Short Communication

Population Dynamics and Acetate Utilization Kinetics of Two Different Species of Phototrophic Purple Nonsulfur Bacteria in a Continuous Co-culture System

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The population dynamics and acetate utilization kinetics of two strains of phototrophic purple nonsulfur (PPNS) bacteria, *Rhodopseudomonas* sp. strain TUT3630 and *Rhodobacter* sp. strain TUT3733, in a continuous co-culture system were investigated. The mixed populations were cultured stepwise at different concentrations of acetate under semi-aerobic conditions in the light and were monitored by 16S rRNA-targeted fluorescence in situ hybridization (FISH) and PCR-denaturing gradient gel electrophoresis (DGGE). Cells of *Rhodobacter* sp. strain TUT3733, having a low affinity for acetate, dominated when the acetate concentration in the feed ranged from 5 to 20 mM. On the other hand, the feeding of less than 1 mM of acetate resulted in an increase in cell numbers of *Rhodopseudomonas* sp. strain TUT3630 due to its high affinity for acetate. These results suggest that the affinity for acetate is one of the most important determinants for the competitiveness among different species of PPNS bacteria in the environment. Our results provide a plausible explanation for why *Rhodobacter* species proliferate as the major PPNS bacteria in wastewater environments containing high levels of lower fatty acids, and the reverse is the case in *Rhodopseudomonas* species.

Key words: phototrophic bacteria, lower fatty acids, substrate affinity, *Rhodobacter*, *Rhodopseudomonas*

Members of the genera *Rhodopseudomonas* and *Rhodobacter*, representing the major groups of phototrophic purple nonsulfur (PPNS) bacteria belonging to the *Alphaproteobacteria*⁽¹⁰⁾, have been extensively studied not only in their ecophysiological aspects but also for their application to wastewater treatment^(6,7,9,11–14,17,19–21), because of their metabolic versatility. These PPNS bacteria are able to grow well using lower fatty acids (LFAs) and many other simple organic compounds as electron donors and carbon sources under both aerobic-dark and anaerobic-light conditions. It has been suggested that organic nutrient strength, especially the bioavailability of LFAs, is one of the most important factors affecting the viability and activity of

PPNS bacteria in activated sludge and photosynthetic sludge systems^(3,4,7,17,22). However, few studies have focused on the relationship between the distribution of different genera or species of PPNS bacteria in the environment and their substrate affinity. Our previous study showed that differences in substrate specificity and affinity for LFAs exist between members of the genera *Rhodopseudomonas* and *Rhodobacter* detected in wastewater treatment systems⁽¹⁷⁾. We also found that pink-colored microbial mats composed of PPNS bacteria as the major constituents developed in a swine wastewater ditch, and suggested that the kind and concentration of LFAs in wastewater are important for the formation and phylogenetic composition of the PPNS bacterial mats⁽¹⁸⁾. In this study, the population dynamics and substrate utilization kinetics of *Rhodopseudomonas* and *Rhodobacter* strains originating from the swine wastewater

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microbial mats and having different substrate affinities for LFAs were investigated using a co-culture system in order to confirm whether the two organisms actually respond to LFA gradients in a mixed continuous culture. The main techniques used for monitoring the population dynamics of the PPNS bacteria were 16S rRNA-targeted fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes. The final goal of our study is to elucidate what kinds of PPNS bacteria in terms of taxonomy and physiology are suitable for wastewater treatment systems loaded with different concentrations of LFAs.

Rhodopseudomonas sp. strain TUT3630 and *Rhodobacter* sp. strain TUT3733, both of which were isolated previously from swine-wastewater pink-colored microbial mats¹⁸, were used. The 16S rRNA gene sequences of strains TUT3630 (DDBJ accession number, AB251405) and TUT3733 (AB251409) have 100% and 99.7% similarity to those of *Rhodopseudomonas palustris* strain DSM 123^T (AB275664) and *Rhodobacter azotoformans* KA25^T (D70846)^{5,6}, respectively. As previously reported, *Rhodopseudomonas* sp. strain TUT3630 has a wider spectrum of LFA utilization and a higher affinity for acetate than *Rhodobacter* sp. strain TUT3733¹⁸. In this study, the two strains were further tested as to their affinity for propionate as well as acetate, because the tested organisms were isolated from a swine wastewater ditch where propionate was detected as a major LFA component in addition to acetate. For this, cells were grown aerobically at full atmospheric tension in darkness or semi-(an)aerobically in the light in a chemically defined medium designated AVS, which contained mineral base RM2³, 10 mM acetate as the sole carbon source and a vitamin solution² (1 ml L⁻¹). After the cultures reached the mid-exponential phase of growth, cells were harvested by centrifugation (11,000×g, 10 min), washed twice with 50 mM phosphate buffer (pH 7.0), and resuspended in this buffer to give an optical density at 660 nm (OD₆₆₀) of 2.0 to 2.5. They were then subjected to oxygen consumption assays. Oxygen uptake was measured at 25°C using an Iijima model B-505 DO analyzer and 0.1 to 10 mM of substrate, and kinetic analyses of substrate utilization were performed as described previously¹⁷. As shown in Fig. 1, *Rhodopseudomonas* sp. strain TUT3630 had smaller Michaelis-Menten K_s values and larger V_{max} values for the two LFAs than did *Rhodobacter* sp. strain TUT3733, thereby confirming the significant difference in substrate affinity between the two organisms. Also, the *Rhodopseudomonas* strain exhibited a higher V_{max} for both the LFAs under semi-aerobic light growth conditions than

