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Short Communication

Identification of a new gene organization related to carbohydrate catabolism from *Citrobacter* sp. strain KCTC 18061P

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A new gene organization related to carbohydrate metabolism of *Citrobacter* species has been identified on about 4.1 kb chromosomal DNA fragment. Sequence analysis revealed four open reading frames (ORFs), and gene orders were determined to be PTS system mannitol-specific transporter subunit EIICBA (MtlA), L-sorbose-1-phosphate reductase (SorE), class II fructose-1,6-biphosphatase (GlpX), and mannitol repressor (MtlR). The deduced amino acid sequences of these ORFs showed more than 80% identities to MtlA, SorE, GlpX, and MtlR of *Escherichia coli*, *Shigella,* and *Salmonella enterica*, respectively. The *sorE* gene was heterologously expressed in *E. coli*. The recombinant SorE clearly showed the L-sorbose-1-phosphate reductase activity.

Key words: *Citrobactor* sp., gene organization, carbohydrate catabolism, L-sorbose 1-phosphate reductase, gene expression.

INTRODUCTION

It is well known that carbohydrate uptake in bacteria is mediated via the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) which involves both the transport and phosphorylation of a large number of carbohydrates (Postma et al., 1993). These PTS-carbohydrates include glucose, fructose, mannose, sorbose, mannitol, glucitol and galactitol. This system is composed of three catalytic proteins, enzyme I (EI), enzyme II (EII) and histidine-containing protein (HPr) (Barabote and Saier, 2005). EI and HPr are cytoplasmic proteins that participate in the phosphorylation of all PTS carbohydrates. EII protein is a single membrane-bound protein composed of three distinct domains (EIIA, EIIB and EIIC), and promotes the transport of one or a few sugars in a given bacterium. Seven EII families have been classified according to their specific sugar substrates (Barabote and Saier, 2005). The structural clustered in a *pts* operon, whereas the genes for the genes *pts*H and *pts*I for HPr and EI, respectively, are substrate-specific EIIs are clustered in an operon or regulon together with the structural genes for the corresponding catabolic enzymes (Barabote and Saier, 2005; Postma et al., 1993). In addition, previous studies have demonstrated that the genes involved in carbohydrate catabolism in bacteria are clustered in an operon (Postma et al., 1993).

Citrobacter is a genus of Gram-negative bacteria in the Enterobacteriaceae family and can be found in soil, water, wastewater, and human intestine. Although the genes of carbohydrate catabolic operons in enteric bacteria are known, no information has been obtained on the genes involved in carbohydrate catabolism of *Citrobacter* species. In the previous study, we have isolated a gene (*ctg* 2) showing the significant similarity (86% identity) to the sequence of putative oxidoreductase of *Escherichia coli*,from *Citrobacter* sp. MY-5 (Kim et al., 2005). The nucleotide sequences of *ctg* 2 gene have been deposited in the GenBank database under accession number AY859495. Very recently, we found that the sequence of a L-sorbose-1-phosphate reductase (SorE) gene from *Enterobacter cancerogenus* ATCC

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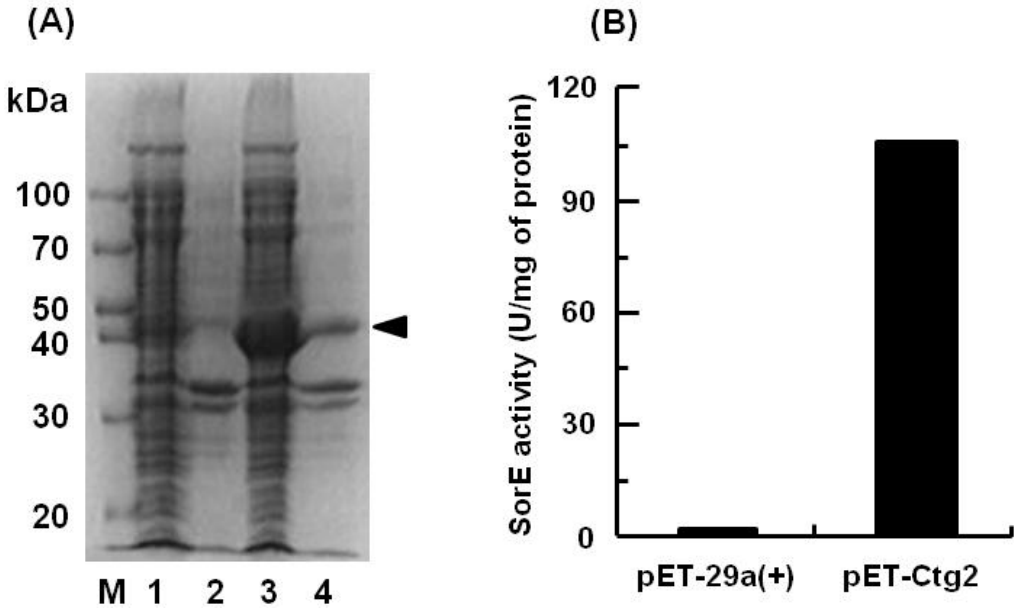

