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Characterization of the Purple Nonsulfur Bacterium, *Rhodopseudomonas palustris* Strain P-1, Degrading Ferulate

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Photosynthetic bacteria which can utilize ferulate as a sole carbon source for their metabolic activities were isolated from soils by liquid enrichment culture technique. The strain P-1 was selected by the highest capability of degrading ferulate in aerobic and anaerobic conditions. The strain P-1 was rod-shaped with its motility, stained gram negatively and could not utilize sulfur compounds. This strain has the bacteriochlorophyll a, group I carotenoid and membrane structures like lamellae. As the results of physiological, morphological and cultural characteristics, the isolate was identified as *Rhodopseudomonas palustris*, one of the purple nonsulfur bacteria. The strain P-1 utilized 2 mM/day in aerobic condition and 0.86 mM/day in anaerobic condition.

KEY WORDS □ **photosynthetic bacteria, ferulate, purple nonsulfur bacteria, *Rhodopseudomonas palustris*.**

Large amounts of organic compounds with aromatic structures have been produced for industrial and agricultural purposes. Many of these synthetic compounds are released into the biosphere and are eventually accumulated in the environment. This accumulation threatens the normal balance in the carbon cycle. Therefore it is necessary to reduce the amount of synthetic organic compounds up to an adequate application level. Some aromatic compounds are found in higher plants as monomeric components in the plant polymer lignin and as various plant secondary products. During their biodegradation, presumably the complex polymers are broken down to simple aromatic lignin derivatives, the fate of which are not well understood. Several groups have studied the aerobic metabolism of these aromatic derivatives (5, 16). Apart from the detailed investigation of anaerobic degradation process about benzoic acid (6), relatively little attention has been paid to the anaerobic degradation of these aromatic organic compounds. The anaerobic transformation of methoxylated aromatic compounds which are released during the aerobic catabolism of lignin has been increased its attention recently (3).

In order to study the degrading activity of ferulate (4-hydroxy-3-methoxycinnamic acid, $C_{10}H_{10}O_4$) in both aerobic and anaerobic conditions, which is an aromatic lignin derivative and a more complexed compound than benzoic acid, we have isolated a purple nonsulfur photosynthetic bacterium and have studied its biochemical and physiological characteristics.

MATERIALS AND METHODS

Bacterial strains and enrichment cultures

Some photosynthetic bacteria from soils were isolated from the enriched culture media supplemented with ferulate (4-hydroxy-3-methoxycinnamic acid, $C_{10}H_{10}O_4$) and cultured anaerobically under light conditions. Enrichment cultures were performed in the bottles completely filled with the enriched media in order to sustain the anaerobic conditions (14). Isolation and purification were achieved by successive transfer and repeated restreaking onto the solid agar with enriched media. One strain P-1 was selected by the highest capability of degrading ferulate.

Conditions of media and cultures

The microorganism was cultured under both aerobic and anaerobic conditions at 30°C in the dark and light, respectively. The modified culture methods of Hungate (11) were applicated for anaerobic cultures. Bottles for anaerobic cultures were sealed with solid rubber stoppers aseptically, while a stream of N_2-gas was substituted for air in the bottle. Anaerobic cultures were performed at 30°C with 60-W incandescent lamps at a distance from 20 to 30 cm. Aerobic cultures were incubated with shaking (100 rpm) at 30°C by a

gyratory environmental shaker. The basal media had the following compositions (values in grams per liter of distilled water): NH_4Cl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.4; NaCl, 0.4; $CaCl_2 \cdot 2H_2O$, 0.05; KH_2PO_4, 1.0; Trace element mixture, 10 ml (18). The pH was adjusted to 7.2 with 3 N-NaOH prior to autoclave. trace element mixture was added aseptically to autoclaved media after filter sterilization. Ferulate was dissolved in absolute ethanol (Merck) and added aseptically to autoclaved media after filter sterilization. Since the bacterial growth in this media was slow, 0.1% (w/v) yeast extract was added if necessary. Agar (1.5%, w/v) was added to the mineral salts solution for solid media.

