Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Anthranilate degradation by a cold-adapted *Pseudomonas* sp.

Dockyu Kim[1], Miyoun Yoo[2,3], Eungbin Kim[2,3] and Soon Gyu Hong[1]

[1] Division of Life Sciences, Korea Polar Research Institute, Incheon, Korea
[2] Department of Systems Biology, Yonsei University, Seoul, Korea
[3] Yonsei/Paris-Sud Project, Yonsei International Campus, Incheon, Korea

An alpine soil bacterium *Pseudomonas* sp. strain PAMC 25931 was characterized as eurypsychrophilic (both psychrophilic and mesotolerant) with a broad temperature range of 5–30 °C both for anthranilate (2-aminobenzoate) degradation and concomitant cell growth. Two degradative gene clusters (*antABC* and *catBCA*) were detected from a fosmid clone in the PAMC 25931 genomic library; each cluster was confirmed to be specifically induced by anthranilate. When expressed in *Escherichia coli*, the recombinant AntABC (anthranilate 1,2-dioxygenase, AntDO) converted anthranilate into catechol, exhibiting strict specificity toward anthranilate. Recombinant CatA (catechol 1,2-dioxygenase, C12O) from the organism was active over a broad temperature range (5–37 °C). However, CatA rapidly lost the enzyme activity when incubated at above 25 °C. For example, 1 h-preincubation at 37 °C resulted in 100% loss of enzyme activity, while a counterpart from mesophilic *Pseudomonas putida* mt-2 did not show any negative effect on the initial enzyme activity. These results suggest that CatA is a new cold-adapted thermolabile enzyme, which might be a product through the adaptation process of PAMC 25931 to naturally cold environments and contribute to its ability to grow on anthranilate there.

Abbreviations: AntDO – anthranilate 1,2-dioxygenase; **C12O** – catechol 1,2-dioxygenase; **PAMC** – Polar and Alpine Microbial Collection

Keywords: Aromatic / Degradation / Cold-adaptation / Thermolabile enzyme

Received: January 24, 2013; accepted: April 13, 2013

DOI 10.1002/jobm.201300079

Introduction

Cold natural environments such as alpine areas, the Arctic, and the Antarctic are contaminated with various anthropogenic hydrocarbons [1–3]. Many cold-adapted bacteria have been isolated and characterized for their ability to degrade various aromatic and aliphatic compounds [2, 4–8]. Current evidence suggests that such cold-adapted indigenous bacteria play a key role in degradation and detoxification of aromatic hydrocarbons in cold environments [9–11].

The Korea Polar Research Institute has been operating a culture collection (the Polar and Alpine Microbial Collection, or PAMC) of cold-adapted bacterial strains from various low-temperature environments including the Arctic, the Antarctic, and Alpine regions [12, 13]. During screening of the PAMC for psychrophilic and/or psychrotolerant microbes with the ability to degrade aromatic hydrocarbons, a bacterial strain designated PAMC 25931 was selected due to its ability to grow on anthranilate as a sole carbon and energy source below 10 °C. As shown in Fig. 1, when compared to well-characterized mesophilic *Pseudomonas putida* mt-2, preliminary growth tests showed that (1) PAMC 25931 grows well over a wide range of temperatures 5–25 °C, (2) is unable to grow (although can survive) at 37 °C, and (3) reaches higher growth rates as the culture temperature decreases, with the highest at 5 °C [14]. The growth characteristics cited above are apparently unique to cold-adapted microorganisms. Subsequent 16S rRNA gene sequencing and a phylogenetic analysis showed that PAMC 25931 belongs to the genus *Pseudomonas* (GenBank accession number JN379461).

Anthranilate (2-aminobenzoate), a carbon- and nitrogen-containing aromatic acid, occurs naturally during

Correspondence: Dr. Dockyu Kim, Division of Life Sciences, Korea Polar Research Institute, (406-840) Room 408-1, Get-pearl Tower, 12 Gaetbeol-ro (Songdo-dong), Yeonsu-gu, Incheon, Korea
E-mail: envimic@kopri.re.kr
Phone: +82 32 260 6360
Fax: +82 32 260 6301

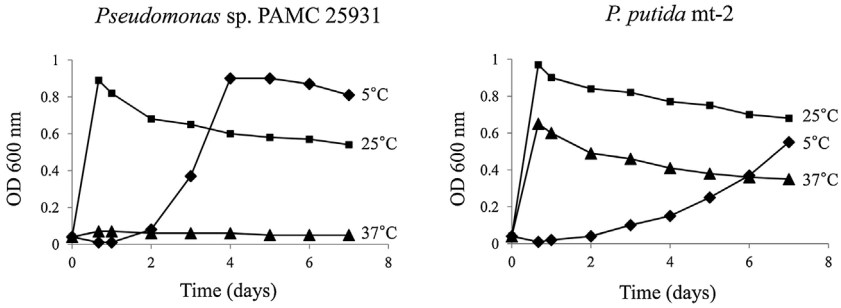

Figure 1. Comparison of growth patterns between *Pseudomonas* sp. PAMC 25931 and *P. putida* mt-2 at different temperatures. PAMC 25931 and mt-2 cells were inoculated in MSB medium containing 5 mM benzoate, the OD 600 nm values of which were measured spectrophotometrically.

tryptophan degradation [15] and is formed as a central metabolite during the degradation of various aromatic compounds such as carbazole, indole, and *o*-nitrobenzoate [16]. Thus, it is not unexpected that anthranilate is used as a source of carbon and nitrogen by many bacterial species isolated from diverse habitats, including *Pseudomonas* [17], *Burkholderia* [15], and *Acinetobacter* [18]. Currently, however, little is known about anthranilate-degrading bacteria in cold environments, which led us to initiate the present study. Here, we characterize the ability of *Pseudomonas* sp. PAMC 25931 to degrade anthranilate.

