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Degradation of polyvinyl alcohol by *Sphingomonas* sp. SA3 and its symbiote

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Abstract A total of 800 samples was taken from Taegu province, Korea, where many textile factories provide a source of polyvinyl alcohol (PVA) waste. These samples were screened for PVA-degrading bacteria. A new strain, SA3, was discovered which formed yellow colonies and used PVA as the sole carbon and energy source. Strain SA3 was identified as a *Sphingomonas* sp., based on the partial nucleotide sequence analysis of 16S ribosomal RNA, the presence of 2-hydroxymyristic acid (14:O 2-OH) and sphingolipids with d-17:0, d-18:0, d-19:1, and d-20:1 as the main dihydrosphingosines. This genus has not previously been reported as a PVA-degrading bacterium. *Sphingomonas* sp. SA3 needs a symbiote strain, SA2, for PVA degradation as a growth factor producer. In mixed cultures of these strains, the optimum temperature for PVA biodegradation ranged from 30°C to 35°C. The optimum pH was 8.0 and the most effective nitrogen source was NH_4^+ .

Keywords Biodegradation · Polyvinyl alcohol · *Sphingomonas* sp. SA3 · Symbiosis

Introduction

Polyvinyl alcohol (PVA), a water-soluble synthetic polymer, has commercial applications in the adhesive,

paper-coating and textile industries and in biodegradable polymers [5]. However, it is one of the major water pollutants in industrial wastewater, especially in textile factory wastewater, which contains large amounts of PVA. In the environment, PVA is not easily decomposed. Therefore, biological methods were investigated as a means of degradation of PVA [3].

There have been reports on the biodegradation of PVA [3, 4, 5, 6, 7, 9, 10]. *Pycnoporus cinnabarinus* is reported to enhance degradation of pre-oxidized PVA [5], but almost all PVA-utilizing bacteria require two strains to accomplish its degradation. Biodegradation may be applicable to the biological treatment of wastewater containing PVA [4].

Among the models to explain obligate mixed bacterial degradation of a recalcitrant compound, two have been proposed for co-degradation of PVA. The first, reported by Mori et al., is co-metabolism by two strains, in which neither produces a growth factor [6]. The second model, by Sakazawa et al., is degradation by two strains, in which one produces a growth factor and the other utilizes this growth factor for polymer degradation [9].

In the work reported here, two strains were isolated from environmental samples. By studying each strain's role in PVA utilization, one was confirmed as a PVA-degrading bacterium. This was identified as a novel strain of *Sphingomonas*, a genus that has not previously been reported as a PVA-degrading bacterium. In addition, several factors for PVA degradation were investigated.

Materials and methods

Screening of PVA-degrading bacteria, medium and culture conditions

PVA-degrading bacteria were isolated from environmental samples from Taegu, Korea by repeated enrichment culturing in a PVA liquid medium and on Luria Broth (LB) agar plates.

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The PVA medium used in this work was the same as that used by Suzuki et al. [12]. It contained the following (per liter of distilled water): 5.0 g PVA (Junsei Chemical Co., Osaka, Japan; degree of polymerization: 500), 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g KH_2PO_4 , 8.0 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NaCl , 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g FeSO_4 , 0.5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 mg MnSO_4 , 0.4 mg Ca-pantothenate, 0.2 mg inositol, 0.2 mg *p*-aminobenzoate, 0.4 mg pyridoxine, 0.4 mg thiamine, 2 μg biotin and 0.5 μg vitamin B12. The pH was adjusted to 7.5. The PVA medium base substituted with other nitrogen or carbon sources was used to study nitrogen and carbon utilization by the isolated bacterium. The isolated strains were maintained in pure cultures in LB: 1% polypeptone (Showa Chemicals, Tokyo, Japan), 0.5% yeast extract (Becton Dickinson Microbiology Systems, Md., USA) and 0.5% NaCl , at pH7.2. For the solid medium, 15 g of agar was added to 1 l of the medium. The liquid culture was grown in a test tube with 5 ml of medium or in a 250-ml Erlenmeyer flask with 25 ml of medium at 30°C on a rotary shaker at 180 rpm. Growth was estimated by reading the optical density at 660 nm (OD_{660}).

