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The *pva* operon is located on the megaplasmid of *Sphingopyxis* sp. strain 113P3 and is constitutively expressed, although expression is enhanced by PVA

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Abstract The upstream and downstream regions of the tentative *pva* operon including genes encoding oxidized polyvinyl alcohol (PVA) hydrolase (*oph*), PVA dehydrogenase (*pvaA*) and cytochrome *c* (*cytC*) from *Sphingopyxis* sp. strain 113P3 were sequenced. The resultant 7.9 kb sequence contained *orf1* in the upstream region and *orf2* and *orf3* in the downstream region. Reverse transcription-polymerase chain reaction (PCR) analyses revealed that the intergenic regions between *orf1* and *oph* or between *cytC* and *orf2* were expressed neither in PVA medium nor glucose medium, indicating that the *pva* operon consists of three genes. A transcription start site was determined by 5′-rapid amplification of cDNA ends to be 428 bp upstream of the start codon of the *oph*. The stop codon of *cytC* was followed by a sequence of inverted repeats that could function as a factor-independent transcription terminator. Strain 113P3 had one megaplasmid including the *pva* operon. Northern blot hybridization for the three genes revealed that mRNA size was approximately 3 to 4 kb and expression was elevated in PVA medium compared to glucose medium.

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Introduction

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer that is used in a variety of industrial, commercial, medical, agricultural, and food applications. PVA is the only known xenobiotic carbon-chain polymer able to biodegrade at high molecular masses (Kawai 1999). After being used, PVA eventually enters wastewater or streams, and therefore, PVA is neither recycled nor incinerated. Therefore, microbial degradation seems to be the only means of decomposing this group of polymers in sewage and in nature. A variety of microorganisms able to assimilate PVA have been reported so far: *Pseudomonas* sp. O-3 (Suzuki et al. 1973); *Pseudomonas vesicularis* PD (Watanabe et al. 1975); *Pseudomonas* sp. VM15C (Sakazawa et al. 1981); *Pseudomonas* sp. 113P3 (Hatanaka et al. 1995), which was reidentified as *Sphingopyxis* sp. strain 113P3 (Hu et al. 2007); *Bacillus megaterium* (Mori et al. 1996); *Alcaligenes faecalis* KK314 (Matsumura et al. 1999); *Sphingomonas* sp. SA3 (Kim et al. 2003); *Penicillium* sp. WSH02–21 (Qian et al. 2004); and *Sphingopyxis* sp. PVA3 (Yamatsu et al. 2006).

Genus *Sphingopyxis* is one of four genera derived from genus *Sphingomonas* and designated as Sphingomonads together with three other genera (*Sphingomonas*, *Sphingobium*, and *Nobosphingobium*; Takeuchi et al. 2001). Sphingomonads belong to the α-4 subgroup of the Proteobacteria and comprise Gram-negative rods, which are strictly aerobic, chemo-organotrophic, yellow or whitish-brown pigmented, and catalase-positive. They contain glycosphingolipids as cell envelope components instead of

the lipopolysaccharides common to Gram-negative bacteria, endowing them with unique features (Laskin and White 1999). Sphingomonads are known for their versatile metabolism of a wide range of natural and xenobiotic compounds (Laskin and White 1999), suggesting that the members of this group have the ability to adapt quickly and efficiently to the appearance of new compounds in the environment (Basta et al. 2004).

From *Sphingopyxis* sp. strain113P3, we have cloned three genes that are relevant to PVA degradation: *oph* (Klomklang et al. 2005), *pvaA* (Hirota-Mamoto et al. 2006), and *cytC* (Mamoto et al. 2007). These genes were hypothesized to consist of the *pva* operon (Klomklang et al. 2005). In addition, we found that a unique dent appeared on the cell surface when *Sphingopyxis* sp. strain 113P3 was grown on PVA, a detail potentially relevant to the uptake of PVA (Hu et al. 2007).

In this paper, we sequenced the upstream and downstream regions of the *pva* operon and determined the size of the operon. The presence of a megaplasmid in *Sphingopyxis* sp. strain 113P3 and the localization of the *pva* operon in the megaplasmid were also examined.

Materials and methods

Materials, bacterial strains, and cultivation conditions

PVA 117 (number-average molecular mass of 75,000) used in this study was a product of the Kuraray (Osaka, Japan). All other reagents were commercial products of the highest grade available. *Sphingopyxis* sp. strain 113P3 (Hu et al. 2007) was used throughout this study. The strain was grown in PVA medium (pH 7.5) as reported previously (Hatanaka et al. 1995). The glucose medium contained the same components as the PVA medium except that glucose was added instead of PVA117. The bacterium was also grown in nutrient broth (NB; Eiken Chemical, Tokyo, Japan). The cells were harvested by centrifugation at 8,000×*g* for 30 min, washed twice with 0.85% NaCl and kept at −80°C until use. The periplasmic fraction was prepared following our previous work (Klomklang et al. 2005). For routine cloning purposes, *Escherichia coli* DH5α or JM109 were used as hosts, and their transformants were grown at 37°C for 12–15 h in Luria–Bertani (LB) medium (Sambrook and Russell 2001) supplemented with 50 μg kanamycin or ampicillin ml^{-1} when necessary.

