Rba. blasticus, Rba. sphaeroides or Rps. palustris as their closest relatives. These Rhodobacter and Rhodopseudomonas species are common among PNSB found in wastewater treatment plants.$^{7,14,17,41}$

Variations in the phylogenetic composition of the PNSB isolates according to increasing and decreasing gradients of BOD strength are shown in Table 1. In the case where BOD values were higher than 590 mg l$^{-1}$, most of the isolates were the Rhodobacter species. In contrast, the Rhodopseudomonas species appeared in large numbers when BOD values in the samples were lower than 290 mg l$^{-1}$. Based on these results, it seemed obvious that not only the population density but also the community structure of PNSB in the wastewater environment is dependent upon organic nutrient gradients.

Utilization of lower fatty acids

Representatives of the isolates as well as authentic PNSB strains were examined for the utilization of LFAs containing different carbon numbers (C2 to C6) by monitoring sub-
strate-dependent oxygen uptake. Table 2 shows oxygen uptake by the PNSB strains grown aerobically in the dark and semiaerobically in light. The test strains of the *Rhodopseudomonas* species were able to aerobically utilize all of the LFAs tested, whereas it was more difficult for the *Rhodobacter* species to utilize LFAs as the number of carbons increased. The addition of 2 mM valerate or caproate had an inhibitory effect on endogenous oxygen uptake in the *Rhodobacter* species. These results indicate that differences in substrate specificity apparently exist between the *Rhodobacter* species and *Rhodopseudomonas* species.

As shown in Table 2, there seemed no marked differences in the oxygen uptake rate between cells grown in aerobic-dark and semi-aerobic-light conditions. It has been shown that the respiratory enzyme activity of phototrophically grown *Rba. capsulatus* increases in response to small amounts of oxygen.

**Kinetics of acetate utilization**

Since acetate is a major organic compound in wastewater as reported here and elsewhere, a kinetic analysis of the utilization of acetate by PNSB strains of the genera *Rhodobacter* and *Rhodopseudomonas* was performed to better understand their responses to nutrient gradients. For all the test strains, oxygen uptake experiments were performed with 0.1 to 10 mM acetate. The Michaelis-Menten’s parameters, *Km* and *Vmax*, calculated from the oxygen uptake curve are summarized in Table 3. The addition of acetate, regardless of the concentration, resulted in an increase in oxygen uptake, independent of the conditions for growth. The main difference between the two generic groups of PNSB was that the affinity for acetate was higher in the *Rhodopseudomonas* strains than in the *Rhodobacter* strains. Also, the apparent maximum velocity (*Vmax*) of oxygen uptake seemed to be higher in the former strains than in the latter, although this property varied markedly among the *Rhodobacter* strains of different origins. These results suggest that wastewater environments containing low concentrations of acetate provide more favorable conditions for the growth of *Rhodopseudomonas* than of *Rhodobacter*.

**Discussion**

In numerous studies, the availability of light and DO
tension have been regarded as the major factors affecting the growth and viability of anoxygenic phototrophic bacteria\(^{21,24,41}\). A sufficient supply of light and a reduction in DO tension enable phototrophic bacteria to perform photosynthesis at a maximal rate\(^{17}\). As reported herein, however, photosynthetic sludge plants with high BOD levels in wastewater yielded high population densities of PNSB (up to \(10^7\) CFU ml\(^{-1}\)), even under highly aerated conditions. The present study has demonstrated that there is a significant correlation between population densities of PNSB and BOD levels in aerobic wastewater treatment systems. These observations support the results of previous studies\(^{15,18}\) and expand our knowledge of the ecology of PNSB in wastewater environments. The PNSB strains recovered from the sludge samples were identified as members of the genera *Rhodobacter* and *Rhodopseudomonas*. The predominant PNSB species changed in relation to in situ BOD levels. Namely, the *Rhodopseudomonas* species became predominant when the BOD level in wastewater decreased, whereas the *Rhodobacter* species increased in number at higher