Estimation of PVA

The PVA concentration was measured spectrophotometrically, using the method of Finley [1]. The method is based on the green color produced by the reaction of PVA with iodine in the presence of boric acid. PVA medium (5 g PVA/l) was used as the standard PVA solution. This was diluted to 0.5 ml with dH_2O , and then 0.75 ml of 4% boric acid and 0.15 ml of I_2 -KI (12.7 g of I_2 and 25 g of KI in 1 l) were added. The solution was mixed well and equilibrated for 30 min at room temperature. Then, the mixture was diluted to a volume of 2.5 ml with dH_2O and analyzed at 660 nm. The standard curve was linear over 0–100 $\mu\text{g}/\text{ml}$.

To measure the PVA degradation, the culture broth was centrifuged at 18,000 *g* for 20 min at 4°C, diluted appropriately and the PVA content was estimated. All measurements were determined in triplicate. The percentage error between replicates was 3% or less.

Confirmation of the role of each strain

Each of the bacteria involved in PVA degradation was cultured in PVA medium for 4 days. Then, the cells in the culture broth were removed by centrifugation, the culture broth was mixed with fresh PVA medium, autoclaved and inoculated with the other bacterium. The growth of both bacteria and their PVA degradation were measured.

16S ribosomal nucleic acid sequence analysis

16S ribosomal RNA was analyzed after PCR amplification of genomic DNA [13]. Strain SA3 was cultured in LB broth. Cells were harvested by centrifugation and chromosomal DNA was extracted. The 1.4-kb 16S ribosomal RNA gene was amplified with two primers: fD1 (5'-CCGAATTCGTCGACA ACAGA GTTTGATCCTGGCTCAG-3') and rD1 (5'-CCC GGG ATC-CAAGCTTAAGGAGGTGATCCAGCC-3'). Gene amplification was performed in a thermal cycler (Techne, Duxford, Cambridge, UK). Conditions consisted of 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, followed by 5 min at 70°C. The amplified PCR product was purified by an AccuPrep gel purification kit (Bioneer Co., Chungbuk, Korea) and cloned into a pT7 blue vector (Novagen Co., Madison, Wis., USA). The PCR product in a clone was sequenced using an ABI Prism 310 genetic analyzer (Perkin Elmer, New Jersey, N.J., USA). The sequence obtained was analyzed with a set of similarity search programs designed to explore all available sequence databases,

using the National Center for Biotechnology Information (NCBI) blast search program (<url><http://www.ncbi.nlm.nih.gov/BLAST/></url>).

Fatty acid methyl ester analysis

Whole-cell fatty acids of the bacteria were analyzed as methyl esters by gas chromatography [8]. The microbial identification system (MIDI, Newark, Del., USA) and Sherlock ver 2.95 were used to perform a comparative analysis.

Sphingolipid analysis

Sphingolipids were extracted and analyzed using the method of Nohynek et al. [8]. Cells (0.5 g wet weight) were harvested and 5 ml of 12 M HCl -methanol (1:3, v/v) were added for acid methanolysis. The resulting suspension was heated for 3 h in a 100°C waterbath. The fatty acid methyl esters were then extracted three times with 2 ml of *n*-hexane-diethyl ether (1:1, v/v). The remaining water phase was made alkaline by dissolving four pellets of solid KOH in it. Sphingosines were extracted three times with 2 ml of *n*-hexane-diethyl ether (1:1, v/v) and the upper phases were collected for sphingosine analysis. For mass spectrometric analyses, sphingosines were silylated as follows: the *n*-hexane-diethyl ether extract was evaporated to dryness under a stream of N_2 and 200 μl of bis(trimethylsilyl)trifluoroacetamide were added. After heating this reaction mixture at 70°C for 2 h, the reagent was evaporated under a stream of N_2 and the residue was dissolved in 200 μl of hexane. Sphingosines were then analyzed with a gas-liquid chromatograph-mass spectrometer (model 5890; Hewlett Packard Co., Palo Alto, Calif., USA) equipped with a HP5 capillary column (Hewlett Packard) and a mass-selective detector (model HP5970). The temperature was increased over 40–290°C at a rate of 8°C/min and the injection temperature was 200°C. DL-Dihydrospingosine (Sigma Chemical Co., St. Louis, Mo., USA) was used as the reference compound.