Materials and methods

Isolation and growth of bacteria

Cold-adapted bacteria had been isolated from alpine soil samples in Grossglockner Hochtor (2,530 m above sea level), Austria, and preserved at −80 °C in 20% glycerol in 96-well microplates [19]. The bacterial strains were revived from the glycerol stock by inoculation onto mineral salts basal (MSB) plates [20] containing succinate (20 mM) and incubation at 10 °C. Bacterial colonies were suspended in saline solution (0.85% NaCl) and dispensed in 96-well microplates for replica plating. The cells were then inoculated onto MSB plates with 5 mM anthranilate as a sole carbon source and incubated at 10 °C for 10 days. The fastest-growing colony, designated PAMC 25931, was selected for further analysis of its ability to degrade various aromatic carboxylic acids and of its metabolic pathways and genes. Stock solutions (0.5 M) of mono-substituted benzoates such as anthranilate and benzoate were prepared in distilled water and, when needed as substrates, were added to MSB medium at a final concentration of 5 mM.

Cloning and sequencing of anthranilate-degrading genes

A degenerate primer set (forward, 5′-TGCASSTWT-CACGGSTGG-3′; reverse, 5′-CTCGACTCCGAGCTTC-CAGTT-3′), designed from conserved regions present in Rieske aromatic oxygenases [21], was used to amplify PCR products from genomic DNA of *Pseudomonas* sp. PAMC 25931. After confirming the nucleotide sequence of the PCR product of the expected size (∼300 bp), a new set of primers (forward, 5′-ATGAGTGGTGCAAGAAGC-3′ and reverse, 5′-GCAGACGGTGCATCATCC-3′) was designed for detection of clones containing the entire *antA*, large subunit gene of anthranilate 1,2-dioxygenase (AntDO), from the genomic DNA library of PAMC 25931, which was constructed using a pCC1FOS fosmid cloning kit (Epicentre, USA), as described by the manufacturer. The library was screened by direct colony-PCR using the above-mentioned primer set for *antA* of PAMC 25931 and a positive clone was selected; the DNA insert was completely sequenced on both strands. Nucleotide sequences were assembled with DNAstar Lasergene software (DNAstar, USA) and analyzed using BLAST software with the GenBank database. The sequences of PAMC 25931 genes for anthranilate degradation were deposited into GenBank with the accession number JN379462-JN379469.

PCR amplification

Cloning of the anthranilate gene by PCR was carried out in a Biometra T3000 Thermal Cycler (Labrepco Inc., USA). The PCR reaction was performed in 100 μl reaction mix containing approximately 500 ng PAMC 25931 genomic DNA, 50 pmol each primer, and MG Taq-*HF* DNA polymerase (Macrogen, Korea). The thermal cycling program was a 5-min hot start (94 °C), 35 cycles of 30 s of denaturation (94 °C), 45 s of annealing (50–52 °C),

1–3 min of extension (72 °C), and a final 5 min of extension (72 °C). For colony PCR, *E. coli* clones were transferred from 96-well plates (12 × 8 strip format) to LB agar plates and grown overnight; all eight colonies from each strip were suspended in 20 μl of water. One-microliter of the cell suspension was used as template in an AccuPower HotStart PCR PreMix (Bioneer, Korea).

For reverse transcriptase (RT)-PCR, PAMC 25931 cells grown in 250 ml MSB/20 mM glucose at 25 °C overnight were harvested, washed with 250 ml MSB, and resuspended in 50 ml MSB. The cell suspension was induced by glucose (20 mM), anthranilate (5 mM), or benzoate (5 mM) at 25 °C for 14 h. Total RNA was extracted from the induced cells using Trizol reagent (Invitrogen, USA) and further purified by spin column and DNase I treatment according to the manufacturer's instructions (QIAGEN, Germany). The integrity of the extracted total RNA was verified by amplifying 16S rRNA genes from all samples (glucose-, anthranilate-, and benzoate-induced cells) as an internal control using the 27F and 1492R universal primers. RT-PCR reactions were performed with 100 ng total RNA and 10 pmol each primer with the ONE-STEP RT-PCR PreMix Kit (iNtRON, Korea) in a final volume of 20 μl. The following primers were designed to amplify target genes: *catBCA* genes (muconate cycloisomerase, muconolactone delta-isomerase, and catechol 1,2-dioxygenase), 5′-AATGTGTCGAAGACGCCTTT-3′ (forward) and 5′-TCCATCAGCAGGTCGAGGTAAT-3′ (reverse) and *antABC* genes (large subunit, small subunit, and reductase of AntDO), 5′-AAAACGGCCTGGACGGTTAT-3′ (forward) and 5′-TCACGCAGGTAGTTGCTCAT-3′ (reverse). The thermocycler program used for the RT-PCR reactions was as follows: 45 °C for 30 min, 94 °C for 5 min, 35 cycles (94 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min), and 72 °C for 5 min.