DNA manipulations, polymerase chain reaction, and nucleotide sequence analysis

DNA cloning and transformation were performed following the standard protocols by Sambrook and Russell (2001). Total DNA of strain 113P3 was extracted by Marmur's (1961) method. Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Bio (Kyoto, Japan) or TOYOBO (Osaka, Japan) and used as specified by the manufacturers. A MagExtractor DNA purification kit (TOYOBO) was used to purify DNA fragments from agarose gels. Oligonucleotides used for polymerase chain reaction (PCR) throughout this study were obtained from Hokkaido System Science (Sapporo, Japan) and are listed in Table 1. PCR mixtures (25 μl) contained 0.4 pmol μl^{-1} each primer, 2 mM $MgCl_2$, 0.25 mM each deoxynucleotide, 1× Ex Taq DNA polymerase buffer, and 0.5 U Ex Taq polymerase (Takara Bio). PCR products were cloned into a pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA, USA). DNA sequencing was done on double-stranded DNA using an ABI3100 Genetic Analyser and a BigDye Cycle Sequencing Kit version 1.1 (Applied Biosystems, Foster City, CA, USA). The gaps between contigs were sequenced using either a primer walking method or a subcloning method, each with appropriately synthesized primers (Table 1). Sequencing and computer analysis of the DNA sequences were done with GENETYX software version 8.2.1.

Cloning of the flanking regions of the tentative *pva* operon from strain 113P3

In our previous paper (Klomklang et al. 2005), a 3.8-kb DNA sequence (accession no. AB190288) containing the tentative *pva* operon was reported. The flanking regions were amplified by inverse PCR (Ochman et al. 1988) using primers designed, based on the 3.8-kb DNA sequence. The total DNA of strain 113P3 was digested with restriction enzymes and self-ligated with ligase (Takara Bio), and used as templates. Inverse PCR was performed using 1870F and 150R primers for the upstream region and cytc-Inv-F and cytc-Inv-R for the downstream region. A 2.5-kb DNA fragment of the upstream region and a 3.5-kb DNA fragment of the downstream region were amplified, ligated into a pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced.

Preparation of total RNA and reverse transcription

Sphingopyxis sp. strain 113P3 was grown at 28°C in PVA medium and glucose medium to an OD_{600} of approximately 0.5. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). The RNA samples were further treated with DNase I (Invitrogen). Reverse transcription (RT)-PCR was performed with a One-Step RT-PCR kit (Qiagen, Victoria, Australia) according to the supplier's instructions. Positive-control experiments were done using genomic DNA as a template, and negative control experiments were done by

Table 1 Oligonucleotide primers used in this research

Primer	Sequence 5′→3′	Note
150R	CAGGGTCTCGCCATCGAGCT	Inverse PCR
1870F	GGCGGAATGGTCTATGTCCA	Inverse PCR
cytc-Inv-F	CGGCATTGTCTGGAACAAGG	Inverse PCR
cytc-Inv-R	GCGTGGCGGCCTTACTCC	Inverse PCR
F-PVADH	ACATGGGAATGCTGCAAGTT	Probe synthesis
R-PVADH	TTTTCCATCGACCGAGAAGGC	Probe synthesis
oph-1	CAAGTACAAGACCGATGCGC	Probe synthesis
oph-2	ACAGTGATCACGACCATCGC	Probe synthesis
pvadh-1	GATGACGGCAAGGTCTATGC	Probe synthesis
pvadh-2	TGGACATAGACCATTCCGCC	Probe synthesis
cytc-1	GATGTGCAGCTTGCCATTCG	Probe synthesis
cytc-2	CGTCAGACAGGCAATGATCG	Probe synthesis
OPH-ex	CGAGCCGCGCACCCTCGTTT	Sequencing
Cytc-ex	CTGTCTGACGGCATCCAAAT	Sequencing
CytC-101ex	ACACCCAGACGACTGTCCCC	Sequencing
1F	GGATCTTCATTCGGTGGAGG	Sequencing
2F	CTGAGCCCGGAACAAATTGC	Sequencing
3F	CGCAACTCTCGATCGCATCC	Sequencing
4F	GAAAACCAGTATCTCGGGCT	Sequencing
150–1R	TGATAAAGTCGAGCTAGAGG	Sequencing
OPH88R	GCTCTTGGCCTGCGCTGTTG	Sequencing
OPH-F	TTGGTTGCCTCGACCGTGCC	Sequencing
OPH-R	TGTTCGATTCCGAATTCTGG	Sequencing
cytc-F1	ATCATTGCCTGTCTGACGG	Sequencing
cytc-F2	GACGTTGTCGCCATATGCG	Sequencing
cytc-F3	ATGCGATGGAATCATGTCG	Sequencing
cytc-F4	GTAAGGTCGCGCTGATTACG	Sequencing
TSP1	GGCGTCTGATGCCCAGCTTT	RT-PCR for *oph*, *pvaA* and *cytC*
OPH-A	TTTGTAATGTACCGTAAATCG	RT-PCR for *oph*
PVADH-A	TTTTCCATCGACCGAGAAGGC	RT-PCR for *pvaA*
Cytc-A	TTTGGATGCCGTCAGACAGGC	RT-PCR for *cytC*
RACE-1	CCATTCGCTCTTGGCCTGC	cDNA synthesis from the *oph* stop codon
RACE-2	GCTACCATCGCCAGACAGC	5′-RACE PCR amplification
Nested-1	TTACGCGCACGGTAATGACG	Nested PCR amplification
AAP	GGCCACGCGTCGACTAGTAC	abridged anchor primer
	GGGIIGGGIIGGGIIG	for 5′-RACE PCR
AUAP	GGCCACGCGTCGACTAGTAC	abridged universal amplification primer for 5′-RACE PCR
P-1	CTATCTCGGGGACTGTGTGA	RT-PCR between *orf1* and *oph*
P-2	TTTACAACAGGTTTGAACAT	RT-PCR between *orf1* and *oph*
P-3	GTCTGACGGCATCCAAATGA	RT-PCR between *cytC* and *orf2*
P-4	GCTCCGCTGTCGGATACCAT	RT-PCR between *cytC* and *orf2*