Fig. 1. Kinetic analyses of oxygen uptake with acetate (C2) and propionate (C3) as the substrate in the *Rhodopseudomonas* sp. strain TUT3630 and *Rhodobacter* sp. strain TUT3733. (a) average K_s obtained with cells grown aerobically at full atmospheric oxygen tension in darkness (solid circles) and semi-aerobically in the light (open circles); the bars between the circles indicate a range of K_s values changeable depending upon DO tension. (b) average V_{max} obtained with cells grown aerobically at full atmospheric oxygen tension in darkness (solid histograms) and semi-aerobically in the light (open histograms); bars=standard deviations. All the experiments were performed in triplicate. The data on acetate uptake are based on information from Okubo *et al.*¹⁸.

under aerobic-dark conditions, whereas the reverse was the case in the *Rhodobacter* strain. Similar results for the affinity and uptake velocity for acetate have been obtained with several other strains of *Rhodopseudomonas* and *Rhodobacter* species^{17,18}. Despite the fact that strains TUT3733 and TUT3630 were isolated from a wastewater environment rich in acetate and propionate, the former strain differed from the latter in failing to grow in the presence of concentrations of propionate higher than 10 mM (data not shown). Therefore, it seemed difficult to construct a system for the co-culture of the two organisms with propionate as the sole carbon source.

Based on the aforementioned results, a continuous co-culture of *Rhodopseudomonas* sp. strain TUT3630 and *Rhodobacter* sp. strain TUT3733 was achieved by feeding AVS medium in which the concentration of acetate was changed in the range of 0.5 to 20 mM. An equal volume (500 ml) of precultures of the two organisms phototrophically grown in AVS medium (OD₆₆₀=0.5 each) was taken and transferred into the continuous culture system which consisted of a 1.5-L culture glass vessel (working volume, 1,000 ml) and a medium reservoir (Fig. 2). The continuous co-culture system was operated at 25°C and a dilution rate of 0.26 day⁻¹ and for 5 weeks under incandescent illumination (ca. 2,000 lx); the culture was not aerated but stirred with a magnetic stirrer at 120 rpm, thus being under semi-aerobic phototrophic growth conditions. The concentration of acetate in the feed was initially 0.5 mM, then increased to

Fig. 2. Schematic diagram of the continuous co-culture system for the phototrophic bacteria. (1) Medium reservoir; (2) medium-feeding pump; (3) co-culture vessel (capacity, 1.5 L; working volume, 1.0 L); (4) incandescent lamp; (5) circulation pump; (6) valve; (7) sampling tube. The culture system was maintained at 25°C and 2,000 lx and supplied with the medium at a flow rate of 260 ml day⁻¹.

20 mM, and subsequently decreased stepwise to 10, 5 and 1 mM (see the top of Fig. 3). Samples from the co-culture vessel were taken on days 0, 5, 7, 11, 14, 18, 21, 26, 28, 32 and 35, and dissolved oxygen (DO) tension, pH, optical density at 660 nm (OD_{660}), the concentration of acetate and K_s for acetate were measured according to protocols reported previously^{17,18}.

As shown in Fig. 3, the chemical and physiological characteristics of the co-culture system changed greatly depending upon the concentration of acetate in the feed. When supplied with 5 mM and higher concentrations of acetate, the co-culture system exhibited a low DO tension (<0.5 mg l⁻¹), an alkaline pH and high population densities (OD_{660} >1.0). In contrast, feeding of 1 mM or less acetate resulted in a much higher DO tension, a neutral pH and low population densities. Acetate was not detected in the effluent as long as the concentration of acetate in the feed was lower than 5 mM. When the acetate concentration in the feed was more than 10 mM, it remained to some extent in the effluent. The K_s value for acetate of the co-culture system varied between 0.11 and 0.23 mM depending upon the concentration of acetate in the feed. This fluctuation appeared to be reflective of the population changes of the two organisms, which exhibited different K_s values for acetate when grown aerobically (see Fig. 1). Also, the changes in the chemical and physiological characteristics seemed to be reversible in response to different acetate-loading rates.

