Polyvinyl alcohol and polyethylene glycol form polymer bodies in the periplasm of Sphingomonads that are able to assimilate them

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Abstract Scanning electron microscopy (SEM) shows remarkable morphological surface changes in *Sphingopyxis* sp. 113P3 cells grown in polyvinyl alcohol (PVA) but not in Luria–Bertani medium (LB) (Hu et al. in Arch Microbiol 188: 235–241, 2007). However, transmission electron microscopy showed no surface changes in PVA-grown cells and revealed the presence of polymer bodies in the periplasm of PVA-grown cells, which were not observed in LB-grown cells. The presence of polymer bodies was supported by low-vacuum SEM observation of PVA- and LB-grown cells of strain 113P3, and the presence of similar polymer bodies was also found when *Sphingopyxis macrogoltabida* 103 and *S. terrae* were grown in polyethylene glycol (PEG). The extraction of PVA and PEG from the periplasmic fraction of cells using a modified Anraku and Heppel method and their analysis by MALDI–TOF mass spectrometry strongly suggested that the polymer bodies are composed of PVA and PEG, respectively, in *Sphingopyxis* sp. 113P3 (PVA degrader) and *Sphingopyxis macrogoltabida* 103 or *S. terrae* (PEG degraders). PEG-grown *S. macrogoltabida* 103 and *S. terrae* showed higher transport of ^{14}C-PEG 4000 than LB-grown cells. Recombinant PegB (TonB-dependent receptor-like protein consisting of a barrel structure) interacted with PEG 200, 4000 and 20000, suggesting that the barrel protein in the outer membrane contributes to the transport of PEG into the periplasm.

Keywords *Sphingopyxis* sp. 113P3 · *Sphingopyxis macrogoltabida* 103 · *S. terrae* · Polymer bodies · PVA and PEG uptake

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Introduction

Polyvinyl alcohol (PVA) and polyethylene glycol (PEG) have a long history of use as representative water-soluble, synthetic polymers in many applications, including fabric sizing, fiber coating, adhesives, films for packaging and agricultural uses (Matsumura and Steinbüchel 2002). In addition, PVA and PEG are biodegradable and are therefore used as components of biodegradable composite polymers. Although they are believed to be non-toxic to organisms,

efficient removal of PVA and PEG is required because they have a strong surface activity and produce large amounts of foam which inhibits oxygen recovery in water. Water-soluble polymers are neither recycled nor incinerated when they were once released into the environment. Thus, their biodegradation, either by degrading microorganisms or enzymes, has attracted much attention as the only means to remove them from water. Many researchers have investigated the microbial degradation of both PVA and PEG, and their metabolism has been well documented (Kawai 2002; Kawai and Hu 2009). Sphingomonads were isolated as xenobiotic polymer-degrading microorganisms (Kawai 1999), including PVA degraders [*Sphingopyxis* sp. 113P3 (Hu et al. 2007), *Sphingomonas* sp. SA3 (Kim et al. 2003) and *Sphingopyxis* sp. PVA 3 (Yamatsu et al. 2006)], PEG degraders [*S. macrogoltabida* and *S. terrae* (Takeuchi et al. 2001)] and the polyaspartate-degrading *Sphingomonas* sp. KT-1 (Tabata et al. 1999). These Sphingomonads metabolize PVA and PEG in the periplasm (Kawai 2010), into which PVA and PEG must enter in order to react with primary metabolic enzymes such as PVA dehydrogenase and PEG dehydrogenase, respectively. The manner of PVA and PEG uptake into the periplasm, however, has not been elucidated. Our previous report (Hu et al. 2007) found that the cellular surface change of *Sphingopyxis* sp. 113P3 was induced with PVA and may be related to its uptake. However, the *pva* operon consists of three genes related to the metabolism of PVA but includes no genes related to the transport of PVA (Klomklang et al. 2005). On the other hand, the *peg* operon, which exists in *S. macrogoltabida* and *S. terrae,* includes two genes encoding TonB-dependent receptor-like protein and permease. The former has a barrel structure in the outer membrane and might be related to uptake of PEG into the periplasm, and the latter might be related to transport of PEG (or depolymerized PEG) into the cytoplasm (Tani et al. 2007).

In this paper, we conclude that the previously reported claims of cell-surface changes found on PVA-grown cells are incorrect, and we determine that changes on the cell surface of *Sphingopyxis* sp. 113P3 are caused by extraction of PVA particles from the periplasm. When PVA is incorporated into the periplasm, it forms a dense polymer body, and the same phenomenon is found in PEG-grown cells of *S. macrogoltabida* and *S. terrae.* The TonB-dependent receptor-like protein in the outer membrane of *S. macrogoltabida* and *S. terra* has been suggested as a transporter for PEG into the periplasm.

Materials and methods

Materials

PVA 117 (average molecular weight of 75,000) is a product of Kuraray Co., Inc. (Tokyo, Japan). PVA 2000 (polymerization degree of ca. 2000) was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). ^{14}C-PEG 4000 was purchased from GE Healthcare (Uppsala, Sweden). Other reagents were commercial products of the highest grade available.

Bacterial strain and cultivation

Sphingopyxis sp. 113P3 (formerly *Sphingomonas* sp. 113P3; FERM P-13483 in the International Patent Organism Depositary (POD), Tsukuba, Japan; Klomklang et al. 2005; Hu et al. 2007) was used as a PVA degrader. The strain was grown on PVA medium (Klomklang et al. 2005). *Sphingopyxis macrogoltabida* 103 as a pure culture and *S. terrae* as a coculture with *Rhizobium* sp. (the mixed culture E-1) were cultivated in PEG 4000 and PEG 6000 media, respectively (Kawai et al. 1985). *Sphingopyxis* sp. 113P3, *S. macrogoltabida* 103 and *S. terrae* (as a single culture) were grown on Luria–Bertani medium (LB). The strains were cultivated in 100 ml of a respective medium in a 500-ml shaking flask at 28 °C. The growth of the strain was expressed by turbidity, which was measured by absorbance at 610 nm (OD$_{610}$) with a UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). Cultivated cells of *Sphingopyxis* sp. 113P3, *S. macrogoltabida* 103 and *S. terrae* were collected by centrifugation at 8,000 rpm for 15 min and washed twice with 50 mM potassium phosphate buffer (pH 7.0) or 0.9 % NaCl.