skipping the reverse transcription step. The sequences and positions of primers used for each PCR amplification are listed in Table 1 and indicated in Fig. 2, respectively.

Identification of the transcription start site of *oph*

We employed 5′-rapid amplification of cDNA ends (RACE; Frohman et al. 1988) to determine the transcription start site of *oph*. cDNA was synthesized using the total RNA (500 ng) of PVA-grown cells as a template, with 2.5 pmol of primer, RACE-1, and SuperScript™ II Reverse Transcriptase (Invitrogen) in a total volume of 26 μl. The reaction was performed following the methods recommended by the manufacturer. PCR amplification was carried out with a gene-specific primer, RACE-2 and an abridged anchor primer, AAP. PCR products were diluted to 1:100 and re-amplified with a Nestd-1 primer and a universal amplification primer, 5′-AUAP. The PCR products were cloned into a pCR2.1 TOPO vector (Invitrogen) following the manufacturer's instructions and then sequenced.

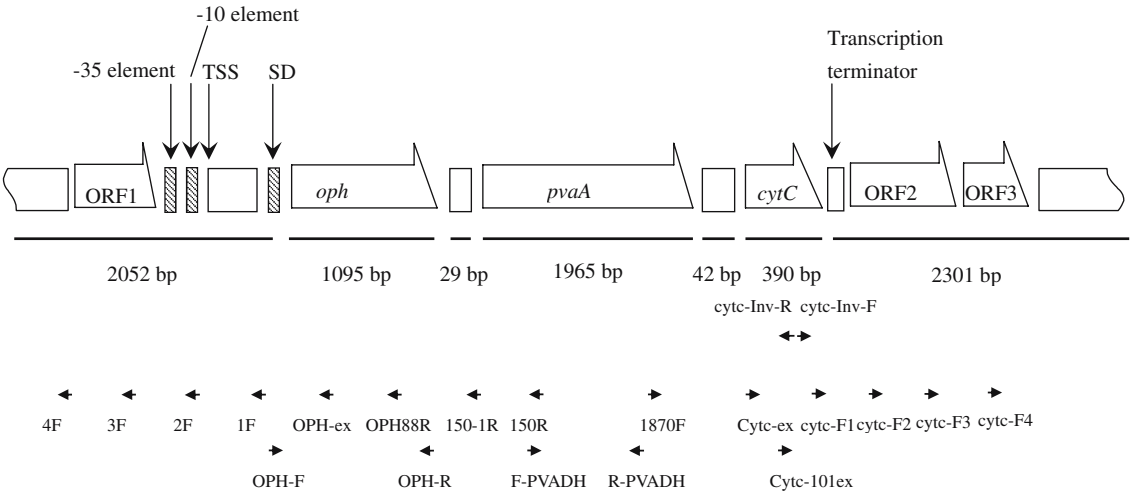

Fig. 1 The gene structure of the tentative *pva* operon and its flanking regions. Cloned DNA fragments are illustrated as *thick lines*, and the primers used for cloning and inverse PCR are shown as *arrows*. ORFs and their orientation are indicated as *thick arrows with gene names*. *TSS* Transcription start site, *SD* Shine-Dalgarno sequence

Detection of a large plasmid

For the extraction and detection of megaplasmid DNA from strain 113P3, the protocol of Kado and Liu (1981) was used with a minor modification. The 113P3 strain was grown in NB at 28°C with 140 rpm reciprocal shaking for 2 days. The cells were collected and washed twice with 50 mM Tris–HCl buffer (pH 8.0) and then resuspended in the same buffer. To the cell suspension (30 μl), 150 μl of lysing solution (50 mM Tris, 3% SDS, pH 12.6) was added, and the mixture was mixed well and incubated at room temperature for 15 min. The lysate was then heated at 65°C for 2 min and mixed with 180 μl of phenol–chloroform solution (1:1, *v*/*v*) saturated with TE buffer [10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. The lysate was incubated at room temperature for 5 min and centrifuged at 13,000×*g* for 20 min at room temperature. The aqueous phase was used as plasmid DNA. The total DNA of 113P3 was used as a positive control. The total DNA of *E. coli* JM109 was used as a negative control. The samples were directly subjected to electrophoresis in a 0.7% agarose gel at 35 V for 24 h. After electrophoresis, the gel was subjected to Southern blot hybridization. Alternatively, the megaplasmid and chromosomal DNA were extracted from the gel, purified using a MagExtractor purification kit, and used as templates for PCR.