The relative abundance of the two PPNS organisms in the co-culture system during the operational period was determined by FISH probing and PCR-DGGE. The plate-counting method might not work well for this purpose, because the cells in the co-culture system were self-aggregated depending upon the substrate concentration. For FISH, we

Fig. 3. Changes in chemical and physiological characteristics of the continuous co-culture system operated at different acetate-loading rates. (a) DO tension; (b) pH; (c) OD_{660} and the concentration of acetate remaining (triangles); (d) Michaelis-Menten K_s for acetate. The concentration of acetate (mM) in the feed is shown at the top of the figure.

designed a Cy3-labeled probe, RPS1422 (5'-CACTGCCTTCAGGTAGAACC-3'), for detecting *Rhodopseudomonas* sp. strain TUT3630 and a FITC-labeled probe, RBA145 (5'-CCGTACCTTTGGGCATGTTTC-3'), for *Rhodobacter* sp. strain TUT3733 based on their 16S rRNA gene sequences. Cells were fixed with 4% paraformaldehyde, hybridized with the probes for 2 h at 46°C, and observed under an Olympus epifluorescence microscope as described previously⁸). In the PCR-DGGE analysis, bulk DNA was extracted and purified from the co-culture system as well as from pure cultures of the two test strains, and the variable V3 region of the 16S rRNA gene (233 bp) was amplified

Fig. 4. 16S rRNA-targeted FISH detection of cells of *Rhodospseudomonas* sp. strain TUT3630 (red) and *Rhodobacter* sp. strain TUT3733 (green) in the continuous co-culture system on days 0 (a), 25 (b) and 35 (c). Scale bars=10 μ m.

with these DNA samples as the template according to protocols described previously^{15,16}. Electrophoresis was carried out for 3 h at a voltage of 200 V and a temperature of 58°C using the Bio-Rad Dcode system (Bio-Rad Laboratories, Hercules, USA).

The cell counts of the two strains TUT3630 and TUT3733 in the co-culture system as measured by FISH probing accounted for 45 and 55% of the total population (7.0×10^8 cells ml^{-1}) at the start of operation, respectively (Fig. 4a). During the first week in which 0.5 mM acetate was continuously added to the system, the cell number of *Rhodospseudomonas* sp. strain TUT3630 increased gradually and accounted for approximately 60% of the total population at the end of this first stage (not shown). In the second phase at which the acetate concentration in the feed was

Fig. 5. PCR-DGGE detection of 16S rRNA genes of *Rhodospseudomonas* sp. strain TUT3630 and *Rhodobacter* sp. strain TUT3733 from the continuous co-culture system. The 1st and 2nd lanes show the PCR products from pure cultures of strain TUT3630 and TUT3733, respectively. The remaining lanes show the PCR products from the co-culture system on days 0 to 35. The DGGE was performed with a 40–60% denaturing gradient.

increased to 20 mM, *Rhodobacter* sp. strain TUT3733 constituted the overwhelming majority of the total population with the concomitant formation of cell aggregates (data not shown) in which extracellular DNA was possibly involved¹⁸. Due to this floc formation, it was difficult to precisely estimate the cell number of both the organisms, although the treatment of samples fixed on slide glasses with a DNase I (TAKARA BIO Inc., Otsu, Japan) solution (5 units ml^{-1}), for 30 min prior to FISH improved the resolution to some degree. The FISH analysis after DNase treatment clearly showed that strain TUT3733 fully dominated as long as the acetate concentration in the feed was more than 5 mM (e.g., Fig. 4b). The cell number of *Rhodospseudomonas* sp. strain TUT3630 increased again with a decrease in the acetate concentration to 1 mM (Fig. 4c).

The DGGE banding patterns of the 16S rRNA genes from the co-culture system were consistent with the results of FISH probing (Fig. 5). When the acetate concentration was sufficiently high (on days 11 to 28), the DGGE bands of *Rhodobacter* sp. strain TUT3733 were clearly detected, whereas those of *Rhodospseudomonas* sp. strain TUT3630 were not. When the acetate concentration in the feed was less than 1 mM (on days 0 to 7 and 32 to 35), the PCR product from strain TUT3630 appeared as a strong DGGE band compared to that of strain TUT3733. One should note that the intensity of DGGE bands of the two organisms noted above might not be directly proportional to their relative populations, because the copy number of chromosomal 16S

rRNA genes of PPNS bacteria differs from species to species.

The present experiments, investigating the competition between two different PPNS bacteria in a co-culture system grown semi-aerobically in light, have provided evidence that the concentration of acetate as the substrate is a key factor controlling the species composition of the PPNS bacterial community. This important determinant is possibly true for other LFAs. In the pink-colored microbial mats from which the two test organisms were previously isolated, *Rhodopseudomonas* species occurred in higher numbers than *Rhodobacter* species¹⁸. This relative abundance of *Rhodopseudomonas* and *Rhodobacter* in the microbial mats may result from the differences between the two in substrate utilization kinetics for acetate and propionate, both of which were the major organic nutrients in the swine wastewater¹⁸. The results of this study, together with those reported previously^{4,7,17}, provide a plausible explanation as to why *Rhodobacter* species have been found in large numbers in aerobic wastewater environments containing high levels of lower fatty acids^{1,7,17}, whereas *Rhodopseudomonas* species have been detected as the major phototrophs mainly in less organic-polluted wastewaters¹⁷. Although several investigators have studied the physiology and growth kinetics of PPNS bacteria, such as *Rhodobacter capsulatus*^{14,19–21}, *Rhodobacter sphaeroides*¹³ and *Rhodopseudomonas palustris*^{11,12}, in connection with their application to wastewater treatment, much more attention should be paid towards the suitability of different PPNS species for wastewater containing different levels of LFAs.

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