Isolation of an Operon Involved in Xylitol Metabolism from a Xylitol-Utilizing *Pantoea ananatis* Mutant

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An operon involved in crypted xylitol metabolism of *Pantoea ananatis* was cloned by transposon tagging. A xylitol negative mutant with a transposon insertion in the xylitol 4-dehydrogenase gene (*xdh*) was isolated and genomic DNA around the transposon was sequenced. Consequently, six consecutive genes, *xytB-G* are located downstream of *xdh* in the same strand. These seven genes are cotranscribed as a single transcript in a *P. ananatis* xylitol-utilizing mutant, suggesting that they comprise an operon. In addition to *xdh*, *xytF* also encodes oxidoreductase that is a member of the short-chain dehydrogenase/reductase family. Recombinant *Escherichia coli* that heterologously expresses the Xdh protein converts xylitol to xylulose as expected. On the other hand, the recombinant XytF protein has activity with L-arabitol but not with xylitol. XytB, XytD and XytE have significant sequence similarities to genes encoding the substrate-binding, ATP-binding and permease subunits, respectively, of ATP-binding cassette transporters. Although the physiological role of the operon remains unknown, the operon appears to be involved in uptake and metabolism of a various sugar alcohols. A gene encoding a DeoR-type transcriptional regulator, *xytR*, is located upstream of the operon in the opposite strand and a single nucleotide substitution that could cause a nonsense mutation is present in the *xytR* gene of the xylitol-utilizing mutant. This result suggests that the product of *xytR* negatively controls expression of the operon like other DeoR regulators.

[**Key words:** DeoR regulator, operon, oxidoreductase, *Pantoea ananatis*, transposon tagging, xylitol metabolism]

Bacteria have developed a great number of metabolic pathways to enable them to utilize a wide variety of natural compounds as growth substrates. The five-carbon sugar alcohols are known as pentitols and four different structures exist in nature. Two of them, ribitol and D-arabitol, are rather common and a large number of bacteria including *Escherichia coli* C strains and many strains in *Klebsiella* have inducible pathways to degrade them (1, 2). In these strains ribitol and D-arabitol are metabolized through the pathways that consist of the oxidation of the pentitols and the subsequent phosphorylation of the pentuloses. The enzymes involved in the pathways form two independently controlled operons. The ribitol operons encode ribitol dehydrogenase and ribulokinase, and the D-arabitol operon encodes D-arabitol dehydrogenase and xylulokinase (3). Some strains of bacteria have been found to utilize a less common pentitol, xylitol as a growth substrate. Three different metabolic pathways of xylitol have been so far reported. Certain strains of *Lactobacillus casei* possess an inducible xylitol pathway consisting of xylitol phosphotransferase system, which transports xylitol as 5-phosphate, and xylitol 5-phosphate dehydrogenase, which oxidizes xylitol 5-phosphate to D-xylulose 5-phosphate (4, 5). The other two pathways consist of the oxidation of xylitol to xylulose by xylitol dehydrogenase and the subsequent phosphorylation of xylulose by xylulokinase. *Morarella mobile* ATCC 25829, *Providencia stauartii* ATCC 25827 and *Serratia marcescens* ATCC 13880 oxidize xylitol at the C-2 position to produce D-xylulose using D-xylulose reductase (6). *Erwinia* sp. 4DP2 oxidizes xylitol at the C-4 position to produce L-xylulose using L-xylulose reductase (6).

Doten and Mortlock isolated a xylitol-utilizing *Pantoea ananatis* (previously known as *Erwinia uredovora*) DM101 by spontaneous mutation of a wild type *P. ananatis* ATCC 19321, which was unable to use xylitol as a sole carbon source for growth (7). *P. ananatis* DM101 showed constitutive synthesis of NADH-dependent L-xylulose reductase and L-xylulokinase. From *P. ananatis* DM101, mutants that were unable to use xylitol as a growth substrate (xylitol negative mutants) were isolated by transposon-mediated mutagenesis and they were classified into two classes represented by *P. ananatis* DM121 and *P. ananatis* DM122 (7). *P. ananatis* DM121 showed L-xylulokinase activity but no L-xylulose reductase activity, whereas *P. ananatis* DM122 showed L-xylulose reductase activity but no L-xylulokinase activity. The xylitol-negative mutants that lack both of the
1-xylulose reductase and 1-xylulokinase activities could not be obtained by the transposon mutation (7). Unlike the genes involved in the ribitol and δ-arabitol metabolism, the 1-xylulose reductase and 1-xylulokinase genes appears to be located in separate operons.