Southern blot hybridization and PCR experiments to detect *pvaA* in a megaplasmid

The megaplasmid and chromosomal DNAs of strain 113P3 were electrophoresed in a horizontal 0.7% agarose gel, transferred to nylon membranes (Hybond-N, Amersam Biosciences) and hybridized with a partial *pvaA* gene, which was amplified from the total DNA of strain 113P3 using F-PVADH and R-PVADH primers. The Southern blot hybridization (Southern 1975) was performed using an AlkPhos Direct Labeling and Detection System (GE Healthcare, Buckinghamshire, UK) for probe synthesis and detection. To detect *pvaA* in a megaplasmid from strain 113P3, F-PVADH and R-PVADH (Table 1) were used as primers for PCR amplification. Megaplasmid and chromosomal DNAs from strain113P3 were used as templates. The PCR products were electrophoresed in a 0.7% agarose gel.

Northern analysis

Each 15 μg of total RNA was electrophoresed in a 1.0% formaldehyde gel using 20 mM MOPS buffer (pH 7.0) containing 1 mM EDTA, 5 mM sodium acetate, and 2.2 M formaldehyde. The gels were stained with 6.7 μg ethidium bromide ml^{-1}. Then, RNA samples were transferred to nylon membranes (Hybond-N+) and heat-fixed at 80°C for 120 min. Probes were labeled as described in the manual for the AlkPhos Direct Labeling and Detection System. The conditions used for pre-hybridization, hybridization, washing, and detection followed the instructions recommended by the manufacturer.

Enzyme assays

Oxidized-PVA hydrolase (OPH) and PVA dehydrogenase (PVADH) activities were measured according to our previous work (Klomklang et al. 2005; Hirota-Mamoto et al. 2006).