Functional analysis of *antABC* genes

The *antABC* genes from PAMC 25931 genomic DNA were PCR amplified and cloned into the expression vector pEXP5-CT/TOPO (Invitrogen). The resulting plasmid, designated pDOC092, was transformed into *E. coli* BL21 Gen-X for expression. Ten-milliliters of recombinant *E. coli* culture grown in LB medium was transferred to 200 ml LB supplemented with ampicillin (100 μg ml^{-1}) and cultured to an OD_{600} = 0.4 (approximately 2 h) at 37 °C. The culture was induced by adding 1.0 mM IPTG and incubated further for 18 h at 20 °C. The culture was then harvested, washed with 50 mM potassium phosphate buffer (pH 7.4), and resuspended in 25 ml of the same buffer containing 20 mM glucose. After the addition of anthranilate (5 mM), the bioconversion reaction was carried out by incubating for 18 h at 30 °C. *E. coli* cells were removed by centrifugation (10,000 × *g*, 20 min, 4 °C) and the supernatant was extracted twice with an equal volume of ethyl acetate followed by concentration in a rotary evaporator. The bioconversion products were analyzed by gas chromatography-mass spectrometry (GC–MS) with a Perkin–Elmer Clarus 500 MS (70 eV) connected to Clarus 500 GC with an Elite-5 capillary column (0.25 mm × 30 m, 0.25-μm film thickness). The following conditions were used for the GC: 1.5 ml He min^{-1}; conventional split/splitless injector (CAP, injector temperature, 250 °C); oven temperature 100 °C for 1 min, increased to 250 °C at a rate of 10 °C min^{-1}, and then held at 250 °C for 10 min. The GC interface inlet line and ionization temperatures were 200 and 180 °C, respectively.

Functional analysis of the *catA* gene

The PAMC 25931 *catA* gene was PCR amplified from genomic DNA and cloned into pEXP5-CT/TOPO generating the recombinant plasmid pDOC132, which was transformed into *E. coli* BL21 (DE3) for expression of recombinant CatA. One-milliliter of recombinant *E. coli* culture grown in LB medium was transferred to 50 ml of LB/ampicillin (100 μg ml^{-1}) and cultured to OD_{600} = 0.6–0.8 (approximately 2.5 h) at 37 °C. Following cooling to 15 °C for 30 min, the culture was induced by adding 1.0 mM IPTG and incubated further for 17 h at 15 °C. Subsequently, the culture was harvested, washed in a half volume of PBS buffer (pH 7.4), and disrupted by sonication in 1.0 ml of the same buffer. Unbroken cells and cell debris were removed by centrifugation (10,000 × *g*, 30 min, 4 °C). The resulting supernatant was used as crude enzyme solution. Catechol 1,2-dioxygenase (C12O) activity of the recombinant CatA was assayed spectrophotometrically and quantified, as previously described [22]. The enzyme assay buffer (50 mM Tris–HCl, 1.3 mM EDTA, pH 8.0) was pre-incubated at a temperature range of 5–37 °C, and then the enzyme solution was added to the reaction buffer containing 0.4 mM catechol or 4-methylcatechol. One unit was defined as the enzyme activity producing 1 μmole ring-cleavage product per min, per milligram of protein.

Results

Identification of the genes encoding anthranilate-degrading enzymes

As an initial effort to identify the genes encoding anthranilate-degrading enzymes, a set of degenerate primers were tested for amplification of the Rieske

iron-sulfur motif region of the potential anthranilate oxygenase. The PCR reaction, using total genomic DNA from PAMC 25931 as template, amplified an approximately 300 bp fragment, the size expected from a potential ring hydroxylation oxygenase gene. Sequencing and analysis of the PCR product revealed that the deduced amino acid sequences were 93% identical to the Rieske region of anthranilate oxygenase from *Pseudomonas fluorescens* MB214 [17].

A genomic library of PAMC 25931 was constructed by using fosmid vector pCC1FOS and then screened by colony-PCR using a specific primer set designed for the anthranilate oxygenase in PAMC 25931. A positive clone, designated pDOC2011, was selected and the DNA insert was sequenced and analyzed. Sequence analysis of the insert DNA revealed 17 open reading frames (ORFs) in a 33,409-bp region, of which eight were predicted to be directly involved in the degradation of anthranilate (Table 1). A homology search against the NCBI protein database revealed that (1) three ORFs, designated *antA*, *antB*, and *antC*, encode a two-component anthranilate dioxygenase enzyme, (2) three ORFs, designated *catA*, *catB*, and *catC*, encode catechol 1,2-dioxygenase, muconate cycloisomerase, and muconolactone delta-isomerase, respectively, and (3) two ORFs, designated *antR* and *catR*, encode transcriptional regulators. Overall, the genes showed high identities (79–95%) with those from benzoate- or anthranilate degrading *Pseudomonas* spp. When tested for various aromatic carboxylic acids other than anthranilate, PAMC 25931 was able to grow on benzoate, but not on the other isomers of anthranilate (3- and 4-aminobenzoate), phthalate (2-carboxybenzoate), salicylate (2-hydroxybenzoate), and *o*-toluate (2-methylbenzoate). Above results of growth substrate tests were in good accordance with those of the analyses for PAMC 25931 degradative genes.

Anthranilate-mediated induction of the *ant* and *cat* genes

The two gene clusters, *catBCA* and *antABC*, are closely linked within an approximately 7.8 kb region and are transcribed in opposite directions (Fig. 2A). To determine if *catBCA* and *antABC* are in operon structures and specifically expressed in response to anthranilate and/or benzoate, RT-PCR experiments were performed with total RNA extracts from anthranilate- and benzoate-induced cells of PAMC 25931 which were previously grown at 25 °C. Oligonucleotide primers were designed to generate PCR products as follows: *catBCA*, 982 bp and *antABC*, 1922 bp.