Recently, the xylitol 4-dehydrogenase (a synonym of 1-xylulose reductase) gene has been cloned from \textit{P. ananatis} DM101 based on the N-terminal sequence of the purified enzyme (8). However, the structure of the operons involved in the xylitol metabolism remains unclear. We attempted the inverse PCR method to isolate the operon from \textit{P. ananatis} DM121 in which the 1-xylulose reductase gene was expected to be disrupted by the transposon insertion, but we could not find any oxidoreductase genes in genomic DNA flanking the transposon sequence. Southern blot analysis indicated that the transposons appeared to be inserted into multiple sites of its genome (unpublished data).

In this study, we reconstructed a xylitol dehydrogenase-defective mutant of \textit{P. ananatis} according to the method by Doten and Mortlock (7) and successfully cloned the full-length sequence of the xylitol (\textit{xyt}) operon, which contains seven genes including the 1-xylulose reductase gene. Moreover, a putative regulator was found to lie adjacent to the operon. The regulation mechanism of the operon is also discussed.

**MATERIALS AND METHODS**

**Strains** \textit{P. ananatis} NRRL B-14773, identical to \textit{P. ananatis} ATCC 19321, was obtained from the ARS culture collection (National Center for Agricultural Utilization Research, Peoria, IL, USA). A wild type \textit{E. coli} K-12 strain, AB707 was obtained from the Coli Genetic Stock Center (Yale University, New Haven, CT, USA) and used for heterologous expression of \textit{P. ananatis} genes. \textit{E. coli} BW25141 (9) was used for cloning of \textit{P. ananatis} genomic DNA containing a transposon element. \textit{E. coli} DH5α purchased from Invitrogen (Carlsbad, CA, USA) was used for plasmid preparation.

**Mutant isolation** A xylitol-utilizing mutant of \textit{P. ananatis}, designated \textit{P. ananatis} YXT1, was generated from \textit{P. ananatis} NRRL B-14773 by incubating at 25°C on a minimal salt agar (10) supplemented with 5 g/l xylitol as described by Doten and Mortlock (7). Xylitol-negative mutants were then generated from \textit{P. ananatis} YXT1 by random gene disruption using an EZ-TN <R6K<\textit{yori}/KAN-2> Tnp transposome kit (Epiphenics, Madison, WI, USA). One ml of overnight culture of \textit{P. ananatis} YXT1 was inoculated into 100 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl) and incubated at 30°C. The culture was allowed to grow to a density of OD\textsubscript{600}=0.9, placed on ice for 10 min and centrifuged at 5000×g for 10 min. The cells were washed three times with 10% (v/v) glycerol and then resuspended in 500 μl of 10% (v/v) glycerol after the final centrifugation. One μl each of the EZ-TN <R6K<\textit{yori}/KAN-2> Tnp transposome and a TypeOne restriction inhibitor (Epiphenics) was added to 40 μl of the cell suspension, stored on ice for 1 min, transferred to a 1 mm-wide cuvette, and subjected to a pulse from a Gene Pulser electroporater (Bio-Rad, Hercules, CA, USA). The settings were 1.8 kV, 25 μF and 200 Ω, which resulted in a pulse time of 4.0 ms. Then, the cells were suspended in 1 ml of SOC medium (20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl\textsubscript{2}, 10 mM MgSO\textsubscript{4} and 20 mM d-glucose) and allowed to recover for 1 h at 30°C with shaking. One hundred μl- aliquots of the electroporated cells were plated onto MacConkey agar plates (BD Diagnostic Systems, Sparks, MD, USA) supplemented with 10 g/l xylitol and 50 μg/ml kanamycin and incubated at 30°C for 2 d. Pale pink colonies were selected as xylitol-negative mutants and restreaked onto the fresh xylitol-MacConkey agar plates to isolate single colonies. The xylitol-utilizing and xylitol-negative mutants were incubated in LB medium with or without 10 g/l xylitol at 30°C for 20 h with shaking (250 rpm). Cells were harvested from a 1-ml aliquot of each culture by centrifugation at 16,000×g for 10 min and then suspended in 50 μl of a BugBuster protein extraction reagent containing 0.2% (v/v) Lysonase Bioprocessing reagent according to the supplier’s manual (Novagen, Darmstadt, Germany) to prepare the soluble cell fractions. Xylitol dehydrogenase activities in the cell lysates were assayed as described below. A xylitol-negative mutant that showed no xylitol dehydrogenase activity, designated \textit{P. ananatis} AXDH, was used for cloning of the \textit{xyt} operon. \textit{P. ananatis} XYT1 and \textit{P. ananatis} AXDH have been deposited in the ARS culture collection under accession nos. NRRL B-41502 and NRRL B-41503, respectively.