<table>
<thead>
<tr>
<th>Type of sludge and wastewater</th>
<th>BOD (mg ml(^{-1}) of sample)</th>
<th>No. of colonies isolated/tested</th>
<th>No. of isolates identified as:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthetic sludge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean curd wastewater</td>
<td>1100</td>
<td>20</td>
<td><em>Rba. azotoformans</em> 0 0 0 0 0</td>
</tr>
<tr>
<td>Soybean curd wastewater</td>
<td>590</td>
<td>17</td>
<td><em>Rba. blastoicus</em> 0 0 0 0 0</td>
</tr>
<tr>
<td>Soybean curd wastewater</td>
<td>290</td>
<td>20</td>
<td><em>Rba. sphaeroides</em> 0 0 0 0 0</td>
</tr>
<tr>
<td>Soybean curd wastewater</td>
<td>200</td>
<td>18</td>
<td><em>Rps. palustris</em> 0 0 0 0 0</td>
</tr>
<tr>
<td>Activated sludge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swine wastewater</td>
<td>3100</td>
<td>2</td>
<td><em>Rhodopseudomonas</em> 0 0 0 0 0</td>
</tr>
<tr>
<td>Municipal sewage</td>
<td>240</td>
<td>5</td>
<td><em>Rhodobacter</em> 0 0 0 0 0</td>
</tr>
<tr>
<td>Municipal sewage</td>
<td>170</td>
<td>5</td>
<td><em>Rhodobacter</em> 0 0 0 0 0</td>
</tr>
</tbody>
</table>

All isolates were obtained from SAYS agar media used for the enumeration of PNSB, except those from the swine wastewater sludge, which were isolated by enrichment with SAYS medium and streaking on MY5 agar medium.

Table 2. Oxygen uptake with different lower fatty acids (C2 to C6) by the PNSB strains grown aerobically in the dark and semiaerobically in light.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Growth condition(^a)</th>
<th>Oxygen uptake rate (mg min(^{-1}) g(^{-1}) dry cells) with: (^b)</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Valerate</th>
<th>Caproate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodobacter azotoformans</em> KA25(^T)</td>
<td>A</td>
<td>117±14.2</td>
<td>-42.3±9.8</td>
<td>-42.3±9.8</td>
<td>-36.4±1.1</td>
<td>-20.3±4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>79.9±2.7</td>
<td>-38.5±6.2</td>
<td>-62.1±2.1</td>
<td>-62.1±2.1</td>
<td>-65.0±3.1</td>
<td></td>
</tr>
<tr>
<td>*Rhodobacter capsulatus DSM 1710(^T)</td>
<td>A</td>
<td>105±7.7</td>
<td>98.0±2.8</td>
<td>63.2±6.7</td>
<td>23.5±4.3</td>
<td>12.4±1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>64.4±4.5</td>
<td>73.1±3.1</td>
<td>25.6±1.6</td>
<td>28.9±1.9</td>
<td>-27.2±1.5</td>
<td></td>
</tr>
<tr>
<td>*Rhodobacter sphaeroides DSM158(^T)</td>
<td>A</td>
<td>85.3±9.5</td>
<td>24.9±7.4</td>
<td>73.3±13.5</td>
<td>6.06±2.1</td>
<td>-18.6±2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>184±20.8</td>
<td>-21.2±1.5</td>
<td>88.3±2.0</td>
<td>9.84±0.6</td>
<td>-21.2±1.5</td>
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<tr>
<td>*Rhodobacter sphaeroides SA38</td>
<td>A</td>
<td>101±11.7</td>
<td>-38.0±4.5</td>
<td>-47.9±6.5</td>
<td>-47.0±12.3</td>
<td>-76.9±7.6</td>
<td></td>
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<tr>
<td></td>
<td>B</td>
<td>95.4±4.5</td>
<td>-53.5±6.9</td>
<td>0±0</td>
<td>-74.7±8.7</td>
<td>-77.2±5.7</td>
<td></td>
</tr>
<tr>
<td>*Rhodobacter sphaeroides LW-BR</td>
<td>A</td>
<td>253±13.8</td>
<td>-79.4±6.5</td>
<td>-70.3±3.8</td>
<td>-121±8.8</td>
<td>-121±8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>206±8.5</td>
<td>-61.2±1.6</td>
<td>-29.0±0.4</td>
<td>-64.4±12.1</td>
<td>-77.2±9.6</td>
<td></td>
</tr>
<tr>
<td><em>Rhodopseudomonas</em> sp. PL29</td>
<td>A</td>
<td>409±3.3</td>
<td>279±11.2</td>
<td>270±6.6</td>
<td>262±13.8</td>
<td>297±12.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>312±9.9</td>
<td>227±19.9</td>
<td>251±21.4</td>
<td>287±12.6</td>
<td>209±19.7</td>
<td></td>
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<tr>
<td><em>Rhodopseudomonas</em> palustris LW-RE</td>
<td>A</td>
<td>306±21.5</td>
<td>262±6.0</td>
<td>223±11.4</td>
<td>352±19.7</td>
<td>274±22.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>426±18.2</td>
<td>364±9.5</td>
<td>287±18.0</td>
<td>332±4.1</td>
<td>312±11.4</td>
<td></td>
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</tbody>
</table>