Figure 1. SDS polyacrylamide gel electrophoresis (A) and enzyme activity (B) of recombinant SorE produced in *E. coli* cells harboring pET-Ctg2. Lane M, molecular weight marker; lane 1, soluble fraction of pET-29a(+) vector (control); lane 2, insoluble fraction of pET-29a(+) vector; lane 3, soluble fraction of recombinant SorE; lane 4, insoluble fraction of recombinant SorE. One unit of SorE is defined as the amount that catalyzes the oxidation of 1 nmol of NADH per minute in the standard assay (Anderson and Simkins, 1982).

35316 showing very remarkable sequence similarity to that of c*tg* 2 gene described here, with an identity of 92% at amino acid level, was deposited in GenBank database with accession no. ZP_05970186.

Based on this finding, the present study was undertaken to investigate a gene organization related to carbohydrate catabolism of *Citrobacter* species and we found a new gene organization. This is the first report on molecular genetic analysis of carbohydrate catabolism in *Citrobacter* species. To investigate whether the protein encoded by the *ctg* 2 gene (1278 bp) has the enzyme activity of SorE, an 1.3 kb fragment including open reading frame (ORF) of *ctg* 2 was amplified by polymerase chain reaction (PCR) using Pyrobest DNA polymerase (Takara Shuzo, Japan) and a combination of forward (5'-TACAT*ATG*AAAACGAAAGTGGCTG-3') and reverse (5'-TACTCGAG*TCA*TATCGCCGCCTCC-3') primers, where the underlines represent the recognition sites of *Nde*I and *Xho*I, respectively, and translation codon ATG and stop codon TGA of ORF are shown in italics. The amplified 1.3 kb fragment was digested with *Nde*I and *Xho*I and then inserted into the corresponding sites of pET-29a(+), resulting in expression plasmid pET-Ctg2 which was finally transformed into *E. coli* BL21(DE3). *E. coli* BL21(DE3) cells harboring pET-Ctg2 were grown in LB medium containing 50 μg/ml of kanamycin at 37°C with shaking and protein expression was induced by the addition of 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) when the culture turbidity at 600 nm reached 0.8. After induction for 3 h, the cells were collected by centrifugation at 5000 × g at 4°C for 10 min, and then washed with 50 mM MES buffer (pH 6.2). After disruption by sonication, the cell components were separated into soluble and insoluble fractions by centrifugation at 12,000 × g for 20 min, and then analyzed by SDS-PAGE. A predominant band corresponding to the expected size (46 kDa) of recombinant protein was also observed in soluble fraction of induced cells (Figure 1A, lane 3). This molecular weight is in close to 45,825 Da calculated from the amino acid sequence of Ctg2. Enzyme assay of L-sorbose-1-phosphate reductase was done by the method of Anderson and Simkins (1982), using D-fructose-1-phosphate as a substrate. As shown in Figure 1B, SorE activity was not detectable in *E. coli* BL21 (DE3) cells harboring pET-29a(+) vector plasmid, whereas it was significantly increased in cells harboring pET-Ctg2 plasmid. This result indicates that *ctg*2 gene in this study encodes SorE gene of *Citrobacter* sp. strain KCTC 18061P.