**Cloning of transposed genomic fragment** Genomic DNA was isolated from overnight culture of \textit{P. ananatis} AXDH using a QIAGEN Genomic tip 100/G (Qiagen, Valencia, CA, USA). One μg of the genomic DNA was fragmented by EcoRI (New England Biolabs, Ipswich, MA, USA) digestion and self-ligated using 2 U of T4 DNA ligase (New England Biolabs) in 20 μl of reaction for 1 h at room temperature. \textit{E. coli} BW25141 was transformed with 1 μl of the ligation reaction and incubated on LB agar plates containing 50 μg/ml kanamycin, which allows only cells carrying the EZ-TN <R6K<\textit{yori}/KAN-2> transposon to grow. The transposed genomic DNA was isolated from kanamycin-resistant colonies using a Spin Doctor Miniprep kit (Germ Biotech, Oxford, OH, USA).

**Nucleotide sequence analysis** Nucleotide sequence was analyzed using a BigDye Terminator cycle sequencing kit and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Transposon-specific primers (Epiphenics) were used for sequencing immediately adjacent to the transposon insertion. For further sequencing, a HyperMu <\textit{CHL-1}> insertion kit (Epiphenics) was used to make priming sites in the cloned genomic DNA. The ORF Finder provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) was used for detection of genes in the genomic DNA sequenced. Products of the genes (\textit{xdh}, \textit{xytB-G} and \textit{xytR}) were assigned based on sequence similarity analyzed with the BLAST homology search engine and the Conserved Domain Database (NCBI). Subcellular localization of the gene products was predicted with the PSORTb v.2.0 program (http://www.psort.org/) (11). A helix-turn-helix motif in the \textit{XytR} protein was predicted by the method of Dodd and Egan (12).

Multiple sequence alignment of DeoR proteins including \textit{XytR} was performed by using the Clustal W program (13).

The \textit{xdh}, \textit{xytR} genes and their intergenic region were amplified from genomic DNA of \textit{P. ananatis} NRRL B-14773 and \textit{P. ananatis} XYT1 by PCR using two primer pairs, 1156F/1950R and -105F/1161R (Table 1). Each PCR reaction was performed in 50 μl of reaction containing 1× \textit{PfuUltra} HF reaction buffer with 0.4 μM each of the primers, 200 μM each of dNTP and 2.5 U of \textit{PfuUltra} Hotstart High-Fidelity DNA polymerase (Stratagene, Cedar Creek, TX, USA). The PCR conditions were as follows: 2 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, and then 10 min at 72°C. Each PCR product was then cloned into a TA cloning vector, pCR-XL-TOPO (Invitrogen). Sequencing analysis was performed using primers complementary to the sequences in the multi-cloning site of the vector.