\(^a\) A, aerobic-dark; B, semi-aerobic-light.

\(^b\) Negative values indicate the degree of inhibition of the endogenous oxygen uptake.
BOD levels. These results indicate that the concentration of organic nutrients in addition to light intensity and DO tension is an important factor affecting PNSB populations in terms of both quantity and quality. In the wastewater treatment plants studied, volatile organic acids, especially acetate, were the major source of BOD, suggesting that the concentration of lower fatty acids is the key determinant in this respect.

In general, LFAs have been thought to be good carbon and energy sources for phototrophic bacteria. However, the results of oxygen uptake experiments in this study have revealed significant differences in the utilization of LFAs between members of the genera Rhodobacter and Rhodopseudomonas. LFAs with larger carbon numbers, such as valerate and caproate, exerted serious inhibitory effects on endogenous oxygen uptake by the Rhodobacter species, while such effects were not found in the Rhodopseudomonas species. Growth inhibition by propionate has been reported in Rhodobacter sphaeroides. Inhibitory effects of LFAs on oxygen uptake and ATP synthesis have also been found in activated sludge and low BOD-loaded photosynthetic sludge. Thus, the test strains of Rhodopseudomonas have proved to be more nutritionally versatile with respect to the utilization of LFAs. This ability may give the Rhodopseudomonas a competitive advantage over the Rhodobacter in the environment.

Comparative kinetic analyses of oxygen uptake with acetate have revealed that the Rhodopseudomonas species have a greater affinity for the substrate than do the Rhodobacter species. This finding provides circumstantial evidence as to why Rhodobacter species are more abundant in high BOD-loaded wastewater, while Rhodopseudomonas species predominate in wastewater with lower BOD levels. Because of their greater affinity for acetate, the Rhodopseudomonas species are better suited to the competitive consumption of organic acids in low BOD environments. The differences between the Rhodopseudomonas and Rhodobacter species in affinity for acetate may be partly derived from the difference in the metabolic pathway for acetate, e.g., the glyoxylate cycle. The key enzymes of this pathway, isocitrate lyase and malate synthase, could be detected in Rhodopseudomonas palustris grown on acetate but were not detectable in Rhodobacter sphaeroides.

As mentioned above, our previous study showed that oxygen uptake by low BOD-loaded photosynthetic sludge, in which Rhodopseudomonas species rather than Rhodobacter were possibly the predominant PNSB, was inhibited by LFAs with larger carbon numbers. This may be a reflection of the negative effects of the LFAs on oxygen uptake by the chemotrophic bacteria which occurred in much greater numbers than the phototrophic bacteria.

In conclusion, the present study indicates that the population density and structure and ecological roles of PNSB in wastewater environments differ at different trophic levels. However, our results might contain an enrichment bias and exclude the organisms present in small numbers due to the selective conditions of cultivation and isolation procedures used. The additional quantitative and qualitative estimation of PNSB populations by culture-independent methods, such as cloning and sequencing of functional genes universally distributed among PNSB, is necessary to confirm our conclusion. Also, one should note that the utilization of carbon sources by microorganisms, including phototrophic bacteria, is related not only to the concentrations, but also the combinations of different organic compounds existing together in wastewater. Rha. sphaeroides usually grows slowly on propionate as a sole carbon source but assimilates it rapidly in the presence of acetate. Other environmental
factors, such as gradients of light, DO tension and micro-
habitat variability, cause significant changes in the metabol-
ic activities of PNSB. How these environmental fluctua-
tions in wastewater influence the population dynamics of
PNSB and co-existing chemotrophic bacteria is still an im-
portant subject awaiting further investigation.

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