Identification of bacteria

Physiological and cultural characteristics of the strain P-1 were investigated according to the "Manual of Methods for General Bacteriolgy" (8) and the "Bergeys Manual of Determinative Systematic Bacteriology (8th ed)" (2). The type of flagellation was determined by the staining procedure of Leifson (8). Specimens were prepared for transmission electron microscopy by staining with 1% of uranyl acetate and examined with a Hitachi 600 transmission electron microscope.

Absorption spectrum

The absorption spectra of whole cells and bacteriochlorophylls were obtained by suspending in 60% sucrose (w/v) and by extracting into acetone-methanol mixture (7:2, v/v), respectively (17). Carotenoids were extracted from cells in the early stationary growth phase by using benzene-ethylether mixture (7:3, v/v), and they were separated by thin-layer chromatography (TLC) on kieselgel 60 F_{254} (Merck, Art. 5735) with n-hexane-ethylether-acetone solvent mixture (8:2:1, v/v/v).

Substrate utilzation

Bacterial growth were measured either by determining absorbance at 660 nm or by measuring dry weight after washing the cells with 10 mM K-Na phosphate buffer (pH 6.8). The washed cells were dried in aluminium cups at 105°C for 6 hrs (13). Generation times were calculated by measuring Optimal Density (O.D.) from the linear stage in the bacterial growth curve. The disappearance of ferulate was quantitatively monitored by UV spectrophotometry (Beckman, DU-40). Absorption spectrum from 240 to 340 nm was scanned, since the ferulate is absorbed maximally at 310 and 290 nm (9). Different values of the absorption suggest the transformational changes of the substrate.

RESULTS

Isolation of the microorganisms

Enrichment cultures were conducted in the bottles completely filled with media in order to sustain the anaerobic conditions. Isolated colonies were transferred to the same minimal media successively and cultured in Luria Bertani media for further purification. Some isolates having similar characteristics of the culture and morphology were detected. They showed relatively similar growth rate, but they showed different characteristics under aerobic and anaerobic conditions. One isolate was chosen by its highest capability of degrading ferulate and was designated as strain P-1.

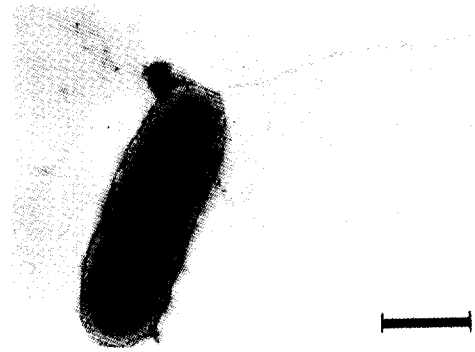

Fig. 1. *Transmission electron micrograph of the isolated strain P-1 showing polar flagellum. The specimen was stained with 1% of uranyl acetate. The bar represents 1.0 μm (x12,000).*

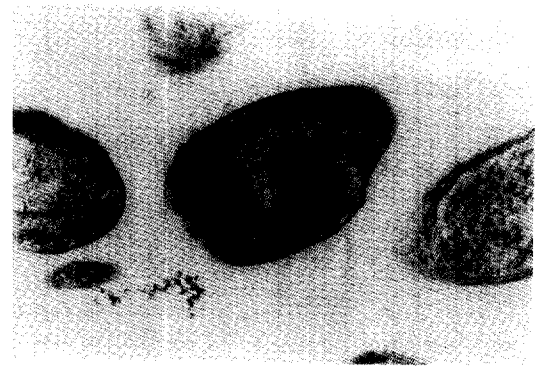

Fig. 2. *Electron micrograph of the intracytoplasmic membrane system of the strain P-1 grown anaerobically in the light conditions. (x60,000)*

Morphology of the strain P-1

Cells of the strain P-1 were rod with round ends, 0.9~1.1 μm wide and 2.6~3.0 μm long.

The strain P-1 showed single cell and active motility. This strain had monotrichous flagellum (Fig. 1) and classified as gram negative. Ultrathin sections of photosynthetically grown cells revealed the intracytoplasmic system consisting of lamellae which were pararelly underlying along with the cytoplasmic membrane (Fig. 2).