Analysis of PVA and PEG in the periplasm

The periplasmic fraction was prepared from cells grown on PVA, PEG or LB medium, according to the modified Anraku and Heppel method (1967), as previously described (Klomklang et al. 2005). Molecular weights of PVA and PEG in the periplasmic fractions were confirmed by MALDI–TOF mass spectroscopy (MALDI–TOF–MS). The molecular weight and amount of PVA and PEG in the periplasmic fraction was also measured by high-performance liquid chromatography (HPLC). HPLC was performed with Tosoh CCPM-II liquid chromatograph (Tosoh Corporation, Tokyo, Japan); detection, Tosoh RI-8020. HPLC conditions were as follows: columns, Tosoh TSK-GEL2500PW connected with TSK-GEL3000PW; eluent, 0.3 M NaNO$_3$; flow rate, 1 ml/min and column temperature, 40 °C.

Transmission electron microscopy (TEM) and low-vacuum scanning electron microscopy (LV-SEM)

Sphingomonas sp. 113P3 was grown on PVA medium or LB at 30 °C for 3 and 2 days, respectively. *S. macrogoltabida* 103 was grown on PEG 4000 medium or LB at

30 °C for 3 and 2 days, respectively. The mixed culture E-1 was grown on PEG 6000 medium at 30 °C for 4 days, and *S. terrae* was grown on LB at 30 °C for 2 days. The cells were collected, washed thoroughly in 10 mM potassium phosphate buffer (pH 7.0) and then examined with a transmission electron microscope (Model H-700H, Hitachi Ltd., Tokyo, Japan) after negative staining with phosphotungstic acid dissolved in 2.0 % KOH. The washed cells were also directly examined by low-vacuum scanning electron microscope (S04300SE/N, Hitachi Ltd., Tokyo, Japan) after being lightly coated with Pd-Pt at 70 Pa.

Intact PVA and LB-grown cells of *Sphingomonas* sp. 113P3 were fixed in Epon, following the conventional fixation by Hobot et al. (1985), based on the Ryter-Kellenberger procedure (1964). Thin section were cut to 80–90 nm with a diamond knife on a Bromma 2088 Ultratome V (LKB-Produkter AB, Sweden), stained with uranyl acetate and lead citrate and examined in a JEM1200EX electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV.

MALDI–TOF–MS analysis

MALDI–TOF–MS was performed using an Ultraflex MALDI–TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The N_2 laser was used at 50 Hz. Analytical samples were prepared by the dried-droplet method: 2, 5-Dihydroxybenzoic acid was dissolved to a concentration of 10 mg/ml in TA solution (acetonitrile: 0.1 % trifluoroacetic acid = 1:2, v/v), which was used as a matrix solution. To make a domed water droplet, 1 μl of 0.1 % NaCl solution was placed on the AnchorChip target (Bruker Daltonics). The matrix solution and sample were mixed in a 200-μl Eppendorf tube at a 1:1 ratio, and 1 μl of this mixture was injected into the salt solution droplet and air-dried at room temperature. Calibration was performed by the peptide calibration standard with a molecular mass of 1–4 kDa (Bruker Daltonics). All samples were analyzed in linear or reflector mode to obtain spectra.

Uptake of ^{14}C-PEG 4000

Washed cells of *S. macrogoltabida* 103 and *S. terrae* were suspended in approximately one-tenth volume (compared with culture size) of potassium phosphate buffer (pH 7.0) and used for an uptake reaction with ^{14}C-PEG 4000. A reaction mixture consisted of the 6-ml cell suspension (final OD_{610} of ca. 0.5) in 100 mM potassium phosphate buffer (pH 7.0) and 6.0 ml 0.2 % PEG 4000 aqueous solution freshly prepared, which was kept on ice. After the addition of 600 pCi of ^{14}C-PEG 4000 (12 μl of ca. 50 μCi/ml), the reaction mixture was incubated at 30 °C

with gentle shaking. Each 100 μl amount (in triplicate) was withdrawn at intervals, which was filtered on a nitrocellulose membrane of 0.45 μm pore size. Radiolabeled cells collected on a membrane were washed twice with 500 μl of 0.9 % NaCl and dried. Dried cells were subjected to counting of radioactivity. The amount of radioisotope was expressed as disintegration per min (DPM).

Interaction of PEG with recombinant PegB and PegD

Recombinant PegB and PegD were prepared using the same protocol as was used for the expression of genes within the *peg* operon (Tani et al. 2007). Recombinant PegB and PegD (15 μg each) were incubated with PEG 200, 4000 and 20000 (1 mg) in 100 μl of 50 mM potassium phosphate buffer (pH 7.0) at 28 °C for 10 min. The reaction mixtures were then subjected to HPLC analysis. HPLC measurements were performed with a Tosoh (Tokyo, Japan) Liquid Chromatograph 8020 equipped with an RI detector, a Tosoh TSK GEL GMPW column, MilliQ water, at a column temperature of 40 °C and an elution rate of 1.0 ml/min.