As shown in Fig. 2B, the *catBCA* primers amplified the expected size cDNAs from both anthranilate- and benzoate-induced cells, showing that *catBCA* is specifically induced by either anthranilate or benzoate. In contrast, the *antABC*-specific primers amplified the expected fragment from the genomic DNA of the anthranilate-induced cells, but failed to amplify any product from the benzoate-induced cells. In addition, no RT-PCR products were detectable using total RNA extracts from the cells grown on glucose. These RT-PCR results indicate that the expression of the *catBCA* genes is induced by either anthranilate or benzoate while *antABC*

Table 1. Predicted ORFs identified in a 33,409 bp region from fosmid pDOC2011.

Gene	Representative homolog[a]	Identity (%)[b]	Organism	GenBank accession no.
catA	Catechol 1,2-dioxygenase	79	*Pseudomonas reinekei* MT1	ABI93947
catC	Muconolactone delta-isomerase	84	*P. reinekei* MT1	ABI93946
catB	Muconate cycloisomerase	90	*P. reinekei* MT1	ABI93945
catR	LysR type transcriptional regulator	85	*P. reinekei* MT1	ABI93944
antR	Transcriptional activator	94	*P. fluorescens* MB214	ABA06558
antA	Large subunit of anthranilate 1,2-dioxygenase	95	*P. fluorescens* MB214	ABA06559
antB	Small subunit of anthranilate 1,2-dioxygenase	94	*P. fluorescens* MB214	ABA06560
antC	Reductase of anthranilate 1,2-dioxygenase	92	*P. fluorescens* MB214	ABA06561
orf1	Aromatic amino acid permease	97	*P. fluorescens* SBW25	YP_002874701
orf2	Methylmalonate-semialdehyde dehydrogenase	98	*P. fluorescens* WH6	ZP_07777518
orf3	Transcriptional regulator, LysR family	97	*P. fluorescens* WH6	ZP_07777519
orf4	Signaling-related membrane protein	92	*P. fluorescens* SBW25	YP_002874713
orf5	Exonuclease V subunit alpha	94	*P. fluorescens* SBW25	YP_002874715
orf6	D-Aminoacylase	91	*P. fluorescens* SBW25	YP_002874681
orf7	Integral membrane sulphate transporter	98	*P. fluorescens* SBW25	YP_002874685
orf8	Hypothetical protein	No match		
orf9	Hypothetical protein	94	*P. fluorescens* SBW25	YP_002874691

[a]Homolog searches for putative anthranilate-degradative genes were based on the comparison with functionally characterized proteins.

[b]Percentage of identity was obtained by aligning the deduced amino acid sequences using BlastP.

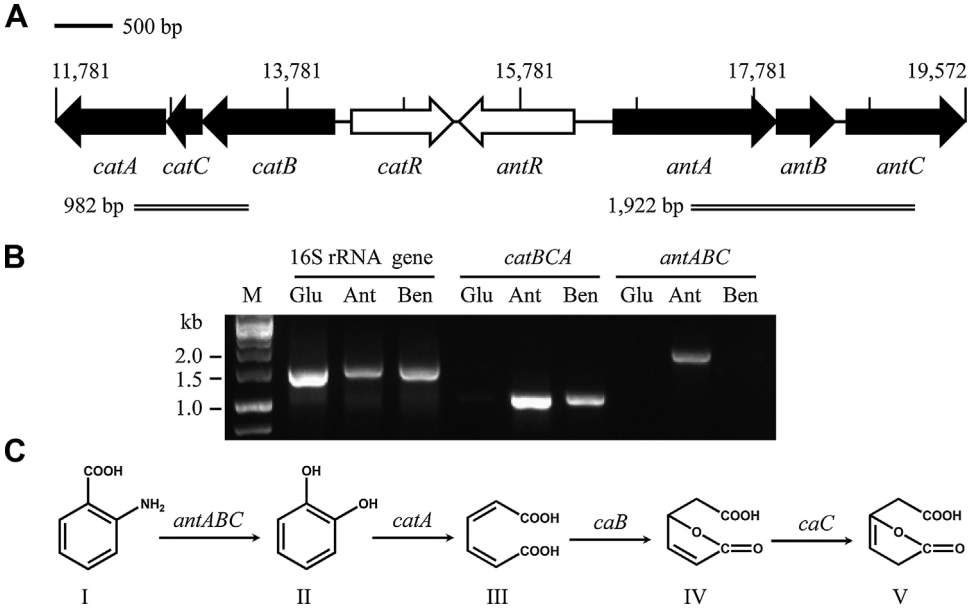

Figure 2. Organization of genes encoding anthranilate-degrading enzymes in *Pseudomonas* sp. PAMC 25931. The direction of transcription is indicated by arrowheads (black, degradative genes; white, transcriptional regulatory genes) in panel A. The expected PCR products for *catBCA* and *antABC* are indicated as double lines with the size indicated. Panel B shows the agarose gel electrophoresis of the RT-PCR products. Glu, glucose; Ant, anthranilate; Ben, benzoate. The proposed degradative pathway for anthranilate in *Pseudomonas* sp. PAMC 25931 is shown in panel C. Chemical designations: (I) anthranilic acid; (II) catechol; (III) *cis*,*cis*-muconic acid; (IV) muconolactone; (V) β-ketoadipic acid enol-lactone.

genes are induced only by anthranilate. Therefore, it is suggested that PAMC 25931 possesses a common ring-cleavage pathway for the degradation of benzoate and anthranilate, although the initial oxidation reactions are catalyzed by separate oxygenases.