**RT-PCR** Each pair of oligonucleotide primers was synthesized to amplify two (1725F/2468R, 4222F/5741R, 5650F/6242R and 6711F/7290R) or three (2194F/4046R) successive genes (illustrated in Fig. 1). Nucleotide sequences of the primers are listed in...
After restriction digestions (200 μM of each dNTP, 2.5 mM MgCl₂, 0.5 mM each of sense and antisense primers, 200 μM of each dNTP and 2.5 U of PfuUltra Hotstart High-Fidelity DNA polymerase). Primer pairs of 1156F/1950R and 6232F/7026R were used for the amplification of xytF-G and 5650F/1161R. The resulting plasmids, pTTQ18-xytF and pTTQ18-xytF, were transformed into E. coli AB707 to obtain strains AB707/Xdh and AB707/XytF. AB707 was also transformed with the plasmid pTTQ18 to construct the control strain.

Each strain was inoculated from a single colony into 3 ml of LB medium containing 10 g/ml ampicillin and incubated overnight at 30°C with shaking (250 rpm). An aliquot of the precultures was diluted 100-fold into 20 ml of LB medium containing 10 g/l xylitol and 200 μg/ml ampicillin. After 2-h incubation at 37°C with shaking (250 rpm), expression of the recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures were further incubated at 37°C with shaking (250 rpm), and aliquots of the cultures were removed at various time points. Cell densities were monitored by measuring the optical density of the appropriately diluted cultures at 600 nm (OD₆₀₀). The soluble cell fractions were prepared by using the BugBuster protein extraction reagent as described above. These fractions were loaded into a 12% SDS–PAGE gel and electrophoresed to confirm expression of the recombinant proteins. The aliquots taken from the cultures were centrifuged at 16,000×g for 10 min and concentrations of xylitol and xylulose in the supernatants were analyzed by high performance liquid chromatography (HPLC) as described below.

**Enzyme assay**

To determine pentitol dehydrogenase activities in the cell lysates, 10 μl of each cell lysate was mixed with 990 μl of reaction mixture (100 mM Tris–Cl [pH 9.0], 0.5 mM MgCl₂, 2 mM NAD⁺ or NADP⁺ and 100 mM pentitol) in a quartz cuvette at 30°C. Activity was measured by following the increase in absorbance at 340 nm using a spectrophotometer (model 8453; Agilent Technologies, Santa Clara, CA, USA). Total protein amount in absorbance at 30°C was determined using bovine serum albumin as standard. One unit was defined as the amount of enzyme that caused the reduction of 1.0 μmol NAD(P)⁺ to NAD(P)H per min.

**Carbohydrate analysis**

Xylitol and xylulose were quantified by HPLC using an Aminex HPX-87P column with a deashing Micro-Guard precolumn (Bio-Rad). The Aminex HPX-87P col-
The xylitol-utilizing ability of \( \gamma \)EZ-TN <R6K> strain had no detectable activity of xylitol dehydrogenase in constitutively low level and significantly induced the enzyme in the presence of xylitol. In contrast, the wild type strain had no detectable activity of xylitol dehydrogenase in the absence and presence of xylitol. To isolate xylitol dehydrogenase-deficient mutants, the EZ-TN <R6K> operon was introduced into \( \gamma \)XTN. Xylitol-negative mutants were screened using MacConkey agar plates supplemented with 10 g/l xylitol and 50 μg/ml kanamycin based on change of the color of colonies. On the xylitol-MacConkey agar plates, \( \gamma \)XTN produced red colonies, whereas \( \gamma \)XYT1, exhibiting xylitol-negative mutants were assayed to isolate xylitol dehydrogenase-deficient mutants. One of the 13 mutants, designated \( \gamma \)ananatis \( \Delta \)XDH, did not show xylitol dehydrogenase activity.