Results

TEM and LV-SEM observation of *Sphingopyxis* sp. 113P3, *S. macrogoltabida* 103 and *S. terrae*

To gain insight into the surface change induced by PVA and observed by SEM (Hu et al. 2007), PVA- and LB-grown cells of strain 113P3 were investigated by TEM. Contrary to our expectations, neither a dent nor any surface changes were found with PVA- or LB-grown cells. Instead, circular, thicker-density zones were detected only with PVA-grown cells, as shown in Fig. 1A. These zones were not detected with LB-grown cells. To confirm the localization of the zones, a thin section of PVA- and LB-grown cells were microscopically examined, as shown in Fig. 1B. No corresponding existence was found in the cytoplasm of PVA-grown cells. When observed by LV-SEM, no particular morphological difference was found between PVA- and LB-grown cells. However, white circles appeared and became flatter and bigger only with PVA-grown cells, as the cells gradually dried up, as shown in Fig. 1C. The final shape of some circles was bigger than thicker-density zones detectable by TEM, suggesting that circles are not hard solid materials. The different sizes of circles were probably due to the residual amounts of PVA in the cells. Considering these observations and the sample preparation process for regular SEM, we assumed that the thicker-density circles were polymer particles, and the dent previously observed by regular SEM was artificially formed
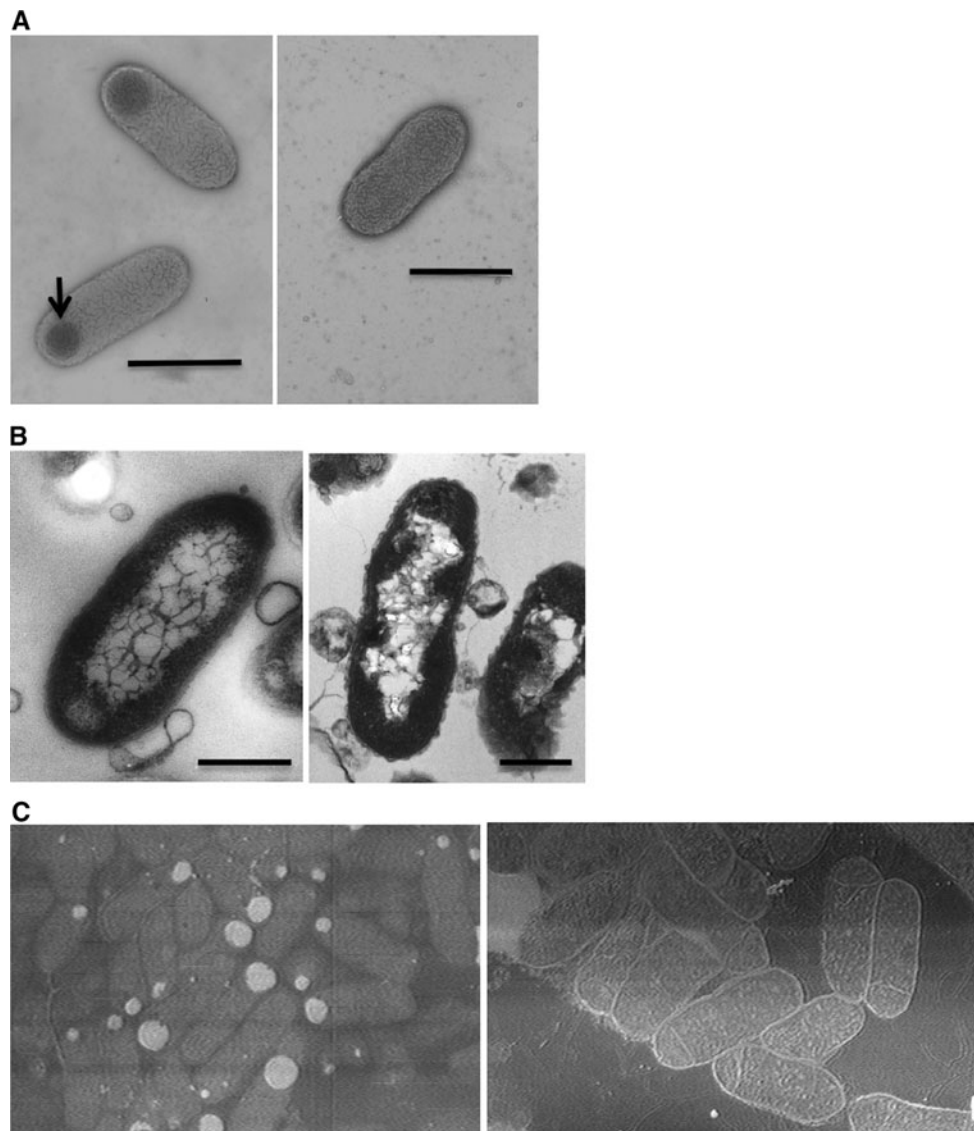
Fig. 1 TEM and LV-SEM observation of PVA- and LB-grown cells of *Sphingopyxis* sp. 113P3. **A** TEM observation of PVA-grown cells (*left*) and LB-grown cells (*right*). *Bar* 1 μm. *Arrows* indicate polymer bodies. **B** TEM observation of a thin section of PVA-grown cells (*left*) and LB-grown cells (*right*). *Bar* 0.5 μm. **C** LV-SEM observation of PVA-grown cells (*left*) and LB-grown cells (*right*). TEM and LV-SEM observation were performed, as described in "Materials and methods"

by the extraction of polymer particles from the periplasm with organic solvents used during SEM processing.

To confirm whether this is a common phenomenon, we investigated *S. macrogoltabida* 103 and *S. terrae* grown in PEG 4000 or 6000 medium plus LB. As shown in Fig. 2, the thicker-density bodies were observed with PEG-grown cells, and no bodies were found with LB-grown cells. This result strongly supports the hypothesis that polymers incorporated into cells make polymer bodies.