Results

Screening of a PVA-degrading strain

Eight hundred environmental samples, such as soil, water and sludge, were collected and subjected to enrichment-culturing in liquid PVA medium for 10 days. One of the sludge samples, SA, showed PVA degradation and growth of bacteria. The SA culture was plated onto PVA agar and cultivated for 6 days at 30°C. Small white colonies, all having the same shape, were formed on the PVA agar plate, but this strain could not degrade PVA in liquid PVA medium. Therefore, a portion of the SA enrichment culture in PVA medium was diluted in sterile liquid PVA medium and appropriate dilutions were plated on LB agar. After 4–8 days of incubation, seven different bacteria were isolated independently on the LB agar plates. None of these strains, SA1–SA7, showed decomposition of PVA. However, in the mixed culture of these strains, PVA did degrade. For PVA degradation in PVA medium, strains SA2 and SA3 were required.

Strain SA2 grew and formed white colonies, less than 1 mm diameter, on PVA agar plates after cultivation for 6 days at 30°C; and only very slight growth was

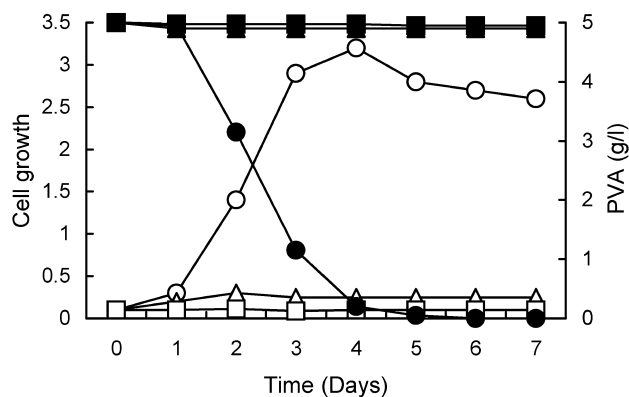


Fig. 1 Polyvinyl alcohol (PVA) concentration and bacterial growth in pure and mixed cultures of strain SA2 and *Sphingomonas* sp. SA3. *White triangles* Growth of strain SA2, *white squares* growth of strain SA3, *white circles* growth of strains SA2 and SA3 in co-culture, *black triangles* PVA concentration in strain SA2 culture medium, *black squares* PVA concentration in SA3 culture medium, *black circles* PVA concentration in SA2 and SA3 co-culture medium. Cell growth was measured by optical density at 660 nm

observed without PVA degradation in the PVA liquid cultures (Fig. 1). Sakazawa et al. reported a similar result, showing growth of non-PVA-utilizing colonies on PVA agar medium by utilization of impurities in the solid medium [9]. The slight growth of strain SA2 ($OD_{660}=0.18$) may be due to utilization of impurities such as sodium acetate in PVA [3]. PVA is made from polyvinyl acetates by replacing the acetate groups with hydroxyl groups; and sodium acetate is a good carbon source for growth of strain SA2 (data not shown). Strain SA3 formed yellow colonies on LB agar plates; but no growth on PVA agar plates or in PVA liquid cultures was observed.

PVA degradation by the co-culture

PVA degradation by strains SA2 and SA3 in pure or mixed cultures was examined. Fresh cultures of strains SA2 and SA3 from LB medium were inoculated at 1% (v/v) into PVA medium.

The pure culture of strain SA2 or SA3 showed no PVA degradation, but the co-culture of strains SA2 and SA3 showed abundant growth and full PVA degradation. After 4 days in mixed cultures, cell growth reached a maximum and about 95% of the PVA was degraded (Fig. 1).

Roles of strains SA2 and SA3 in PVA degradation

Culture supernatant was prepared from a 4-day-old culture of each strain incubated in PVA medium. The culture of strain SA2 showed slight growth, as shown in Fig. 1. After removal of the cells by centrifugation, 3 ml

Table 1 Effect of strains SA2 and SA3 growing in polyvinyl alcohol (PVA) medium (see Materials and methods). Cell growth was measured by optical density at 660 nm after 5 days of growth

Strain	Medium	Cell growth	PVA removal (%)
SA3	SA2 culture broth + PVA medium	3.63	85
SA2	SA3 culture broth + PVA medium	0.12	5

of the supernatant was mixed with 1 ml of fresh PVA medium, autoclaved at 121°C for 15 min and then the other strain, freshly cultured from LB medium, was inoculated at 1% (v/v) into this medium.

The culture of strain SA2 in the medium containing SA3-culture broth showed little PVA degradation, but the culture of strain SA3 in the medium containing SA2-culture broth showed 85% PVA degradation and growth of strain SA3 after 5 days (Table 1). As a result, strain SA3 was confirmed as the biodegrader of PVA and strain SA2 as a growth factor producer.