Structure of \( \gamma \)xyn operon
Genomic DNA prepared from \( \gamma \)ananatis \( \Delta \)XDH was digested with EcoRI, ligated intramolecularly and transformed into \( E. coli \) BW25141, which expressing the II protein. Only the genomic DNA fragments containing the transposon was propagated in \( E. coli \) due to R6K<\textit{Kan}) that was integrated into the transposon. Transformants were selected for kanamycin resistance and an 18-kb plasmid consisting of the transposon (2 kb) and insertion-flanking regions of the genomic DNA (16 kb) was obtained. Sequencing analysis around the transposon insertion revealed that the transposon interrupted a gene, which was identical to the xylitol 4-dehydrogenase gene (\( xdh \)) isolated from \( \gamma \)ananatis DM101 (8). The wild type \( xdh \) gene cloned from \( \gamma \)ananatis NRRL B-14773 by using PCR consisted of 795 nucleotides and encoded a 264-amino acid protein, which had sequence similarity to members of the short-chain dehydrogenase/reductase (SDR) family. Comparison of the \( xdh \) sequences between the wild type and \( \Delta \)XDH strains indicated that the insertion site of the transposon element was located at nucleotide position 319 and 10-bp duplication was generated by the transposon insertion at both sites of the transposon.

The sequencing analysis also indicated that six successive genes (\( \gamma \)Xyn-G) were located downstream of \( xdh \) (Fig. 1). Their characterization is given in Table 2. \( \gamma \)XynB, \( \gamma \)XynD and \( \gamma \)XynE had high sequence similarity to periplasmic sugar-binding proteins, ATP-binding proteins and transmembrane proteins, respectively, of ATP-binding cassette (ABC) transporters. Between \( \gamma \)XynB and \( \gamma \)XynD, \( \gamma \)XynC was located and its product was predicted to be a periplasmic lipoprotein and showed no significant similarity to already-known proteins present in the database. As well as \( xdh \), \( xynF \) had significant similarity to members of the SDR family, but their deduced amino acid sequences were only 28% identical to each other. \( \gamma \)XynG was predicted to encode a 490-amino acid protein and had partial sequence similarity to xynin proteins. The next open reading frame was found 424-bp apart on the opposite strand.
FIG. 2. Cotranscription of the genes. The RT-PCR was performed with the Access RT-PCR kit (Promega) as described in materials and methods. The RT-PCR products and a DNA molecular weight marker (Promega, lane M) were loaded onto a 1.0% agarose gel. One hundred ng of total RNA prepared from P. ananatis XYT1 that was incubated with (lanes 1–5) or without (lane 6) xylitol, or from P. ananatis NRRL B-14773 (lane 7) was used as a starting template. The oligonucleotide primers used and the expected sizes of the products are as follows: 1725F and 2468R, 744 bp (lanes 1, 2, 3, 5, 6, and 7); 1294F and 4046R, 1853 bp (lane 2); 4222F and 5741R, 1520 bp (lane 3); 5650F and 6242R, 593 bp (lane 4); 6711F and 7290R, 580 bp (lane 5).

Cotranscription of genes Putative −35 (TTGTTT) and −10 (TAAAT) promoter sequences were found 43 and 19-bp upstream from the initiation codon of xdh. To test the cotranscription of the genes, we performed RT-PCR with primer pairs designed to amplify two or three successive genes (Fig. 1, Table 1). As shown in Fig. 2, the RT-PCR amplified DNA fragments with the expected sizes from total RNA prepared from P. ananatis XYT1 that was incubated with xylitol (lane 1: xdh-xytB, 744 bp; lane 2: xytB-D, 1853 bp; lane 3: xytD-E, 1520 bp; lane 4: xytE-F, 593 bp and lane 5: xytF-G, 580 bp). No fragments were detected in the control reactions without reverse transcription, indicating that the PCR products were amplified from mRNA but not genomic DNA. A putative transcription termination structure (15-bp after the stop codon of xytF) comprising an 8-bp palindrome (CTGGCCGCCgacGACCGGCcAG) and the putative −35 (ATGAA) and −10 (TAAAT) promoter sequences (64 and 42-bp upstream of the initiation codon of xytG) were found in the intergenic region between xytF and xytG. The results of the RT-PCR, however, suggested that xytG was expressed due to readthrough transcription from the putative xdh promoter.