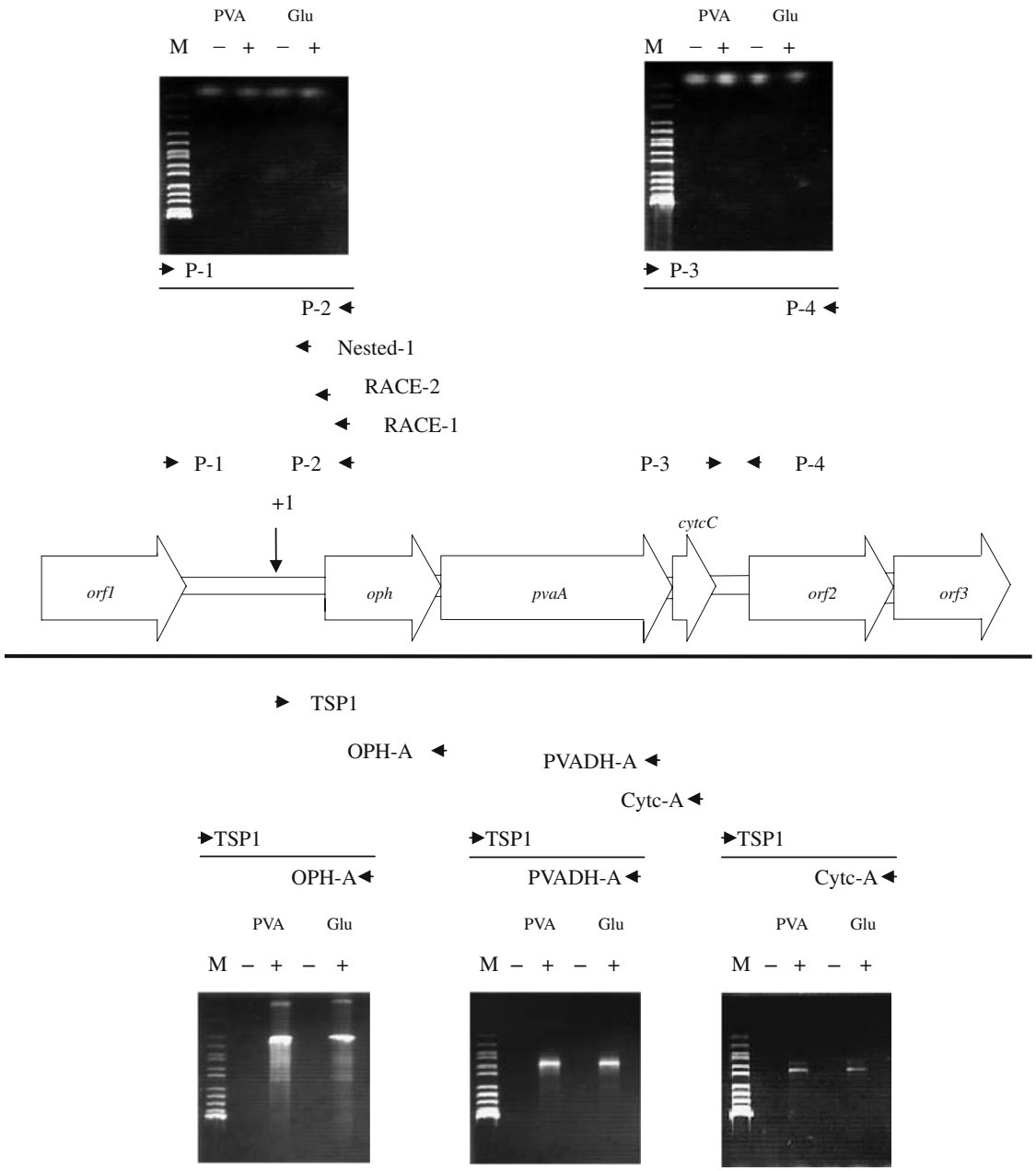

Fig. 2 RT-PCR analysis of the *pva* operon and its flanking regions. ORFs and their orientation are indicated as the *thick arrows with gene names*. RT-PCR analyses of *oph*, *pvaA*, and *cytC* are shown below the ORFs. RT-PCR analyses of the intergenic regions between *orf1* and *oph* and between *cytC* and *orf2* are shown above the ORFs. The positions and sizes of the detected PCR bands are illustrated by *thin lines* under or above the picture of each gel; *M* λ-EcoT14/*Bgl* marker, + positive control, − negative control, *PVA and Glu* RNA samples isolated from PVA- and glucose-grown cells, respectively. *Arrowheads* indicate the primer positions used in this study

Results

Upstream and downstream sequences of a tentative *pva* operon revealed the structure of the *pva* operon

We have designated three genes (*oph*, *pvaA*, *cytC*) encoding OPH, PVADH, and cytochrome *c*, respectively, as a tentative *pva* operon (Klomklang et al. 2005). We extended the upstream and downstream sequences of the tentative operon by inverse PCR. Consequently, we obtained a 7.9-kb fragment that included the tentative *pva* operon and its upstream and downstream sequences, as shown in Fig. 1. The 7.9 kb sequences were deposited in DDBJ under the accession number AB190288. In the upstream region, one open reading frame (ORF; *orf1*) was predicted to have homology to short-chain dehydrogenase/reductase from marine gamma proteobacterium HTCC2143 (39%). In the downstream region, two ORFs (*orf2* and *orf3*) were

Fig. 3 Nucleotide sequence of the upstream and downstream regions of the *pva* operon. The transcription start site was marked as +1 (*right-angled arrow*). The putative ribosomal binding site (SD), −10, and −35 element sequences are *underlined*. The stop codon (TGA) of *cytC* is shown in *bold and underlined*. An inverted repeat is indicated in *bold with an arrow and underlined*. The putative palindrome sequence is shown as *light gray background*. The putative transcription termination site was shown in *italicized bold and double underlined*

predicted to have homology to AMP-dependent synthase/ligase from *Rhodopseudomonas palustris* BisA53 (59%) and short-chain dehydrogenase/reductase from marine gamma proteobacterium HTCC2143 (52%), respectively.

Oph, *pvaA*, and *cytC* formed a PVA-degrading operon

We have shown that three genes (*oph*, *pvaA*, and *cytC*) and their intergenic regions were transcribed as a single operon (Hirota-Mamoto et al. 2006). To further assess whether the upstream and downstream regions are included in the operon, we performed RT-PCR, as shown in Fig. 2. Intergenic regions between *orf1* and *oph* and between *cytC* and *orf2* were not amplified in either PVA or glucose medium, suggesting that the upstream and downstream ORFs are not transcribed as a single operon. Thus, we concluded that the *pva* operon consists of three genes. These genes, *orf1–3*, seemed to have no relevance to the formation of a dent on the cell surface (Hu et al. 2007). A transcription start site was determined by 5′-RACE. Transcription starts 428 bp upstream of the *oph* start codon (ATG). The putative ribosomal binding site (RBS; Shine Dalgarno sequence), GGAGA, was found 5 bp upstream of *oph*. No gene or ORF was found in long sequence between

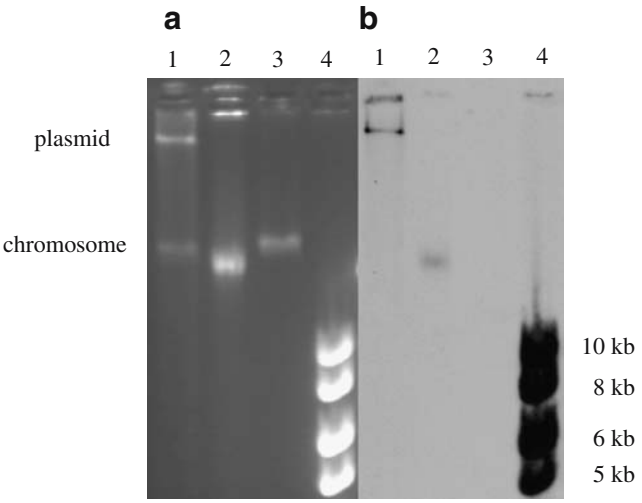

Fig. 4 Detection of a megaplasmid and Southern blot hybridization with a *pvaA* probe. **a** Electrophoresis of plasmid DNA and chromosomal DNA samples prepared from *Sphingopyxis* sp. strain 113P3 cells in a 0.7% agarose gel; **b** Southern blot hybridization pattern with a *pvaA* probe. *Lane 1* Megaplasmid DNA fraction of strain 113P3, *lane 2* total DNA of strain 113P3, *lane 3* total DNA of *E. coli* JM109, *lane 4* 1 kb DNA marker