Strict specificity of the anthranilate dioxygenase encoded by *antABC*

The above gene expression data indicate that *antABC* together encodes an AntDO. To confirm the role of AntDO in anthranilate degradation, the *antABC* genes were cloned, creating plasmid pDOC092, and expressed in *E. coli*. Induced resting cells of *E. coli* BL21 Gen-X harboring pDOC092 or vector alone (negative control) were incubated in the presence of anthranilate to allow for conversion of the substrate to an oxidized product by the recombinant AntDO. The potential oxidized product derived from anthranilate was analyzed by GC–MS, revealing a major peak with a molecular weight of *m*/*z* 110 at 3.72 min in GC chromatogram, and complete loss of anthranilate (*m*/*z* 137) at 6.41 min-retention time. The major product showed the same fragmentation pattern and retention time as authentic catechol (Fig. 3), while catechol was not detected from the control experiment carried out with BL21 Gen-X (pEXP5-CT/TOPO). This result clearly shows that AntDO in PAMC 25931 catalyzes the oxidation of anthranilate to catechol.

It is well known that AntDOs from *Acinetobacter* sp. ADP1, which was recently renamed *A. baylyi* strain ADP1 [23], and *P. aeruginosa* PAO1 are closely related to benzoate dioxygenase [15, 24]. It has also been reported that an *E. coli* expressed AntDO, originated from the anthranilate and benzoate-degrading *Acinetobacter* sp. ADP1, was able to convert benzoate to benzoate 1,2-diol [18]. Thus, an additional bioconversion experiment was performed to see whether the PAMC 25931 AntDO was able to hydroxylate benzoate as was shown for anthranilate. The GC–MS analysis of the culture supernatant showed that the starting amount of benzoate remained intact and hydroxylated benzoate metabolites were undetected (data not shown). This observation demonstrates that AntDO from PAMC 25931 is strictly specific for anthranilate.

We investigated the phylogenetic relationship among aromatic dioxygenase enzymes to characterize AntDO's strict specificity toward anthranilate. The amino acid sequence of the large subunit (AntA) of PAMC 25931 AntDO was aligned with those of close relatives of AntA, using the GenBank database by BLAST search and other representative aromatic dioxygenase large subunits. Currently, aromatic dioxygenase large subunits are phylogenetically classified in five groups [25, 26]. As shown in Fig. 4A, the PAMC 25931 AntA is affiliated with group II, clearly separated from other groups in the classification system. Furthermore, within group II,

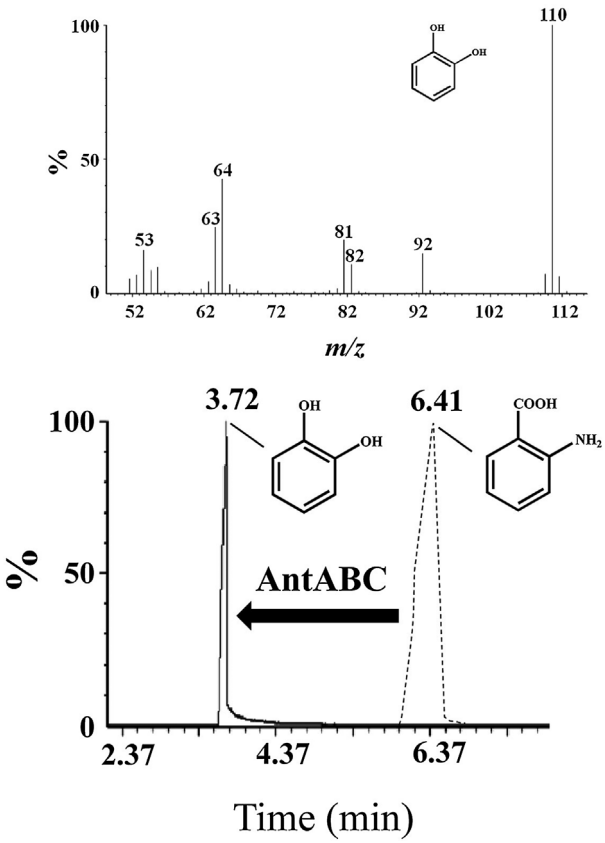

Figure 3. GC–MS analysis of the anthranilate metabolite converted by anthranilate dioxygenase (AntDO) from *E. coli* BL21Gen-X harboring the *antABC* genes. Total ion chromatogram (lower) and MS/MS fragmentation pattern (upper) of anthraniliate bioconversion product, catechol.

PAMC 25931 AntA, along with three other sequences, forms a subgroup in which it is the only member with a confirmed function (this work).

Thermolabile characteristics of the common ring-cleavage dioxygenase encoded by *catA*