Expression of the xdh gene was also detected in P. ananatis XYT1 cells that were incubated in the absence of xylitol (Fig. 2, lane 6), and the level of the expression was lower than that in the cells that was incubated with xylitol. No fragment was amplified from total RNA isolated from XYT1 that was incubated with xylitol (lane 1: xdh-xytB, 744 bp; lane 2: xytB-D, 1853 bp; lane 3: xytD-E, 1520 bp; lane 4: xytE-F, 593 bp and lane 5: xytF-G, 580 bp). No fragments were detected in the control reactions without reverse transcription, indicating that the PCR products were amplified from mRNA but not genomic DNA. A putative transcription termination structure (15-bp after the stop codon of xytF) comprising an 8-bp palindrome (CTGGCCGCCgacGACCGGCcAG) and the putative −35 (ATGAA) and −10 (TAAAT) promoter sequences (64 and 42-bp upstream of the initiation codon of xytG) were found in the intergenic region between xytF and xytG. The results of the RT-PCR, however, suggested that xytG was expressed due to readthrough transcription from the putative xdh promoter.

Upstream region of xyt operon A gene, xytR, was located 373-bp upstream of the xdh initiation codon in the opposite reading sense and the deduced amino acid sequence of xytR showed significant similarity to members of the DeoR transcriptional regulator family (Figs. 1 and 3). DeoR regulators usually include a helix-turn-helix DNA-binding motif in the N-terminal part. In the XytR protein the helix-turn-helix motif was also detected at amino acid position 18–39 (Fig. 3).

The xytR gene isolated from P. ananatis XYT1 was 447 bp in length and encoded a 148-amino acid protein, which was about 100-amino acids smaller than other DeoR proteins reported. Sequencing analysis of xytR of P. ananatis NRRL B-14773 showed that the wild type reading frame consisted of 783 nucleotides and encoded a 260-amino acid protein, which were similar in size to other DeoR proteins. A comparison of the wild type and mutant xytR genes revealed that cystosine at nucleotide position 446 was substituted with adenine in the mutant xytR, which caused a nonsense mutation of a serine residue at amino acid position 149 (Fig. 3).

The sequencing analysis also revealed that nucleotide sequence of the intergenic region between xdh and xytR of P. ananatis XYT1 was identical to that of the wild type. This indicates that no mutations were occurred in the promoter or operator region(s) during the spontaneous mutation.

Substrate specificities of the oxidoreductases To test the xylitol dehydrogenase activities of the xdh and xytF products, they were heterologously expressed in E. coli. Each of the xdh and xytF genes was cloned behind the tac promoter in a pTQ18 vector. A wild type E. coli K-12 strain, AB707 was transformed with pTQ18-xdh (strain AB707/Xdh) or pTQ18-xytF (strain AB707/XytF) and grown in LB medium containing 10 g/l xylitol and 200 µg/ml ampicillin. SDS–PAGE analysis displayed over-production of the recombinant proteins in a soluble fraction of E. coli cells with expected molecular weights (28 kDa) (Fig. 4). As shown in Fig. 5, AB707/Xdh consumed 3.3 g/l of xylitol and accumulated 3.0 g/l xylulose in the culture medium after 24 h. In contrast, AB707/XytF did not consume xylitol or accumulate xylulose in the culture medium (Fig. 5). These results showed that only the xdh gene encoded xylitol dehy-
Xdh and XytF activities with various pentitols were tested using lysates of the recombinant cells (Table 3). The lysates of AB707/Xdh oxidized xylitol (4.10 U/mg of total protein). Xdh also oxidized D-arabitol (0.46 U/mg of total protein) though the activity was about 9-fold lower than that with xylitol. On the other hand, the lysates of AB707/XytF oxidized only L-arabitol (0.16 U/mg of the total protein) in the pentitols tested. XytF had no detectable activity with xylitol. Both enzymes showed a strong preference for NAD$^+$ as the reducing cosubstrate. No dehydrogenase activities were detected when NADP$^+$ was used as the cofactor. The cell lysates from the control strain harboring the vector-only plasmid showed no pentitol dehydrogenase activities.