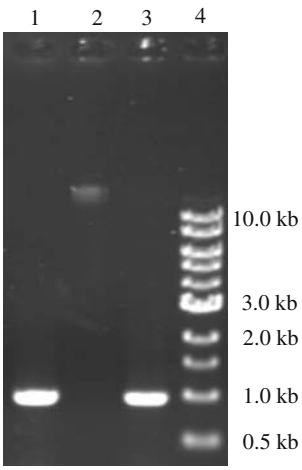

Fig. 5 PCR amplification of *pvaA* using total DNA, chromosomal DNA, and megaplasmid DNA as templates. *Lane 1* Total DNA of strain 113P3, *lane 2* purified chromosomal DNA of strain 113P3, *lane 3* purified megaplasmid DNA of strain 113P3, *lane 4* 1 kb DNA marker

Table 2 Periplasmic enzyme activities from cells grown in PVA medium, glucose medium, and NB

Medium	Specific activity (U mg^{-1})	
	OPH	PVADH
PVA	0.10±0.01	0.28±0.02
Glucose	0.03±0.004	0.09±0.004
NB	0.01±0.001	0.02±0.001

RBS and the transcription start site. The putative −35 (TTGAAG) and −10 (GAAAAT) promoter sequences were observed 31 and 8 bp upstream of the transcription start site (Fig. 3). The stop codon (TGA) of *cytC* was followed by a sequence of inverted repeats (CCGACATCCCGGCTGA and TCAGGCGCAGGAATTCGG), in which a GC-rich stem-loop structure and the following T-rich sequence would yield a thermodynamically unstable RNA–DNA hybrid and function as a factor-independent transcription terminator. All the data supported that only three genes (*oph*, *pvaA*, and *cytC*) comprised the PVA-degrading operon in *Sphingopyxis* sp. strain 113P3.

The *pva* operon was localized in a large plasmid detected in strain 113P3

Basta et al. (2004 and 2005) showed the presence of two to five circular plasmids, which had sizes of about 50–500 kb, in 17 xenobiotic-degrading Sphingomonads. They suggested that these large plasmids are ubiquitous in xenobiotic-degrading Sphingomonads and important for dissemination of degradative abilities among organisms. One megaplasmid was also detected in *Sphingopyxis* sp. strain 113P3 by Kado's methods as well (Fig. 4a). Southern blot hybridization with *pvaA* suggested that the *pva* operon was located in the megaplasmid of *Sphingopyxis* sp. strain 113P3 (Fig. 4b). Circular megaplasmids should be cleaved by Murmer's method and included in the same fraction as chromosomal DNA. To further confirm the localization of the operon in the megaplasmid, PCR was performed for the purified megaplasmid DNA using primers designed based on *pvaA*. As shown in Fig. 5, bands of the same size appeared for total DNA and megaplasmid DNA, but no band was detected for the purified chromosomal DNA. These data strongly supported that the *pva* operon is located in the megaplasmid of strain 113P3.

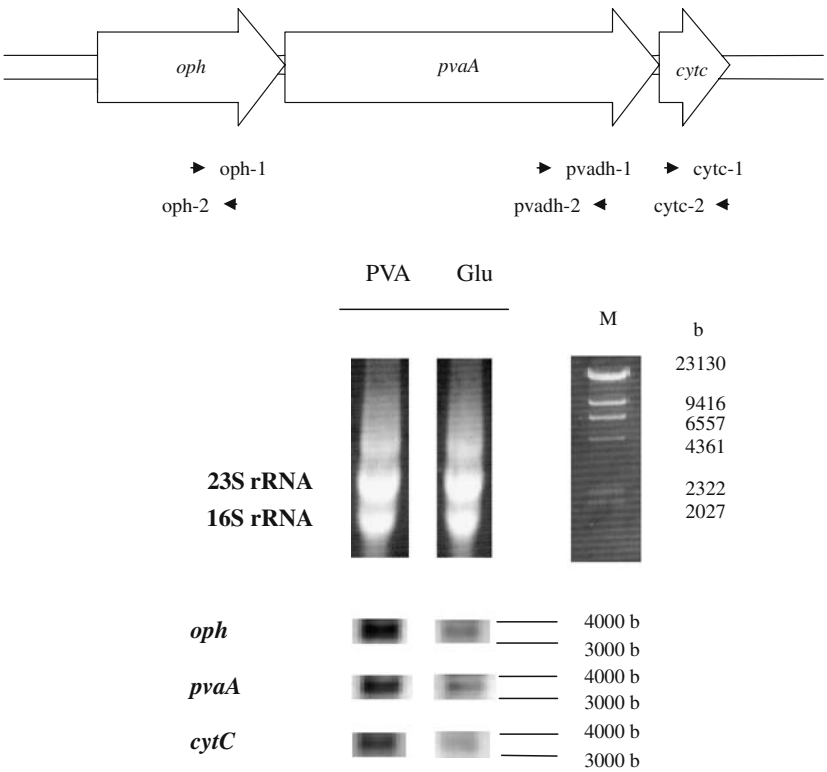

Fig. 6 Northern blot hybridization of the *pva* operon. Northern blot hybridization was performed using gene-specific probes generated by PCR. The quality of the isolated RNA samples was confirmed by electrophoresis. PVA and Glu, RNA samples isolated from PVA- and glucose-grown cells, respectively. *Arrowheads* indicate the primer positions used in this study