Characteristics of the strain

The morphological, physiolgical and cultural

Table 1. *Morphological, physiological and cultural characteristics of the isolated strain P-1*

Characteristics	Results
Morphology	
shape	rod
motility	motile
Gram staining	negative
Flagellar staining	positive
Growth	
anaerobic, light	positive
anaerobic, dark	positive
aerobic, light	positive
aerobic, dark	positive
Pigment	
anaerobic, light	purplish red
anaerobic, dark	none
aerobic, light	pink
aerobic, dark	none
Bacteriochlorophyll of living cell	a (873, 803, 589, 374 nm)
Carotenoid of living cell grown under anaerobic, light	group I
Starch hydrolysis	positive
Gelatine liquefaction	negative
Casein hydrolysis	positive
Catalase	positive
Hydrogenase	positive

characteristics of the strain P-1 were described in Table 1.

The optimal growth conditions of the strain P-1 were 30~35°C, pH 7.0~7.5 and anaerobic light condition. Although the anaerobically cultured cells were purplish red and colorless under the light and dark conditions, respectively, the aerobically cultured cells were pink or colorless under the same conditions of anaerobic cultures. No color-change has been observed with the addition of air into anaerobically grown cultures. The utilization of electron donors and organic compounds of strain P-1 was shown in Table 2.

The cells were cultured well in the minimal media containing 0.1% yeast extract and 10 mM various electron donors of Na-acetate, casamino acid, glycerol, glutamate, succinate. But they could not utilize well the media containing with arginine, aspartate, glucose, methanol, maltose, tartate. In order to confirm the requirement of growth factors, the strain P-1 was cultured in the media containing various vitamines of biotin, Ca-pantothenate, p-aminobenzoate, nicotinate, thiamine-HCl. The strain required not any vitamin for its growth.

Absorption spectrum

The absorption spectrum of whole cells (Fig. 3) showed the peaks at 374, 589, 803, 873 nm with

Table 2. *Utilization as carbon sources and/or electron donors of various compounds by the Rhodopseudomonas palustris strain P-1*

Carbon or Electron donor	Cell growth (μg dry weight/ml)
Control	85
Arginine	73
Na-Acetate	273
Asparatate	68
Casamino acid	235
Na-Citrate	86
Ethanol	200
Fructose	201
Formate	90
Glycerol	260
Glutamate	283
Gluconic acid	107
Glycolate	104
Glucose	73
Lactate	106
Methanol	73
Malic acid	145
Malonic acid	95
Malenic acid	82
Maltose	59
Mannitol	80
Propionic acid	106
Pyruvate	110
Sorbitol	82
Succinate	306
Sulfide	57
Tartarte	64
Thiosulfate	60

the same characteristics to bacteriochlorophyll a and the peaks at 464, 497, 530 nm similar with the carotenoid absorption patterns. The photopigments extracted with acetone-methanol mixture (7:2, v/v) showed the absorption peaks at 361, 465, 495, 765 nm.

The carotenoids were extracted with benzene-ethyl ether mixture (7:3, v/v) after the extraction of bacteriochlorophylls from the cells cultured anaerobically in the complete media for four days under the light conditions. The extracted carotenoids separated into three bands with the Rf value of 0.63, 0.33 and 0.24 on the TLC plate. The absorption spectra of three bands were as shown in Fig. 4. The three bands were similar to the group I carotenoid including sprilloxanthin (465, 495, 525 nm) and lycopene (472, 504 nm) (2, 12).

Substrate transformation

Figure 5 showed the correlation between cell growth and transformation of ferulate under the aerobic and anaerobic conditions. Three mM of ferulate was completely degraded in 36 hrs and

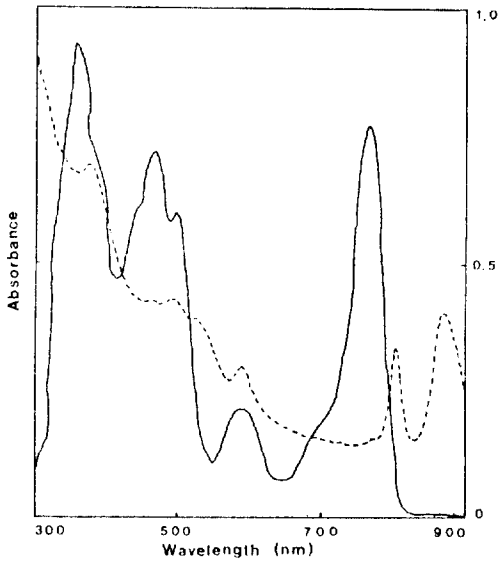

Fig. 3. *The absorption spectra of whole cells and extracted photopigments from the strain P-1 grown anaerobically under the light. The dotted line and continued line represent the spectra of whole cells and photopigments respectively.*