MALDI–TOF–MS analysis of the periplasmic fractions

Periplasmic fractions of strain 113P3 (grown on PVA), *S. macrogoltabida* 103 and *S. terrae* (grown on PEG 4000

and 6000, respectively) were extracted using the procedure described in "Materials and methods." Fractions were subjected to MALDI–TOF–MS analyses to identify PVA, PEG and their metabolites. As previously described (Hu et al. 2007), PVA was depolymerized by strain 113P3 into low molecular weights ranging from the original molecular mass of approximately 75,000 to lower molecular mass of 3–4,000 by HPLC, as previously reported (Hu et al. 2007). The amount of PVA recovered from the periplasm of day 2 cultures (molecular mass of 75,000–3,000 fractions) was approximately 15 % of the PVA added to the culture medium. PVA must be highly concentrated in the periplasm, comparing the amount of PVA and the space in the culture volume and the periplasm of the strain
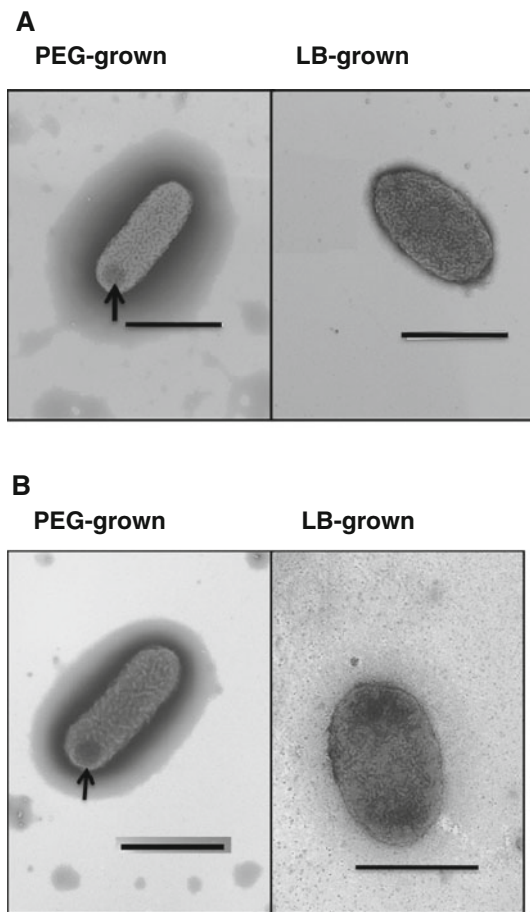
A

PEG-grown   **LB-grown**

B

PEG-grown   **LB-grown**

Fig. 2 TEM observation of PEG- and LB-grown cells of *S. macrogoltabida* 103 and *S. terrae*. **A** *S. macrogoltabidus* 103; **B** *S. terrrae*. *Bar* 1 μm. *Arrows* indicate polymer bodies. TEM observation was performed, as described in "Materials and methods"

113P3. However, MALDI–TOF–MS analysis of the periplasmic extract showed a top peak of approximately 800 (far lower than values by HPLC) (Fig. 3). The peak difference of 44 m/z corresponds to CH_2CHOH in PVA. To confirm whether this corresponds to the right molecular mass of the periplasmic PVA, standard PVA was analyzed under the same conditions. PVA 2000 and PVA 117 showed approximately the same peak signal as that of the periplasmic fraction. Therefore, we concluded that the mass signals that were lower than the molecular weight of PVA were due to fragmentation by laser irradiation.

The residual PEG in the culture supernatant of the mixed culture E-1 (*S. terrae*) is shown in Fig. 4. The amount of PEG bound to cells was only at the trace level (<1 %). The amounts of PEG recovered from the periplasmic fraction of day 2 and day 4 cultures were approximately 7 and 8 %, respectively. The culture supernatant and periplasmic fractions were analyzed by MALDI–TOF–MS. A small peak of PEG 6000 was detected in the periplasmic extracts at day 2 and day 4 of the mixed culture E-1 grown on PEG

6000. A large portion of PEG 6000 was depolymerized into lower molecular masses (the top peak is approximately 4,000) (Fig. 5A). The PEG 6000 peak area was decreased in the culture supernatant, and the top peak was slightly shifted to higher masses while a depolymerized peak was not detected. In addition, the peaks corresponding to the carboxylated PEG were detected in the periplasmic fraction (Fig. 5B). We concluded that a PVA degrader (*Sphingopyxis sp.* 113P3) and PEG degraders (*S. macrogoltabida* 103 and *S. terrae*) take up PVA and PEG, respectively, into the periplasm, and once there, the incorporated polymers produce dense circular bodies (termed "polymer bodies") and are depolymerized.

Uptake of ^{14}C-labeled PEG by *S. macrogoltabida* 103 and *S. terrae*

As mentioned above, PEG is incorporated into the periplasm and metabolized there. The presence of a gene for TonB-dependent receptor-like protein is located in the *peg* operon and is conserved in *S. macrogoltabida* and *S. terrae* (Tani et al. 2007). To confirm the presence of the transport system in the outer membrane, uptake of PEG was examined with PEG- and LB-grown cells (Fig. 6). LB-grown cells did not show significant uptake of PEG for 5 h, but PEG-grown cells showed significant uptake of ^{14}C-labeled PEG (Cho and Salyers 2001), which increased with incubation time. To confirm whether *pegB*-encoding TonB-dependent receptor or *pegD*-encoding permease is relevant to the uptake of PEG or not, recombinant PegB and PegD from strain 103 was incubated with PEGs, and their binding to PEGs was analyzed by the elution of PEG as described in "Materials and methods." As shown in Fig. 7, PEGs incubated with PegB were eluted before the original PEGs, indicating that the PEGs formed a PEG-protein complex with PegB. No complex formation was found with PEG when it was incubated with PegD, since each PEG was eluted at the same position of the original PEG. Taken together, Ton B-dependent receptor-like protein is a transporter for PEGs.