The *pva* operon is constitutively expressed, but expression is enhanced by PVA

As described previously (Hirota-Mamoto et al. 2006), the *pva* operon is constitutively expressed. To reconfirm the expression level of the operon, Northern blot hybridization was performed for three genes in the operon. As shown in Fig. 6, all genes were expressed either in PVA or glucose media. The size of the mRNA was approximately 3 to 4 kb, corresponding approximately to the size from the promoter region of *oph* to the transcription terminator of *cytC*. The expression level of the three genes in PVA medium was clearly higher than that observed in glucose medium. OPH and PVADH activities in the periplasmic fractions from PVA and glucose-grown cells showed distinctly higher expression of both enzymes in PVA medium compared with glucose medium or NB (Table 2). The specific activities of OPH and PVADH in glucose medium and NB were only one third and one tenth of those in PVA medium. This result was in well accordance with the result of Northern blot hybridization (Fig. 6).

Discussion

Sphingomonads are known to degrade a variety of xenobiotics including xenobiotic polymers (Laskin and White 1999). Examples of Sphingomonads related to the degradation of xenobiotic polymers are PVA degraders (Hu et al. 2007; Yamatsu et al. 2006; Kim et al. 2003), polyethylene glycol degraders (Kawai 1999) and a polyaspartate degrader (Tabata et al. 1999). A PVA degrader, *Sphingopyxis* sp. strain 113P3 (Hu et al. 2007) and PEG-degrading Sphingomonads (Tani et al. 2007) suggest the presence of a macromolecule-transport system in the outer membrane. Although the transport system for polyaspartate has not been suggested, the presence of polyaspartate hydrolase in the soluble fraction of the crude extract from *Sphingomonas* sp. KT-1 (Tabata et al. 2001) strongly suggests that this is the periplasmic enzyme and, concomitantly, that the strain must have the transport system of polyaspartate into the periplasm. Among the genes included in the *peg* operon, the most probable transporter for PEG was *pegB* encoding a putative TonB-dependent receptor (Tani et al. 2007). This kind of gene was not found in the *pva* operon or its flanking regions, although PVA induced a specific dent supposed to be related to its uptake (Hu et al. 2007). The PVA-induced appearance of a dent and the elevated expression of PVADH and OPH in PVA medium suggest that expression of the PVA degradation system was promoted when cells were exposed to PVA, while the expression of the *pva* operon was constitutive. Appreciable expression of the operon in glucose medium and NB might be useful to start PVA degradation instantly when the cells were exposed to PVA, and resultant depolymerized PVA would enhance the expression of the operon and possibly the formation of dents.

Basta et al. (2005) suggested that almost all Sphingomonads have megaplasmids that are able to degrade a wide range of natural and xenobiotic compounds, such as biphenyl, naphthalene, fluorine, pyrene, furan, carbazole, estradiol, polyethylenglycols, chlorinated phenols, some herbicides, and pesticides. We have detected that PEG-degradative (Tani et al. 2007) and PVA-degradative (this study) genes are located in a megaplasmid from corresponding Sphingomonads. Shimao et al. (2000) suggested that *oph* and *pvaA* in this order, form an operon, but further clarification of the structure of the operon or its localization have not yet been clarified. A PVADH gene from strain 113P3 has 54% identity with *pvaA* from *Pseudomonas* sp. VM15C, both of which share a common structural motif, displaying a novel group of type II quinoheme protein alcohol dehydrogenases (Hirota-Mamoto et al. 2006). The GC contents of the 6.9-kb PCR product and the *pva* operon were 61.7 and 61.5%, respectively. The GC content of 16S rDNA from strain 113P3 (1,402 bp; AB280000) was 54.3%. This suggested that the *pva* operon and its flanking regions might have originated in a different genus or species. No enzymatic and genetic information was available for *Sphingomonas* sp. strain SA3 (Kim et al. 2003). PVA oxidase is the primary enzyme in *Sphingopyxis* sp. strain PVA3, instead of PVA dehydrogenase (Yamatsu et al. 2006). While PVA oxidases from *Pseudomonas* sp. 0–3 (Suzuki 1976) and from *Pseudomonas vesicularis* PD (Morita et al. 1979) are suggested to be inducible, PVADHs from *Pseudomonas* sp. VM15C (Shimao et al. 1986), *Sphingpyxis* sp. strain 113P3 (Hatanaka et al. 1995; Hirota-Mamoto et al. 2006), *Alcaligenes faecalis* KK314, which is a quinohemeprotein as well (Matsumura et al. 1999; personal communication) are constitutive. Taken together, we concluded that the structure of PVADH and probably of the *pva* operon, including *pvaA* is conserved among different genera. However, another possible operon for PVA uptake and the regulation mechanism of the *pva* operon awaits further characterization.