One PVA-degrading bacterium required pyrrolo-quinoline quinone (PQQ), the cofactor of many dehydrogenase reactions [2], as a cofactor for growth and PVA degradation in PVA medium [11]. Thus, we examined whether strain SA3 requires PQQ for PVA degradation. Strain SA3 was cultured without strain SA2 in PVA medium containing PQQ (10 ng/ml); and growth of strain SA3 and degradation of PVA were observed (data not shown).

Identification of the PVA-degrading strain

Cells of strain SA3 were yellow-pigmented, non-fermentative, gram-negative rods. Figure 2 shows the morphology of strain SA3. Physiological characteristics of strain SA3 are shown in Table 2. Strain SA3 utilizes glucose, trehalose, citrate, succinate, sodium acetate and sodium pyruvate, but does not utilize inositol, mannitol, sorbitol, xylitol, arabinose, xylose, glycerin, rhamnose, galactose, fructose, mannose, lactose, maltose, sucrose, cellobiose, salicin, sodium propionate, lactic acid, adonitol, raffinose, inuline, dextran, melezitose and sodium malonate as sole carbon sources.

The whole-cell fatty acids of strain SA3 are: 1.38% 2-hydroxytetradecanoic acid (14:0 2OH), 7.4% 2-hydroxypentadecanoic acid (15:0 2OH), 1.27% 2-hydroxyhexadecanoic acid (16:0 2OH), 6.19% pentadecanoic acid (15:0), 7.12% hexadecanoic acid (16:0), 3.8% heptadecanoic acid (17:0), 1.71% hexadecenoic acid (16:1), 44.29% heptadecenoic acid (17:1), 16.2% octadecenoic acid (18:1), and 10.63% summed feature of hexadecenoic acid (16:1) and 2-hydroxy isopentadecanoic acid (15:0 iso 2OH).

The sphingolipids were obtained from strain SA3. The trimethylsilyl derivatives of dihydrosphingosines were analyzed by gas-liquid chromatography–mass spectrometry and shown to contain dihydrosphingosines

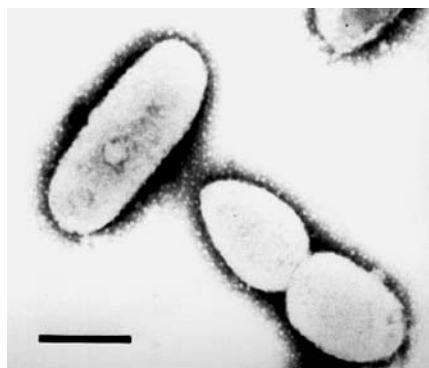


Fig. 2 Transmission electron micrograph of *Sphingomonas* sp. SA3. Cells were incubated in LB medium at 30°C for 40 h on a rotary shaker at 180 rpm, stained with 2% phosphotungstic acid (pH 7.0) and observed using a Philips CM20 electron microscope. Bar 1 μ m

Table 2 Characteristics of *Sphingomonas* sp. SA3. + Positive reaction, – negative reaction

Characteristic	SA3
Cell diameter (μ m)	0.8–1.0
Cell length (μ m)	2.1–2.4
Flagella	None
Gram strain	Negative
Cell form	Rod
Growth at 40°C	+
Growth with NaCl (0%)	+
Growth with NaCl (6%)	–
Diaminopimelic acid	Meso-DAP
Oxidase reaction	–
Catalase reaction	+
Hemolysis	–
Serum requirement	–
Growth on nutrient agar	+
Starch hydrolysis	–
Gelatin hydrolysis	–
Casein hydrolysis	–
Tween 80 hydrolysis	+
Urease reaction	–
Nitrate reduction	–
Indole production	–
Phenylalanine deaminase	–
Lysine decarboxylase	–
Ornithine decarboxylase	–
Arginine decarboxylase	–
H ₂ S	–
O/F test	–

d-17:0, d-18:0, d-19:1 and d-20:1 (number of carbon atoms: number of double bonds). The mass fragmentogram of a commercially available dihydrosphingosine with 18 carbon atoms (d-18:0) was identical to the mass fragmentograms of the d-18:0 dihydrosphingosines of strain SA3 (data not shown).