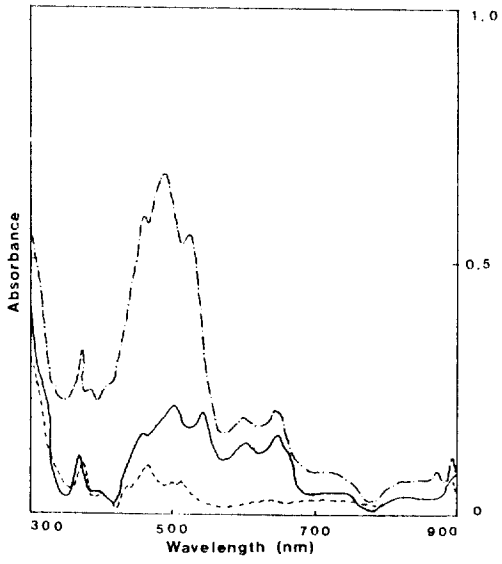

Fig. 4. *Absorption spectra of the pigment components isolated by thin layer chromatography (TLC) after extraction with acetone-methanol mixture (7:2 v/v). The long-dotted, continued and short-dotted line represent sprilloxanthin, lycopene, and unidentified pigments respectively.*

84 hrs under aerobic and anaerobic conditions, respectively.

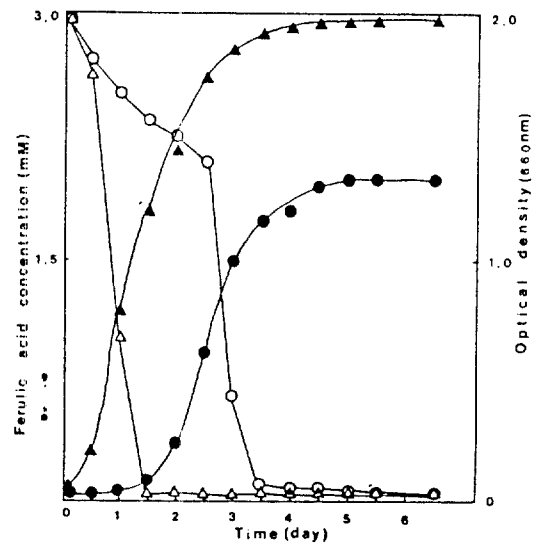

Fig. 5. *Growth curves of the strain P-1 and its degradation rate of ferulate in basal media containing 0.1% (w/v) yeast extract under aerobic and anaerobic conditions.*
▲; aerobic cell density ●; anaerobic cell density △; aerobic, with ferulate ○; anaerobic, with ferulate

Under the aerobic conditions, 10 mM of ferulate was completely degraded in 72 hrs. Activity of ferulate degradation was more effective under aerobic states than under anaerobic states. The strain P-1 showed excellent activity to degrade ferulate under aerobic states, and this strain showed the surpassing activities to degrade ferulate under anaerobic states compared to the other strains (9). In the defined medium with ferulate and yeast extract, the generation times under aerobic and anaerobic conditions were 22 hrs and 10 hrs, respectively. In aerobic incubation with the addition of yeast extract, generation time was 22 hrs compared to 60 hrs the same medium without yeast extract. In anaerobic incubation with yeast extract, generation time was reduced, too. The absorption spectra of ferulate was same in the both culture media with and without the addition of yeast extract. The absorption peaks of ferulate were declined with the lapse of incubation period in the both culture media with and without yeast extract. Yeast extract shortened the bacterial generation time apparantly, but the rate and extent of the ferulate degradation was not enhanced significantly.