Because the initial conversion of anthranilate to catechol by AntABC was elucidated, the biological function of the *catA* gene, encoding the enzyme responsible for catechol degradation, was also investigated using *E. coli* BL21(DE3)-expressed recombinant C12O, PAMC 25931 CatA. The enzyme assay showed that C12O had maximal activity against catechol and was approximately 20% less activity against 4-methylcatecol. The C12O enzyme was active over a broad temperature range (5–37 °C), although the maximal activity was observed at 25 °C. The relative activities measured at 5, 15, 30, and 37 °C were 73, 77, 96, and 92%, respectively, compared to that (100%) at 25 °C. While preincubation of the assay mixture without substrate at different temperatures had a dramatically different effect on enzyme activity; the higher the incubation temperature, the more rapid the enzyme activity decayed (Fig. 5). For example, 60 min preincubation at 25 and 37 °C resulted in 35 and 100% loss of enzyme activity, respectively. A recombinant counterpart C12O_mt-2 was produced using *E. coli* BL21(DE3) with the *catA* gene (GenBank accession no. BAA07036) from mesophilic *P. putida* mt-2 [27] and analyzed under the same conditions. In contrast, the thermostability profile of C12O_mt-2 showed much different data from C12O: when preincubated at 37 °C for 30 and 60 min, its activity rather increased by 130 and 120%, respectively. These experimental data demonstrate that C12O possesses one characteristic property of cold-adapted enzymes opposite to the mesophilic ones: thermolability to rapidly be inactivated at moderate temperatures above 25 °C. This property of C12O enzyme, along with the growth characteristics described above, reinforces our hypothesis that it might have evolved to the cold-adapted thermolabile enzyme through the cold-adaptation process of PAMC 25931, which contributes to its ability to grow on anthranilate in naturally cold environments [28].

We used the classification system for C12Os defined by Murakami et al. [29] for the phylogenetic analysis of the PAMC 25931 CatA. It divides C12Os into three subfamilies (putatively designated Subfamilies A, B, and C) according to the number of C12O amino acid residues and the substrate preference. The amino acid sequence of PAMC 25931 CatA was aligned with those of its close relatives from the GenBank database and representative sequences of the each classification group. Unfortunately, due to the lack of availability of psychrophilic or thermolabile C12Os, we were able to construct a phylogenetic tree only with mesophilic C12Os. As shown in Fig. 4B, PAMC 25931 CatA is affiliated into Subfamily B, in which it is clearly separated from other groups. Also, within the subgroup, PAMC 25931 CatA is the only member with a confirmed function (i.e. cold-adapted thermolabile property). When it is considered that the members of Subfamily B have 309–311 amino acid residues and show high enzymatic activities for catechol only or catechol and 4-methylcatechol, this is in good agreement with our data showing the predicted amino acid content of PAMC 25931 *catA* to be 309 amino acids and that PAMC 25931 CatA demonstrated maximal activity against catechol and a significant activity against 4-methylcatechol.

Discussion

We identified and characterized the genes involved in anthranilate degradation by the cold-adapted *Pseudomonas*