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References

Basta T, Keck A, Klein J, Stolz A (2004) Detection and characterization of conjugative degradative plasmids in xenobiotic-degrading *Sphingomonas* strains. J Bacteriol 186:3862–3872

Basta T, Buerger S, Stolz A (2005) Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic

aromatic compounds and xenobiotics. Microbiology 151:2025–2037

Frohman MA, Dush M, Martin GR (1988) Rapid production of full-length cDNA from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002

Hatanaka T, Asahi N, Tsuji M (1995) Purification and characterization of poly(vinyl alcohol) dehydrogenase from *Pseudomonas* sp. 113P3. Biosci Biotechnol Biochem 59:1813–1816

Hirota-Mamoto R, Nagai R, Tachibana S, Yasuda M, Tani A, Kimbara K, Kawai F (2006) Cloning and expression of the gene for periplasmic poly(vinyl alcohol) dehydrogenase from *Sphingomonas* sp. strain 113P3, a novel-type quinohaemoprotein alcohol dehydrogenase. Microbiology 152:1941–1949

Hu X, Mamoto R, Shimomura Y, Kimbara K, Kawai F (2007) Cell surface structure enhancing uptake of polyvinyl alcohol (PVA) is induced by PVA in the PVA-utilizing *Sphingopyxis* sp. strain 113P3. Arch Microbiol 188:235–241

Kado CI, Liu ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145:1365–1373

Kawai F (1999) Sphingomonads involved in the biodegradation of xenobiotic polymers. J Ind Microbiol Biotechnol 23:400–407

Kim BC, Sohn CK, Lim SK, Lee JW, Park W (2003) Degradation of polyvinyl alcohol by *Sphingomonas* sp. SA3 and its symbiote. J Ind Microbiol Biotechnol 30:70–74

Klomklang W, Tani A, Kimbara K, Mamoto R, Ueda T, Shimao M, Kawai F (2005) Biochemical and molecular characterization of a periplasmic hydrolase for oxidized polyvinyl alcohol from *Sphingomonas* sp. strain 113P3. Microbiology 151:1255–1262

Laskin AI, White DC (1999) Special issue on the genus *Sphingomonas*. J Ind Microbiol Biotechnol 23:231–445

Mamoto R, Hu X, Chiue H, Fujioka Y, Kawai F (2007) Cloning and expression of the soluble cytochrome *c* and its role in polyvinyl alcohol (PVA) degradation by PVA-utilizing *Sphingopyxis* sp. strain 113P3. J Biosci Bioeng (in press)

Marmur JA (1961) A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol 3:208–218

Matsumura S, Tomizawa N, Toki A, Nishikawa K, Toshima K (1999) Novel poly(vinyl alcohol)-degrading enzyme and the degradation mechanism. Macromolecules 32:7753–7761

Mori T, Sakimoto M, Kagi T, Sakai T (1996) Isolation and characterization of a strain of Bacillus megaterium that degrades poly(vinyl alcohol). Biosci Biotech Biochem 60:330–332

Morita M, Hamada N, Sakai K, Watanabe Y (1979) Purification and properties of secondary alcohol oxidase from a strain of *Pseudomonas*. Agric Biol Chem 43:1225–1235

Ochman H, Gerber AS, Hart DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623

Qian D, Du G, Chen J (2004) Isolation and culture characterization of a new polyvinyl alcohol-degrading strain; *Penicillium* sp. WSH02-21. World J Microbiol Biotechnol 20:587–591

Sakazawa C, Shimao M, Taniguchi Y, Kato N (1981) Symbiotic utilization of polyvinyl alcohol by mixed cultures. Appl Environ Microbiol 41:261–267

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Shimao M, Ninomiya K, Kuno O, Kato N, Sakazawa C (1986) Existence of a novel enzyme, pyrroloquinoline quinone-dependent polyvinyl alcohol dehydrogenase, in a bacterial symbiont, *Pseudomonas* sp. strain VM15C. Appl Environ Microbiol 51:268–275

Shimao M, Tamogami T, Kishida S, Harayama S (2000) The gene *pvaB* encodes oxidized polyvinyl alcohol hydrolae of *Pseudomonas* sp. strain VM15C and forms an operon with the polyvinyl alcohol dehydrogenase gene *pvaA*. Microbiology 146:649–658

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Suzuki T (1976) Purification and some properties of polyvinyl alcohol-degrading enzyme produced by *Pseudomonas* O-3. Agric Biol Chem 40:497–504

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

Tabata K, Kasuya K, Abe H, Masuda K, Doi Y (1999) Poly(aspartic acid) degradation by a *Sphingomonas* sp. isolated from freshwater. Appl Environ Microbiol 65:4268–4270

Tabata K, Kajiyama M, Hiraishi T, Abe H, Yamato I, Doi Y (2001) Purification and characterization of poly(aspartic acid) hydrolase from *Sphingomonas* sp. KT-1. Biomacromolecules 2:1155–1160

Takeuchi M, Hamana K, Hiraishi A (2001) Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. Int J Syst Evol Microbiol 51:1405–1417

Tani A, Charoenpanich J, Mori T, Takeichi M, Kimbara K, Kawai F (2007) Structure and conservation of a polyethylene glycol-degradative operon in sphingomonads. Microbiology 153:338–346

Watanabe Y, Morita M, Hamada N, Tsujisaka Y (1975) Formation of hydrogen peroxide by a polyvinyl alcohol degrading enzyme. Agric Biol Chem 39:2447–2448

Yamatsu A, Matsumi R, Atomi H, Imanaka T (2006) Isolation and characterization of a novel poly(vinyl alcohol)-degrading bacterium, *Sphingopyxis* sp. PVA3. Appl Microbiol Biotechnol 72:804–811