It is known that sor operon (5559 bp) involved in metabolism and transport of L-sorbose in *E. coli* and *Klebsiella pneumoniae* contain seven genes clustered behind a single promoter *sorC*, the gene order being *sorCDFBAME* (Figure 2B) (Wehmeier et al., 1992; Wehmeier and Lengeler, 1994). The gene *sorD* encodes a D-glucitol-6-phosphate dehydrogenase, and the genes *sorFBAM* encode four proteins of a phosphoenolpyruvate-

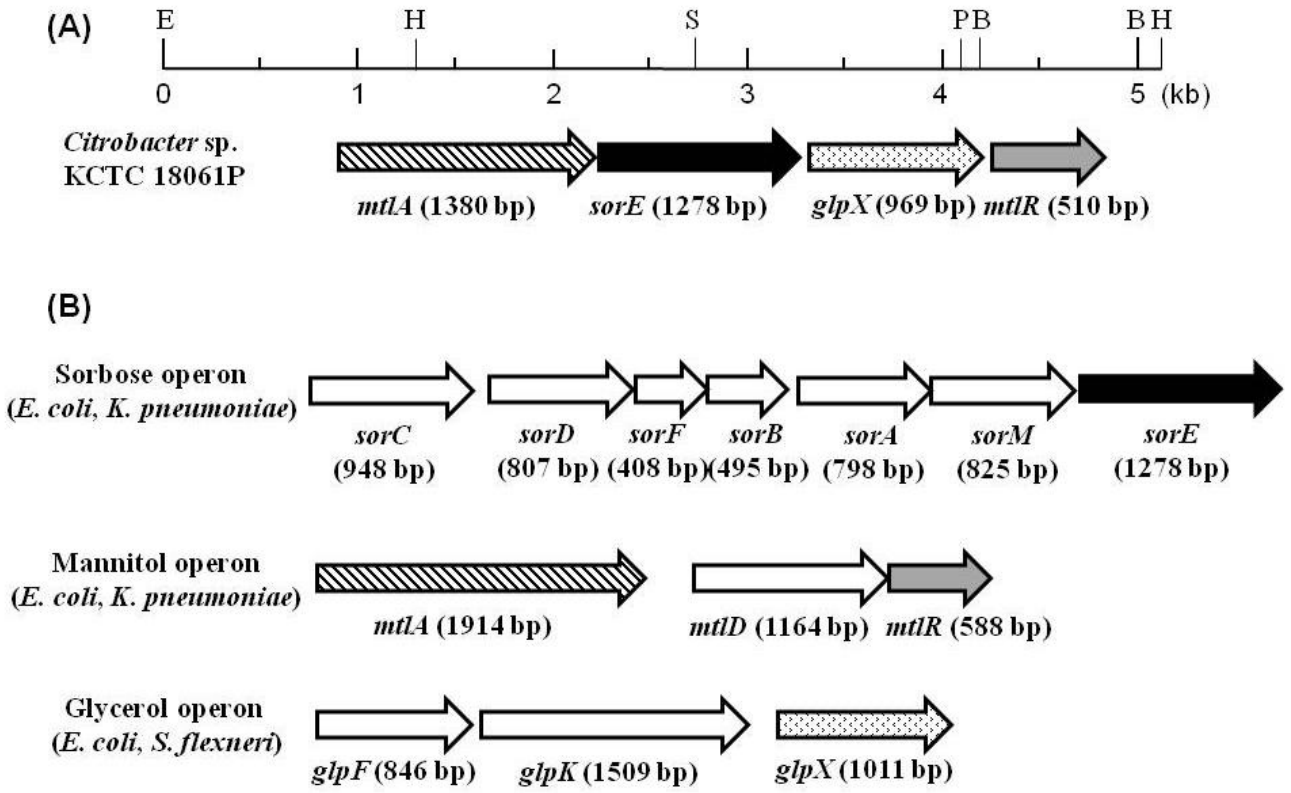

Figure 2. Gene organization of operons related to carbohydrate catabolism. (A) Restriction map of the 5.1 kb fragment from *Citrobacter* sp. strain KCTC 18061P bearing the genes involved in catabolism of sorbose, mannitol, and glycerol. The positions and orientations of the genes are shown below. Restriction sites: E, *Eco*RI; H, *Hind*III; S, *Sal*I; P, *Pst*I; B, *Bam*HI. (B) Gene organization of sorbose, mannitol, and glycerol operons in *E. coli*, *Klebsiella pneumoniae,* and *Shigella flexneri*