**DISCUSSION**

In this study, we have isolated the xyt operon, which is involved in the cryptic xylitol metabolism of *P. ananatis*. Unexpectedly, the operon contains two oxidoreductase genes, *xdh* and *xytF*. Both of them are members of the SDR family but their deduced amino acid sequences are only 28% identical to each other. The nucleotide sequence of *xdh* is identical to that of xylitol 4-dehydrogenase (a synonym of L-xylulose reductase) gene which was cloned from *P. ananatis* DM101 based on the N-terminal sequence of the purified enzyme (8). The recombinant Xdh protein exhibited the activity with xylitol and the side activity with D-arabitol (Table 3), as shown in previous reports (7, 8). The transposon element was inserted into the *xdh* gene of *P. ananatis* ΔXYT that was unable to use xylitol as a sole carbon source for growth. These results clearly indicate that *xdh* encodes L-xylulose reductase and its product is essential for the xylitol utilization of the *P. ananatis* operon. Meanwhile, the recombinant XytF protein displayed the activity with only L-arabitol, not xylitol, among the pentitols (Table 3). However, *P. ananatis* XYT1 was unable to use L-arabitol as a sole carbon source for growth (data not shown), suggesting that only the activation of the xyt operon was not sufficient for degradation of L-arabitol.

Three genes in the xyt operon, *xytBDE* show sequence similarities to the subunits of ABC transporters, suggesting that they consist of a sugar transporter like other known prokaryotic ABC transport systems such as arabino (araFGH), galactose (mglABC), ribose (rbsABC) and xylose (xylFGH) transporters (15–18). The coexistence of two oxidoreductases and an ABC transporter has been found in the smo operon, which is involved in metabolism of a various sugar alcohols including D-sorbitol (D-glucitol) and D-mannitol in *Rhodobacter sphaeroides* Si4 (19). Xdh has very low and no activity with D-sorbitol and D-mannitol, respectively (8), and XytF has no detectable activity with these sugar alcohols (data not shown), suggesting that the xyt operon does not play a role in metabolism of D-sorbitol or D-mannitol.

Although the physiological role of the xyt operon remains to be elucidated, the operon is probably involved in uptake and metabolism of not only xylitol but also other sugar alcohols.

Pentitol metabolism in the *Klebsiella* and *E. coli* C strains that can utilize ribitol and D-arabitol as growth substrates have been well characterized. Their ribitol and D-arabitol pathways consist of the oxidation of the pentitols and the subsequent phosphorylation of the pentuloses (1, 2). The dehydrogenase gene and the corresponding kinase gene are encoded in the single operons (3). In contrast to these operons, the xyt operon that we have isolated contains dehydrogenase genes but not kinase gene, showing that the L-xylulose reductase and the L-xylulokinase that comprise the cryptic xylitol pathway in *P. ananatis* are encoded in separate operons. This is consistent with the observation that the xylitol-negative mutants lacking both L-xylulose reductase and L-xylulokinase activities could not be acquired by the transposon mutation of *P. ananatis* DM101 (7).

Based on sequence similarity, *xytR* encodes transcriptional regulator belonging to the DeoR family. Members of the DeoR family have been shown to regulate the transcription level of structural genes involved in various carbohydrate metabolism of bacteria such as glycerol (GlpR), galac-
and metabolic pathways. Klebsiella pathway to metabolize xylitol by borrowing preexisting DM101 will cause a nonsense mutation or a frame shift in the transcription initiation site to repress transcription efficiently. The loss of the C-terminal part in XytR\textsuperscript{S149X} may affect oligomerization of the protein and result in decrease of the binding ability to the operators and increase of the basal expression level of the protein. RT-PCR analysis showed that the xdh gene was expressed in \textit{P. ananatis} XYT1 but not in \textit{P. ananatis} NRRL B-14773 (Fig. 2). This result supports the hypothesis that wild type XytR negatively regulates expression of the xyt operon. Furthermore, the xylitol dehydrogenase activity and the expression level of the xdh gene (Fig. 2) in \textit{P. ananatis} XYT1 were increased by incubation with xylitol, suggesting that xylitol acts as an inducer of the xyt operon in this mutant. Since the induction by xylitol was not detected in \textit{P. ananatis} NRRL B-14773, the effector-binding property of XytR\textsuperscript{S149X} appears to be different from that of the wild type.