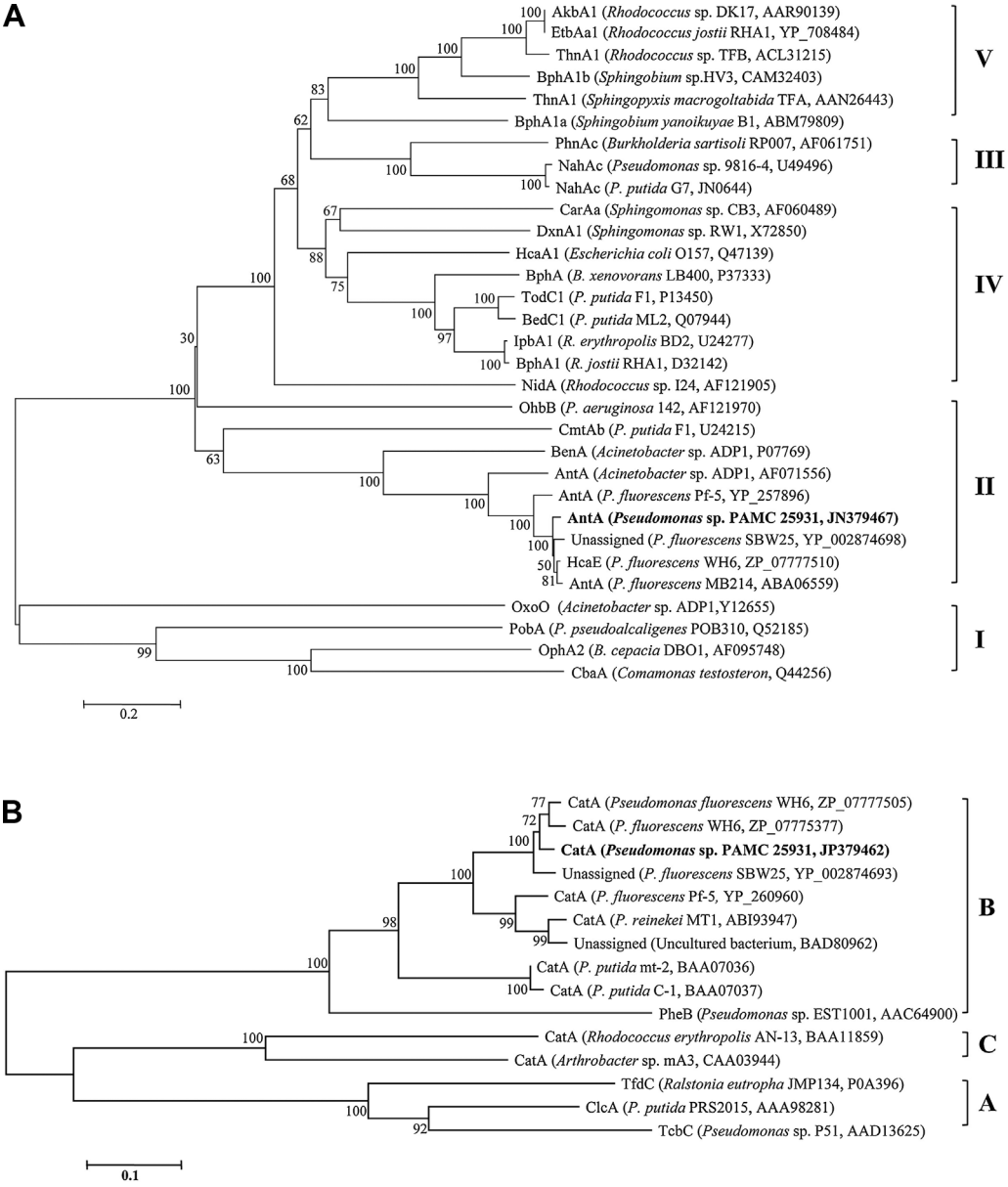

Figure 4. Phylogeny of oxygenase components in aromatic dioxygenase systems (A) and catechol 1,2-dioxygenases (B). The scale bar denotes 0.2 for (A) or 0.1 for (B) substitutions per site and unit time. The names of bacterial strains and GenBank or Swiss-Prot accession numbers are indicated in parentheses after the protein names. The phylogenetic tree was constructed by using MEGA software (version 4.0) with the neighbor-joining method, and bootstrap values (>50) from 1000 replicates are shown for each node.

sp. strain PAMC 25931. As summarized in Fig. 2, the *antABC*-encoded AntDO enzyme, induced specifically by anthranilate (but not benzoate), converts anthranilate (but not benzoate) to its corresponding intermediate catechol, which is further degraded in an *ortho*-cleavage pathway by the cold-adapted C12O, encoded by the *catA* gene. Moreover, the C12O enzyme showed a typical characteristic of psychrophilic enzyme, exhibiting heat labile behavior even at moderate temperatures.

The synthesis of catabolic enzymes specifically adapted to low temperatures is believed to be a cold-adaptation mechanism used by psychrophiles at the enzyme level [28]. In contrast to this explanation, however, it has been suggested that the psychrophilic bacteria are likely conferred not with a unique set of psychrophilic enzyme genes, but with by a collection of synergistic changes in overall genome content and amino acid compositions [30]. PAMC 25931 CatA appears to serve as

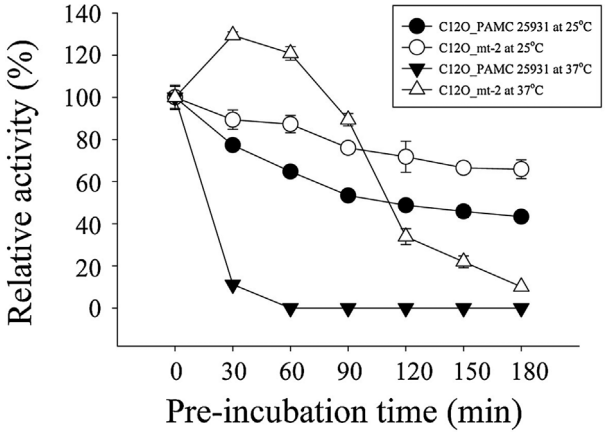

Figure 5. Thermostability of C12Os from cold-adapted *Pseudomonas* sp. PAMC 25931 and mesophilic *P. putida* mt-2. Relative activity was measured following incubating the enzyme solution at different temperatures (25 and 37 °C) for different time intervals.

supporting evidence for the hypothesis of the cold-adaptation mechanism at the enzyme level.

Cold-adapted bacteria are known to play a key role in *in situ* biodegradation in cold environments, and their degradative activities and community composition shift have been well reported [4, 9–11]. Thus, characterizing the catabolic genes in these bacteria could be the basis for a better understanding of physiological cold-adaptation and the ecological role of the aromatic degraders.

Acknowledgments

This work was financially supported by a grant to the Korea Polar Research Institute under project PE13040.

References

[1] Aislabie, J.M., Balks, M.R., Foght, J.M., Waterhouse, E.J., 2004. Hydrocarbon spills on Antarctic soils: effects and management. Environ. Sci. Technol., **38**, 1265–1274.

[2] Ma, Y., Wang, L., Shao, Z., 2006. *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. Environ. Microbiol., **8**, 455–465.

[3] Margesin, R., 2007. Alpine microorganisms: useful tools for low-temperature bioremediation. J. Microbiol., **45**, 281–285.

[4] Whyte, L.G., Bourbonniére, L., Greer, C.W., 1997. Biodegradation of petroleum hydrocarbons by psychrotrophic Pseudomonas strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways. Appl. Environ. Microbiol., **63**, 3719–3723.

[5] Luz, A.P., Pellizari, V.H., Whyte, L.G., Greer, C.W., 2004. A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil. Can. J. Microbiol., **50**, 323–333.

[6] Master, E.R., Agar, N.Y., Gómez-Gil, L., Powlowski, J.B. et al., 2008. Biphenyl dioxygenase from an arctic isolate is not cold adapted. Appl. Environ. Microbiol., **74**, 3908–3911.

[7] Flocco, C.G., Gomes, N.C., Mac Cormack, W., Smalla, K., 2009. Occurrence and diversity of naphthalene dioxygenase genes in soil microbial communities from the Maritime Antarctic. Environ. Microbiol., **11**, 700–714.

[8] Marcos, M.S., Lozada, M., Dionisi, H.M., 2009. Aromatic hydrocarbon degradation genes from chronically polluted Subantarctic marine sediments. Lett. Appl. Microbiol., **49**, 602–608.

[9] Margesin, R., Gander, S., Zacke, G., Gounot, A.M. et al., 2003. Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. Extremophiles, **7**, 451–458.