dependent L-sorbose-phospho-transferase system and *sorE*, finally, an L-sorbose 1-phosphate reductase (Wehmeier and Lengeler, 1994). On the basis of these reports, we tried to isolate a gene cluster involved in metabolism and transport of L-sorbose in *Citrobacter* sp. strain KCTC 18061P. The chromosomal DNA of *Citrobacter* sp. strain KCTC 18061P was digested with several restriction enzymes and analyzed by Southern hybridization using *ctg 2* DNA as a probe. The 3.8-kb *Hin*dIII and 4.2-kb *Eco*RI/*Pst*I fragments showing strong signal were fractionated from 0.7% agarose gels and ligated with the corresponding site of pBluescript II SK (+), and designated pBS4 and pBS5, respectively. After DNA sequencing by subcloning of pBS4 and pBS5, the analyses of nucleotide and deduced protein sequences by using the BLAST (blastx) program of the National Center for Biotechnology Information (NCBI) showed that this 5.1 kb fragments contained four ORFs for the genes (Figure 2A). Unexpectedly, further BLAST analysis revealed that these ORFs have a different genetic organization from sor operon involved in metabolism and transport of L-sorbose (Figure 2B) (Wehmeier et al., 1992; Wehmeier and Lengeler, 1994). The first gene (accession number JX315572) had an open reading frame of 1380 bp encoding a polypeptide comprised of 459 amino acids. The predicted amino acid sequence showed a high similarity (more than 84% identical residues) with PTS system mannitol-specific transporter subunit EIICBA (MtlA) of *E. coli*, *K. pneumoniae,* and *Salmonella enterica*. The second gene (JX315573) had an open reading frame of 1278 bp encoding a polypeptide comprised of 425 amino acids. The predicted amino acid sequence displayed a remarkable similarity (more than 90% identical residues) with SorE of *E. coli*, *K. pneumoniae,* and *S. enterica*. The third gene (JX315574) had an open reading frame of 969 bp encoding a polypeptide comprised of 322 amino acids. The predicted amino acid sequence exhibited a high similarity (more than 82% identical residues) with class II fructose 1,6-bisphosphatase (GlpX) of *E. coli*, *Shigella,* and *S. enterica*. The fourth genes (JX315575) had an open reading frame of 510 bp encoding a polypeptide comprised of 169 amino acids. The predicted amino acid sequence revealed significant similarities (more than 85% identical residues) with mannitol repressor (MtlR) of *E. coli*, *K. pneumoniae,* and *S. enterica*. In addition, the truncated gene downstream from *mtlR* gene revealed a high similarity with putative fructose transport system-related kinase (more than 80% identical residues) of *E. coli*, *Shigella* and *S. enterica*.