A comparison of the partial sequences of 16S ribosomal RNA of strain SA3 (GenBank accession number AF327069) with the sequences of the *Sphingomonas* reference strains showed that *S. adhaesiva* and *S. terrae* are the closest species to strain SA3. Strain SA3 exhibits 98% 16S ribosomal DNA sequence similarity to

S. adhaesiva and *S. terrae*. As a result, strain SA3 was identified as a *Sphingomonas* sp. This genus has not previously been reported as a PVA-degrading bacterium.

Effects of several factors on PVA degradation

To optimize PVA degradation by the co-culture of strains SA2 and SA3, nitrogen sources, the initial pH of the medium and the culture temperature were investigated.

Many kinds of nitrogen sources were added to PVA medium at 0.1% concentration. After shaking the culture at 30°C for 5 days, ammonium salts were proven to be most effective for PVA degradation.

To determine the optimum temperature and pH, mixed cultures were grown in PVA medium for 5 days at pH 7.5 for the temperature studies and at 30°C for the pH studies. Figure 3 shows the optimum pH and temperature for bacterial growth and PVA degradation are pH 8.0 and 30–35°C, respectively.

Sphingomonas sp. SA3 showed growth at 40°C, but the optimum growth temperature was 37°C. Strain SA2 did not grow at 40°C. Its optimum growth temperature

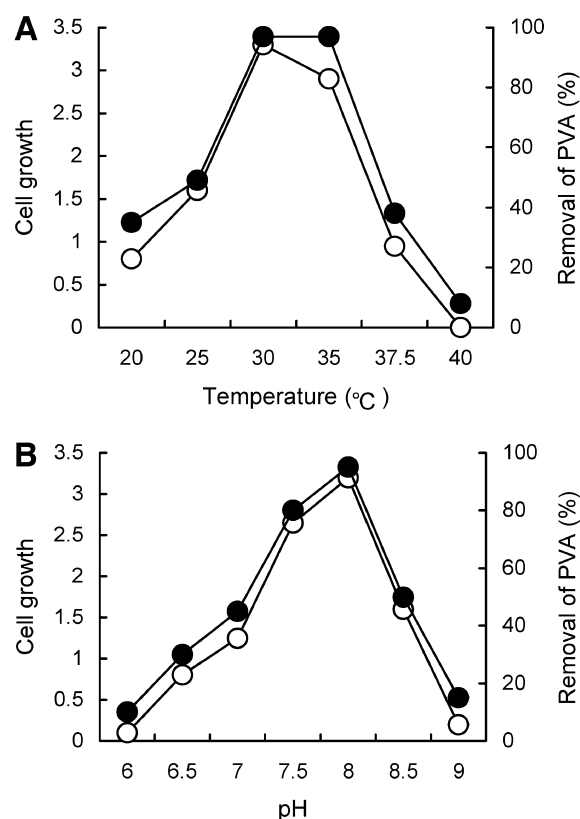


Fig. 3A, B Effect of temperature and pH on PVA degradation in the mixed culture medium of strains SA2 and SA3. White circles Growth of the strains, black circles removal of PVA. Cell growth was measured by optical density at 660 nm after 5 days of growth

was 30°C (data not shown). It seems this difference confirms the range 30–35°C as the optimum temperature for PVA degradation in the co-culture of strain SA2 and *Sphingomonas* sp. SA3.

Discussion

Strains SA2 and SA3 were isolated from environmental samples around Taegu province, where many textile factories are present. PVA decomposition is needed for textile industrial wastewater treatment. Neither of these isolated strains was able to use PVA as their sole carbon and energy source. However, in co-culture, these two strains degraded PVA and bacterial growth was observed.

Two models are reported for the obligate mixed bacterial degradation of PVA: (1) co-degradation by two strains [6] and (2) co-degradation by two strains, a growth factor producer and a PVA-degrader [9].

Strain SA3 degraded PVA in PVA medium containing strain SA2 culture broth. However, strain SA2 did not degrade PVA in PVA medium containing strain SA3 culture broth. It was assumed that a small amount of growth stimulant was produced in the medium during the slight growth of strain SA2, which allowed strain SA3 to degrade PVA by using the growth stimulant. Then, the PVA degradation products were used by both strains SA2 and SA3.