Discussion

Many reports have documented the degradation of PVA in a variety of microorganisms, such as Pseudomonads, Sphingomonads, *Alcaligenes faecalis* KK314, *Bacillus megaterium* BX1 and *Penicillium* sp. WSH02-21 (Kawai and Hu 2009). Extracellular PVA oxidase or periplasmic or membrane-associated PVA dehydrogenase (PVA-DH) is related to PVA depolymerization. When PVA oxidase of *Sphingopyxis* sp. PVA 3 is excreted into the culture supernatant, PVA is depolymerized by extracellular PVA
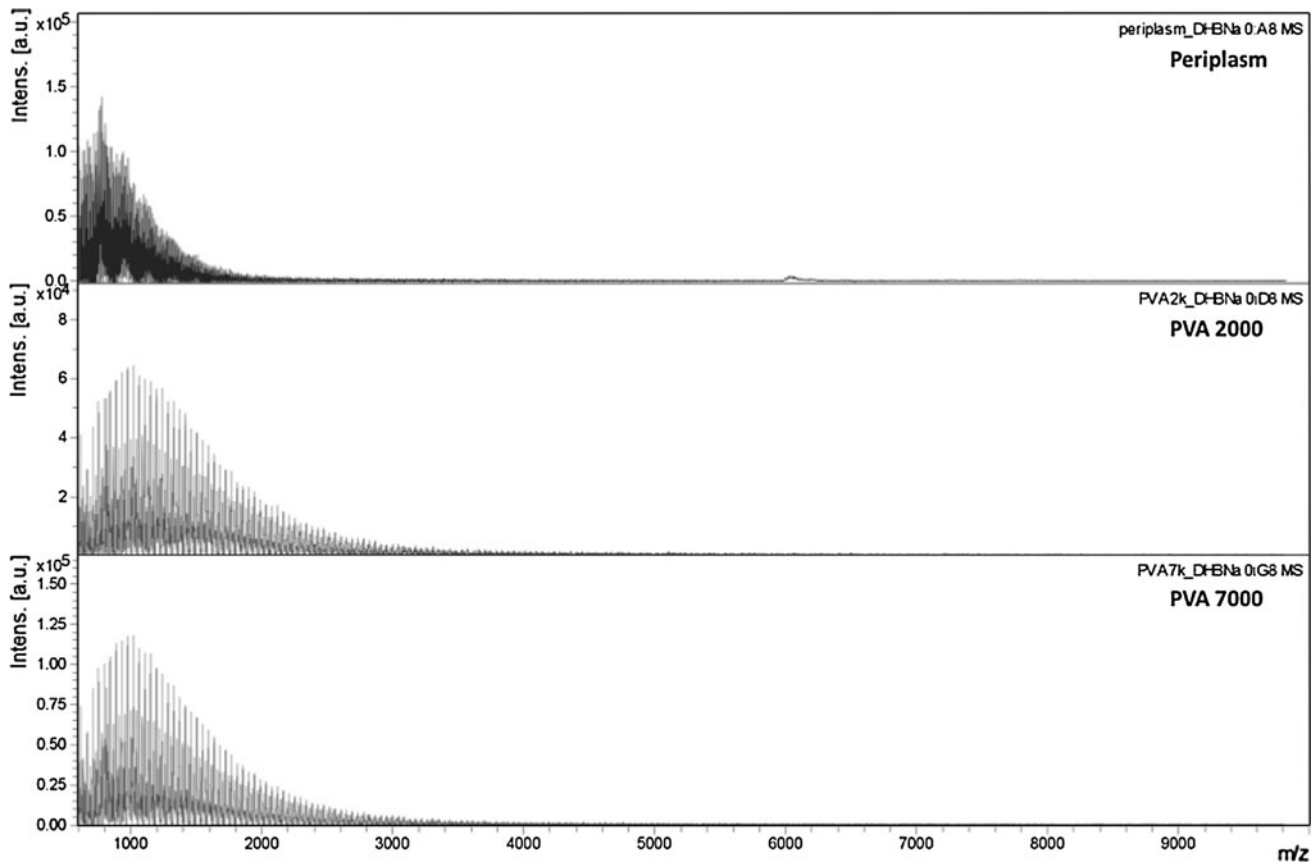
Fig. 3 MALDI–TOF–MS analysis of PVA fraction extracted from the periplasm of *Sphingopyxis* sp. 113P3 grown on PVA 117. MALDI–TOF–MS analysis was performed, as described in "Materials and methods." The top is the periplasmic fraction from day 3 culture. The middle and the bottom are PVA 2000 and PVA 117, respectively
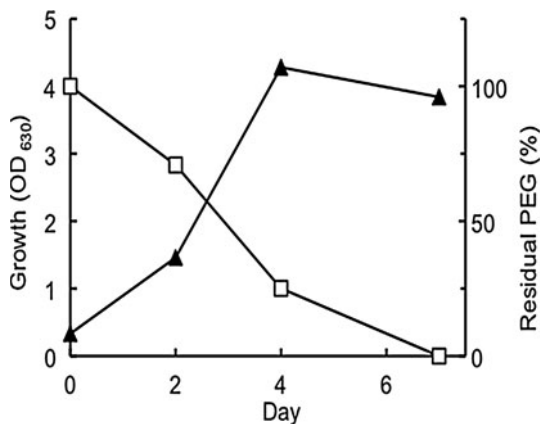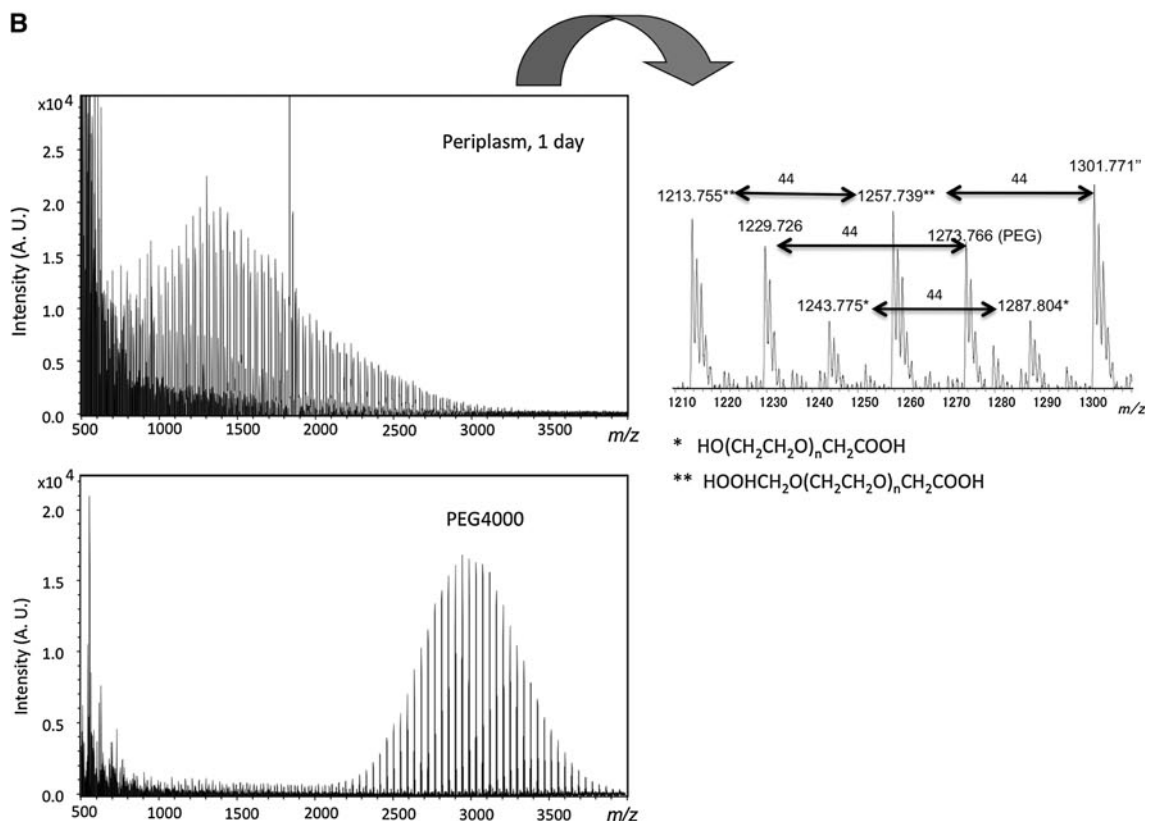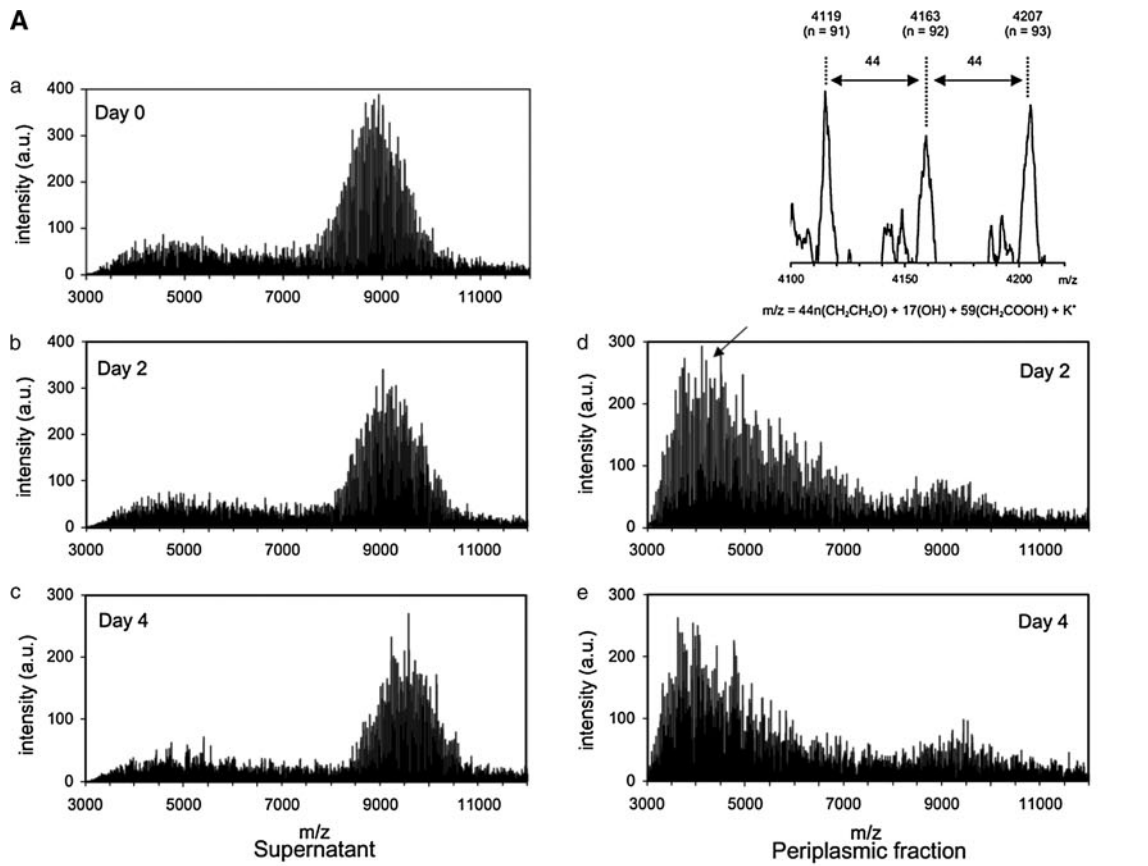