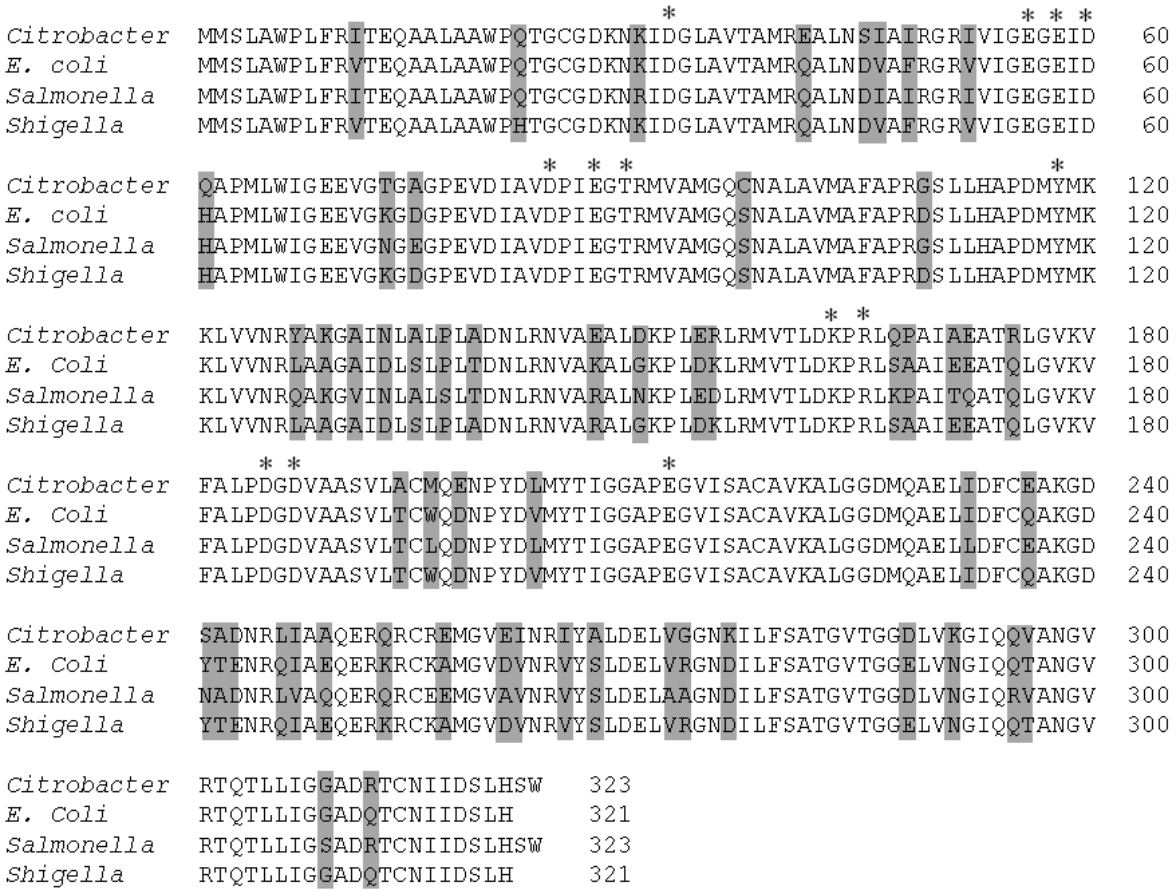

Figure 3. Multiple alignment of amino acid sequence of class II FBPase (GlpX) from *Citrobacter* sp. strain KCTC 18061P with other bacterial GlpXs. Amino acids which are not conserved in at least three of the four sequences are shown in *gray boxes*. Numbers to the right refer to the last amino acid on the line. Amino acids involved in catalytic sites and substrate binding site are marked with an *asterisk above* the alignment. The compared proteins are class II FBPase of *Citrobacter* sp. strain KCTC 18061P, *E. coli* YggF (P21437), class II FBPase of *Salmonella enterica* (ZP_02659378), and class II FBPase of *Shigella flexneri* (EGK19770). *E. coli* YggF is the experimentally verified class II FBPase [3].

Mannitol catabolism has been intensively studied in enteric bacteria (Postma et al., 1993). D-mannitol (Mtl) is also taken up and concomitantly phosphorylated to mannitol-1-phosphate by mannitol-specific PTS. The genes involved in mannitol catabolism have been identified from Gram-negative bacteria, such as *E. coli* (Figge at al., 1994), *Klebsiella* (Otte and Lengeler, 2001), and *Vibrio* (Kumar, 2011). These genes are also clustered in the single operon (3666 bp) composed of three genes (*mtlADR*), encoding (i) EII^{Mtl} (*mtlA*), (ii) a mannitol-1-phosphate dehydrogenase (*mtlD*), and (iii) a mannitol repressor (*mtlR*) (Figure 2B). EIIMtl encoded by *mtlA* participates in phosphorylation and transport of mannitol as mannitol-1-phosphate, which is further oxidized to fructose 6-phosphate by mannitol-1-phosphate dehydrogenase (Kumar et al., 2011). The mannitol repressor MtlR represses the mannitol operon transcription through interaction, either directly or indirectly, with other DNA-binding protein(s), and it was proposed to be a part of a transcriptional complex that regulates the mannitol operon expression of *E. coli*, *Shigella*, *Vibrio*, and other Gram-negative bacteria (Tan et al., 2009). In *E. coli* genome, the *glpFKX* operon (3366 bp) encodes glycerol transport facilitator, glycerol kinase, and class II fructose-1,6-bisphosphatase (FBPase), respectively (Figure 2B). This operon is one of five operons of the *glp* regulon involved in the growth of *E. coli* on glycerol (Brown et al., 2009; Truniger et al., 1992). As shown in Figure 3, amino acid sequence alignment of the experimentally verified GlpX from *E. coli* with those from *Citrobacter* sp. strain KCTC 18061P, *S. enteric*, and *Shigella flexneri* showed more than 82% identity and identified 13 conserved residues involved in substrate binding or catalysis (Brown et al., 2009). On the other hand, the truncated gene downstream from *mtlR* gene in this study revealed a high similarity with putative fructose transport system-related kinase.

In conclusion, we demonstrated that the *sorE* gene

downstream from *mtlA* of *Citrobacter* sp. strain KCTC 18061P encodes L-sorbose-1-phosphate reductase by production of recombinant enzyme and enzyme assay. We also firstly found a new gene organization related to carbohydrate catabolism of *Citrobacter* sp., which is different from the catabolic operons composed of the experimentally verified genes.

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