DISCUSSION

One of the general characteristics of all phototrophic bacteria is to represent the anoxygenic

photosynthesis. The mode of energy production by the phototrophic bacteria can be performed under the light and anaerobic growth conditions or occasionally under the light and much reduced oxygen conditions. The formation of the central pigments of bacterial photosynthetic apparatus, bacteriochlorophyll (Bchl), is enhanced by oxygen inverse-proprotionally. The light increases the inhibitory effect to form the Bchl additionally. However, the sensitivity of Bchl synthesis to oxygen varies among different species of bacteria. This suggests that the synthesis of photopigments, Bchl and carotenoids, in the purple nonsulfur bacteria can be affected by both of light intensity and oxygen tension.

All species of the purple bacteria except *Rhodopseudomonas rubrum* which synthesize Bchl a are characterized by the Bchl formation systems in the presence of B800-850 proteins together with the RC-B 890 protein unit. This protein unit is associated with the reaction center of bacterial photosynthesis. Most species of the purple bacteria show type I B800-850 proteins and they show the absorption spectra in vivo with characteristic bands corresponding to 800 and 850 nm consequently (15). As shown in Fig. 4 of the absorption spectra the strain P-1 has RC-B890 protein and type I B800-850 proteins. From the results of morphological, physiolgical chatacteristics, intracytoplasmic membrane structure and photopigment, the strain P-1 was identified as *Rhodopseudomonas palustris* according to the manual of Bergy (2).

In the anaerobic pathways of bacteria, many aromatic substrates are metabolited into either benzoate or 4-hydroxybenzoate before the cleavage of aromatic rings. The two intermediate forms of benzoate and 4-hydoroxybenzoate indicate the two metabolic processes for aromatic ring cleavage by *R. palustris* (10). Since numerous studies were reported the formation of these two compounds during the anaerobic degradation of aromatic substrates by diverse bacteria (7, 9), the two different metabolic pathways could be recognized generally. Concerning on the transformation of ferulate under anaerobic conditions, *Acetobacterium woodii* (1) was able to demethylate via hydrolysis involving the cleavage of a phenylether bond. This reaction resulted in the formation of caffeate. Since caffeate was found in these experimemts, the demethoxylation had been occurred before side-chain transformation. It was different pathway from those performed by *Pseudomonas acidovorans* (19) and *Rhodococcus erythropolis* (4), in which ferulate was first converted into vanillate. The first reaction of the transformation was the conversion of the side chain into the vanillate and protocatechuate. The degradative fates of vanillate and protocatchuate are not explained clearly. These compounds could be metabolited by the strain P-1, but they are not detected in the liquid culture media during the bacterial growth because of their short existence period. Anaerobic demethoxylation and reductive dehydroxylation of aromatic compounds have been observed also in other bacterial degradation (4, 19). It can be suggested that the vanillate and protocatechuate are converted into 4-hydroxybenzoate, since the 4-hydroxybenzoate has been found in liquid culture media. The ferulate can be converted into 4-hydroxybenzoate via vanillate, protocatechuate anaerobically by the strain P-1 and be continued to degrade further along the pathways of aromatic compounds. Further investigations on their complete characterization including metabolic pathways and genetic studies are in progress.

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초 록: Ferulate를 분해하는 *Rhodopseudomonas palustris* Strain P-1의 특성에 대하여

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대사경로에서 단일탄소원으로 ferulate를 이용하는 광합성세균들을 액체 집적배양법에 의해 토양으로부터 분리하였다. 그 가운데 ferulate의 분해능력이 우수한 균주 P-1을 선택하여 여러가지 형태적, 생리적 특징을 조사한 결과는 다음과 같았다. 균주 P-1은 그람음성이고, 운동성이 있는 간균으로, 황화합물을 이용하지 못하였다. 또한 박테리오클로로필 a와 group I 카로티노이드계 색소를 가지고, lamellae 형태의 막 구조를 가지고 있으므로 홍색 비황세균의 한 종류인 *Rhodopseudomonas palustris*로 동정되었다. 이 균주는 호기적 상태에서 하루에 2 mM의 ferulate를 분해하고 혐기적 상태에서는 하루에 0.86 mM의 ferulate를 분해하였다.