Fig. 4 Growth of the mixed culture E-1 on PEG 6000. The mixed culture was grown in 100 ml PEG 6000 medium in a 500-ml shaking flask. An appropriate amount of culture broth was withdrawn at intervals. The growth was monitored by OD_{610} and residual PEG in the culture supernatant was measured by HPLC, as described in "Materials and methods." Symbols used: *filled triangle* growth on PEG 6000, *open square* residual PEG

Fig. 5 MALDI–TOF–MS analysis of PEG fractions from the culture supernatant and periplasm of *S. terrae* and *S. macrogoltabida* 103. MALDI–TOF–MS analysis was performed, as described in "Materials and methods." *S. terrae* was cultivated on PEG 6000 as a coculture with *Rhizobium* sp., where cell numbers of *Rhizobium* sp. are <1 % of total viable cells (Kawai and Enokibara 1996). *S. macrogoltabida* 103 was grown on PEG 4000. **A** MALDI–TOF–MS analysis of PEG fractions in the culture supernatants of *S. terrae* at day 0 (*a*), day 2 (*b*) and day 4 (*c*) and the periplasmic extracts of day 2 (*d*) and day 4 (*e*). Expanded mass spectrum of the periplasmic extract is shown for day 2 (*d*). **B** MALDI–TOF–MS analysis of PEG fractions in the periplasmic extract of strain 103 at day 1

oxidase, and cells take up the depolymerized products (Yamatsu et al. 2006). Because PVA-DH is either membrane associated (*Pseudomonas* sp. VM-15C; Shimao et al. 1986) or located in the periplasm (*Sphingopyxis* sp. 113P3; Mamoto et al. 2006), PVA must be taken up into the periplasm of these microorganisms. *Sphingopyxis* sp. 113P3 does not produce a significant amount of PVA oxidase (Hatanaka et al. 1995; Hu et al. 2007), and PVA-DH and
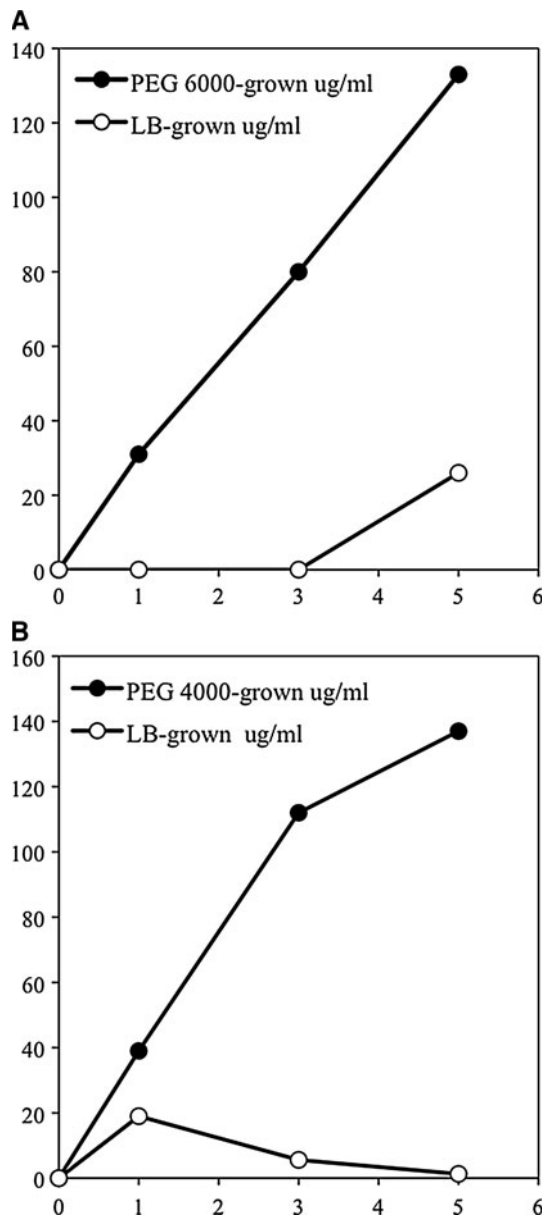
A

$m/z = 44n(CH_2CH_2O) + 17(OH) + 59(CH_2COOH) + K^+$

B

* $HO(CH_2CH_2O)_nCH_2COOH$

** $HOOHCH_2O(CH_2CH_2O)_nCH_2COOH$

Fig. 6 ^{14}C-PEG 4000 uptake by washed cells of *S. macrogoltabida* sp. 103 and *S. terrae*. **A** *S. terrae*. **B** *S. macrogoltabida* sp. 103. The amount of radioisotope of ^{14}C-labeled cells was measured, as described in "Materials and methods." Symbols used: *filled circles* PEG-grown cells, *open circles* LB-grown cells

Fig. 7 Interaction of recombinant PegB and PegD with PEGs. Elution of PEGs with and without PegB and PegD was confirmed by HPLC, as described in "Materials and methods." **A** PEG 20000; **B** PEG 4000; **C** PEG 200. *Dotted line* standard PEG, *solid line* PEG with PegB, *broken line* PEG with PegD

oxidized-PVA hydrolase of strain 113P3 are located in the periplasm (Klomklang et al. 2005; Mamoto et al. 2006). Therefore, the entire PVA molecule has to be incorporated into the periplasm and must react with periplasmic enzymes. The cell-surface structural change was induced by PVA, and FITC-labeled PVA was bound more to cells grown in PVA than to cells grown in LB (Hu et al. 2007). Murata's group has reported the uptake of alginate (average molecular weight, 25,700) through a pit formed on the cell surface of *Sphingomonas* sp. A1 (Hisano et al. 1995).
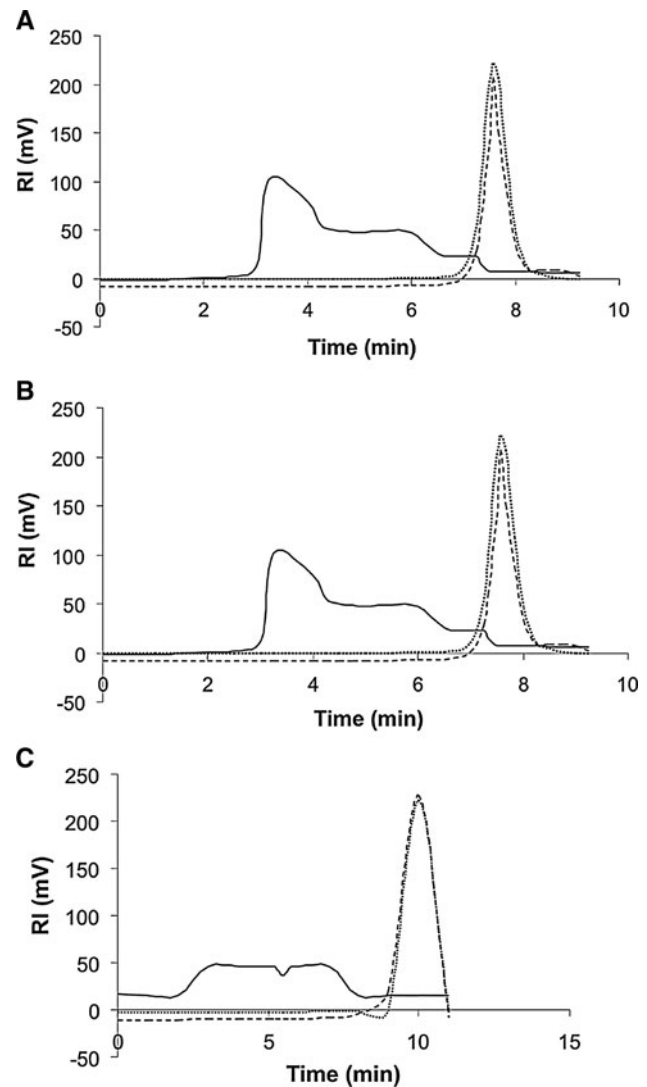
The dent appearing on strain 113P3 seemed to be playing a role similar to that of the pit formed by *Sphingomonas* sp. A1. The present study, however, does not implicate the presence of a specific surface structure in relation to the uptake of PVA. PVA is incorporated by an unknown mechanism into the periplasm, which was confirmed by HPLC. The electron microscopic observation of a thin section of PVA-grown cells does not implicate the presence of a specific cytoplasmic structure and PVA in the periplasm must be highly concentrated, considering the amount of PVA and the periplasmic space. Therefore, highly concentrated PVA in the periplasm most probably forms polymer bodies. These polymer bodies can be extracted with organic solvents used in preparing SEM