A mutation site of the xyt operon in \textit{P. ananatis} DM101, which constitutively synthesizes xylitol dehydrogenase, is likely to be different from that of \textit{P. ananatis} XYT1. Since a \textit{P. ananatis} DM101-specific anti-sense primer (Primer 1492, 5’-AATCATCTCCAGGATCTTTGCTTCCAG-3’) did not amplify a PCR product from wild type genomic DNA, the mutation was suggested to be located in this primer region (8). The sequence comparison between Primer 1492 and the corresponding genomic DNA region cloned from the wild type \textit{P. ananatis} strain NRRL B-14773 (nucleotide positions 36-12 in the complementary strand of xytR, 5’-AATCATCTCCAGGATCTTTGCTTCCAG-3’) suggests that three-base pair substitutions and one-base pair insertion (underlined) occur in \textit{P. ananatis} DM101. The mutations in \textit{P. ananatis} DM101 will cause a nonsense mutation or a frame shift in the N-terminus of the XytR protein, which results in loss of the repressor function (Fig. 3).

Some strains of bacteria that do not normally use xylitol as a growth substrate have been shown to establish a new pathway to metabolize xylitol by borrowing preexisting metabolic pathways. \textit{Klebsiella} strains possess the ribitol and D-arabitol operons to degrade these sugars and their xylitol-utilizing mutants metabolize xylitol by using enzymes included in these operons (26, 27). The new xylitol pathway established in the mutants utilizes the transporter from the D-arabitol operon, the dehydrogenase from the ribitol operon and the kinase from the D-arabitol operon. By using these enzymes, the mutants can transport xylitol into the cells, oxidize xylitol to D-xylulose and phosphorolylate D-xylulose to D-xylulose 5-phosphate, which is a common intermediate in pentose degradation. The D-arabitol operon is weakly induced when wild type cells are incubated with xylitol, but the ribitol operon is not. The ribitol operon is known to be negatively controlled by its regulator gene, \textit{rbcC} (28). Therefore, growth of the xylitol-utilizing mutants on xylitol results from the loss of the function of \textit{rbcC}, which permits the constitutive synthesis of the enzymes in the ribitol operon. Similarly, the xylitol-utilizing mutants of \textit{P. ananatis} have mutations in the putative regulator gene and use dehydrogenase and kinase encoded in separate operons. This leads us to hypothesize that the \textit{P. ananatis} mutants also metabolize xylitol by borrowing metabolic pathways of other sugars, even though the physiological role of the xyt operon remains unknown. Further characterization of each gene product in the xyt operon will provide insights into the function of this operon.

The constituents of the xyt operon could be valuable tools for synthesis of rare sugars by metabolic engineering. Xdh has been shown to convert xylitol to L-xylulose (8). XytF is predicted to convert L-ribitol to L-xylulose by catalyzing the oxidation of L-ribitol at the C-2 or C-4 position. Xylitol and L-arabitol can be easily obtained by oxidation of D-xylene and L-arabinose, which are common components of lignocellulosic materials. In contrast, L-ribulose and L-xylulose are scarce in nature and currently very expensive. Interest for these rare sugars has been growing especially in the pharmaceutical industry, since L-xylulose and L-ribulose can be enzymatically isomerized to L-xylulose and L-ribulose, respectively (29, 30), which are used as raw materials for the production of antiviral drugs (31, 32).

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**REFERENCES**