The role of strains SA2 and SA3 for PVA utilization is similar to that of other PVA-degrading symbiotes, *Pseudomonas putida* VM15A and *Pseudomonas* sp. VM15C, as reported by Shimao et al. [11]. In the presence of PQQ, strain SA3 was able to degrade PVA without strain SA2, as VM15C did without VM15A in mixed culture on PVA. Therefore, it is assumed that strain SA2 may produce PQQ or a PQQ-like cofactor. Further studies on strain SA2 are required to prove that the cofactor produced is PQQ.

Data in this paper show that strain SA3 is a member of the genus, *Sphingomonas* established by Yabuuchi et al. [14]. This genus includes many organisms that degrade xenobiotic compounds, but this is the first isolate detected to degrade PVA.

Figures 1, 3 show that the remaining PVA concentration decreased when the bacterial cell density increased. This means that PVA degradation and growth of bacteria were interrelated in the co-culture of strain SA2 and *Sphingomonas* sp. SA3. In other PVA-degrading bacteria, the same relationship was reported [11].

For an industrial wastewater-treatment system, thermostable strains are preferred [4]. It may be possible

to increase the temperature for PVA degradation by using *Sphingomonas* sp. SA3 together with other thermostable growth factor-producing strains instead of strain SA2.

References

1. Finley JH (1961) Spectrophotometric determination of polyvinyl alcohol in paper coatings. *Anal Chem* 33:1925–1927
2. Goosen N, Horsman HP, Huinen RG, Putte P (1989) Acinetobacter calcoaceticus genes involved in biosynthesis of the coenzyme pyrrolo-quinoline-quinone: nucleotide sequence and expression in *Escherichia coli* K-12. *J Bacteriol* 171:447–455
3. Jeong SY, Jo YL, Cho MW, Kim JM (1992) Isolation and characteristics of polyvinyl alcohol degrading bacteria. *Korean J Appl Microbiol Biotechnol* 20:96–101
4. Kim CK, Choi YJ, Lee CW, Rim YT, Ryu JK (1997) PVA removal from textile wastewater by the symbiotic PVA-utilizing bacteria. *Korean J Appl Microbiol Biotechnol* 25:89–95
5. Larking DM, Crawford RJ, Christie GB, Lonergan GT (1999) Enhanced degradation of polyvinyl alcohol by *Pycnoporus cinnabarinus* after pretreatment with Fenton's reagent. *Appl Environ Microbiol* 65:1798–1800
6. Mori T, Sakimoto M, Kagi T, Sakai T (1996a) Isolation and characterization of a strain of *Bacillus megaterium* that degrades poly(vinyl alcohol). *Biosci Biotechnol Biochem* 60:330–332
7. Mori T, Michio S, Takashi K, Takuo S (1996b) Degradation of vinyl alcohol oligomers by *Geotrichum* sp. WF9101. *Biosci Biotechnol Biochem* 60:1188–1190
8. Nohynek LJ, Nurmiaho-Lassila EL, Suhonen EL, Busse HJ, Mohammadi M, Hantula J, Rainey F, Salkinoja-Salonen MS (1996) Description of chlorophenol-degrading *Pseudomonas* sp. strains KF1T, KF3, and NK1F1 as a new species of the genus *Sphingomonas*, *Sphingomonas subarctica* sp. nov. *Int J Syst Bacteriol* 46:1042–1055
9. Sakazawa C, Shimao M, Taniguchi Y, Kato N (1981) Symbiotic utilization of polyvinyl alcohol by mixed cultures. *Appl Environ Microbiol* 41:261–267
10. Shimao M, Saimoto H, Kato N, Sakazawa C (1983) Properties and roles of bacterial symbionts of polyvinyl alcohol-utilizing mixed culture. *Appl Environ Microbiol* 46:605–610
11. Shimao M, Yamamoto H, Ninomiya K, Kato N, Adachi O, Ameyama M, Sakazawa C (1984) Pyrroloquinoline quinone as an essential growth factor for a poly(vinyl alcohol)-degrading symbiont, *Pseudomonas* sp. VM15C. *Agric Biol Chem* 48:2873–2876
12. Suzuki T, Ichihara Y, Yamada M, Tonomura K (1973) Some characteristics of *Pseudomonas* O-3 which utilizes polyvinyl alcohol. *Agric Biol Chem* 37:747–756
13. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173:697–703
14. Yabuuchi E, Yano I, Oyaizu H, Hashimoto Y, Ezaki T, Yamamoto H (1990) Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiol Immunol* 34:99–119