[10] Margesin, R., Labbé, D., Schinner, F., Greer, C.W. et al., 2003. Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine Alpine soils. Appl. Environ. Microbiol., **69**, 3085–3092.

[11] Labbé, D., Margesin, R., Schinner, F., Whyte, L.G. et al., 2007. Comparative phylogenetic analysis of microbial communities in pristine and hydrocarbon-contaminated Alpine soils. FEMS Microbiol. Ecol., **59**, 466–475.

[12] Kim, E.H., Cho, K.H., Lee, Y.M., Yim, J.H. et al., 2010. Diversity of cold-active protease-producing bacteria from arctic terrestrial and marine environments revealed by enrichment culture. J. Microbiol., **48**, 426–432.

[13] Lee, Y.M., Kim, G.H., Jung, Y.J., Choe, C.D. et al., 2012. Polar and Alpine Microbial Collection (PAMC): a culture collection dedicated to polar and alpine microorganisms. Polar Biol., **35**, 1433–1438.

[14] Kim, D., Yoo, M., Hong, S.G., Park, H.J. et al., 2011. Anthranilate degradation by an eurypsychrophilic *Pseudomonas* sp. from Alps. In: Proceedings: 2011 International Meeting of the Federation of Korean Microbiological Societies Abstr. B003, p. 211.

[15] Chang, H.K., Mohseni, P., Zylstra, G.J., 2003. Characterization and regulation of the genes for a novel anthranilate 1,2-dioxygenase from *Burkholderia cepacia* DBO1. J. Bacteriol., **185**, 5871–5881.

[16] Urata, M., Miyakoshi, M., Kai, S., Maeda, K. et al., 2004. Transcriptional regulation of the *ant* operon, encoding two-component anthranilate 1,2-dioxygenase, on the carbazole-degradative plasmid pCAR1 of *Pseudomonas resinovorans* strain CA10. J. Bacteriol., **186**, 6815–6823.

[17] Retallack, D.M., Thomas, T.C., Shao, Y., Haney, K.L. et al., 2006. Identification of anthranilate and benzoate metabolic operons of *Pseudomonas fluorescens* and functional characterization of their promoter regions. Microb. Cell Fact., **5**, 1.

[18] Eby, D.M., Beharry, Z.M., Coulter, E.D., Kurtz, D.M., Jr. et al., 2001. Characterization and evolution of anthranilate 1,2-dioxygenase from *Acinetobacter* sp. strain ADP1. J. Bacteriol., **183**, 109–118.

[19] Lee, Y.M., Kim, S.Y., Jung, J., Kim, E.H. et al., 2011. Cultured bacterial diversity and human impact on alpine glacier cryoconite. J. Microbiol., **49**, 355–362.

[20] Stanier, R.Y., Palleroni, N.J., Doudoroff, M., 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol., **43**, 159–271.

[21] Kitagawa, W., Suzuki, A., Hoaki, T., Masai, E. et al., 2001. Multiplicity of aromatic ring hydroxylation dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1 demonstrated by denaturing gradient gel electrophoresis. Biosci. Biotechnol. Biochem., **65**, 1907–1911.

[22] Kim, D., Kim, Y.S., Kim, S.K., Kim, S.W. et al., 2002. Monocyclic aromatic hydrocarbon degradation by *Rhodococcus* sp. strain DK17. Appl. Environ. Microbiol., **68**, 3270–3278.

[23] Vaneechoutte, M., Young, D.M., Ornston, L.N., De Baere, T. et al., 2006. Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*. Appl. Environ. Microbiol., **72**, 932–936.

[24] Bundy, B.M., Campbell, A.L., Neidle, E.L., 1998. Similarities between the *antABC*-encoded anthranilate dioxygenase and the *benABC*-encoded benzoate dioxygenase of *Acinetobacter* sp. strain ADP1. J. Bacteriol., **180**, 4466–4474.

[25] Nam, J.W., Nojiri, H., Yoshida, T., Habe, H. et al., 2001. New classification system for oxygenase components involved in ring-hydroxylating oxygenations. Biosci. Biotechnol. Biochem. **65**, 254–263.

[26] Kim, D., Yoo, M., Choi, K.Y., Kang, B.S. et al., 2011. Differential degradation of bicyclics with aromatic alicyclic rings by *Rhodococcus* sp. strain DK17. Appl. Environ. Microbiol., **77**, 8280–8287.

[27] Nakai, C., Uyeyama, H., Kagamiyama, H., Nakazawa, T. et al., 1995. Cloning,DNA sequencing, amino acid sequencing of catechol 1,2-dioxygenases (pyrocatechase) from *Pseudomonas putida* mt-2 *Pseudomonas arvilla* C-1. Arch. Biochem. Biophys., **321**, 353–362.

[28] Collins, T., Roulling, F., Piette, F., Marx, J.C. et al., 2008. Fundamentals of cold-adapted enzymes, in: Margesin, R., Schinner, F., Marx, J., Gerday, C. (Eds.), Psychrophiles: From Biodiversity to Biotechnology, Springer-Verlag, Berlin, Heidelberg, 211–227.

[29] Murakami, S., Kodama, N., Shinke, R., Aoki, K., 1997. Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from Gram$^+$ aniline-assimilating *Rhodococcus erythropolis* AN-13. Gene, **185**, 49–54.

[30] Methé, B.A., Nelson, K.E., Deming, J.W., Momen, B. et al., 2005. The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. Proc. Natl. Acad. Sci. USA, **102**, 10913–10918.