samples, and the removal of polymer bodies must have left the vacant space, which may have produced the previously observed dent (Hu et al. 2007). When observed by LV-SEM, no particular morphological difference was found between PVA- and LB-grown cells. However, white circles appeared only with PVA-grown cells, as the cells gradually dried up. White circles are most probably PVA, as the similar spot appears when PVA solution was dried up. Interestingly, similar polymer bodies were found in PEG-grown Sphingomonads. HPLC and MALDI–TOF–MS analyses of periplasm extracts from PVA- and PEG-grown cells, respectively, showed depolymerized PVA, PEG and their metabolites, supporting the hypothesis that PVA and PEG are the constituents of polymer bodies, respectively, and are metabolized in the periplasm. Sphingomonads are attracting attention as degraders of hydrophobic xenobiotic pollutants including synthetic polymers (PEG, PVA and polyaspartate), (Kawai 1999), which may be attributable to the ability of Sphingomonads to take up these polymers into the cells. Transport or uptake of various compounds through outer membranes is well documented (Nikaido 2003). Salyers' group has reported that *Bacteroides* appears to degrade polysaccharides in the periplasm using cell associated rather than extracellular enzymes (1980, 1989), and outer membrane proteins are essential for growth on chondroitin sulfate and starch (1995, 2001). Different outer membrane proteins bind the polysaccharides, and both binding proteins have sequence similarity with TonB-dependent receptors (Cheng et al. 1995; Reeves et al. 1996). The *peg* operon includes a TonB-dependent receptor-like gene (*pegB*). Although a TonB-dependent receptor-like gene has not been found in the vicinity of the *pva* operon, the probable presence of a PVA-specific transporter is predicted by the uptake of PVA described in this paper. Several cytoplasmic granules are found in eukaryotic cells: melanosomes, microbodies, glyoxysomes and Weibel–Palade bodies. The presence of metachromatic bodies is found in genus *Corynebacterium*, mycobacteria, yeasts and algae. Polymer bodies occupy a large space, localized in rods, compared with metachromatic bodies, and they exhibit a thicker density (or different density from cell materials) detectable by TEM, suggesting that these polymers are highly concentrated and might be surrounded by proteinous materials including metabolic enzymes localized in the periplasmic space (Kawai 2010). Since polymers extracted from the periplasm showed reasonable molecular weight patterns, compared with original polymers, they are not strongly bound to proteinous materials. Rather than expanding polymers throughout the whole periplasm, microbes concentrate and utilize them as needed. Condensed forms of polymers may therefore be efficient for metabolism. ^{14}C-PEG 4000 uptake by washed PEG-grown Sphingomonads was quicker than by LB-grown cells, supporting the induction of transporter genes in the *peg* operon. The induction of *pegB* in the *peg* operon by PEG has already been confirmed (Charoenpanich et al. 2006). PEG incubated with PegB was eluted quicker by gel filtration than PEG alone, suggesting complexation of PEG with PegB. Elution positions of PEG 200, 4000 and 20000 complexed with PegB are approximately the same, strongly indicating that the elution position corresponds to the size of PegB and not to the sizes of the PEGs. In addition to the probable localization of both PegB and PegD and the fact that PegB consists of a barrel structure, PegB must be a transporter for PEGs. *S. macrogoltabida* 103 and *S. terrae* have different degradation abilities toward PEGs with different molecular sizes. The former assimilates PEG up to 4000, and the latter (as a mixed culture E-1 with *Rhizobium* sp.) can assimilate PEG up to 20000 (Kawai 2002; Tani et al. 2007). However, PEG dehydrogenases from strains 103 and E-1 did not display the significant difference between their substrate specificities toward PEGs (Kawai et al. 1985; Yamanaka and Kawai 1989), suggesting that the different assimilability is probably caused by different transportability of PEGs. Unexpectedly, purified recombinant PegB from strain 103 showed interaction with PEG 20000, which is not assimilated by strain 103. Therefore, the different assimilabilities of PEGs by strains 103 and E-1 are most likely due to the different barrel sizes of PegB when buried in the outer membrane.

Unlike the *peg* operon, which includes transporter genes such as *pegB* and *pegD*, a *pva* operon consists of three metabolic genes (*cytc, oph* and *pvadh*) and does not include any transporter genes. In the future, we will further investigate and propose whether the transporter involved in PEG uptake is also relevant for the uptake of PVA.

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