이학석사학위논문

인체 장내 유익균인 *Faecalibacterium prausnitzii* A2-165균의 당수송 신호전달계의 역할 규명

Characterization of the PTS in the beneficial human gut bacterium *Faecalibacterium prausnitzii* A2-165

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Abstract

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Faecalibacterium prausnitzii, an extremely oxygen sensitive Gram-positive bacterium, is known to be one of the most abundant bacteria in the human intestinal microbiota of healthy adults. This obligate anaerobe produces substantial amounts of butyrate, which has anti-inflammatory effects in the gut. The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is the predominant mechanism used for the efficient uptake of carbohydrates in many bacteria. The phosphorylation status of the PTS components reflects the availability of carbohydrates and the energy conditions of the cell. While studies have been conducted on this bacterium's importance for human health, little is known about its PTS. In this research, we identify the PTS components of *F. prausnitzii* A2-165 and establish the phosphorelay of the general PTS components, EI and HPr, and the EII complexes of the glucose-glucoside family. A unique feature of the *F. prausnitzii* PTS is that it possesses two paralogs of the general PTS proteins EI (EI-1 and EI-2) and HPr (HPr-1 and HPr-2), and the glucose-glucoside specific transporters, EIIBC-1 and EIIBC-2, hereafter named NagE and PtsG, respectively). Through *in vitro* phosphorylation assays, we found only EI-2 with phosphotransferase activity. Furthermore, in

Gram-positive bacteria, HPr can be phosphorylated at two different sites: on Histidine-15 via phosphoryl group transfer from enzyme I and on Serine-46 via HprK and ATP. While both HPr-1 and HPr-2 can be phosphorylated at Histidine-15, only HPr-2 can be phosphorylated at Serine-46. Lastly, we found that NagE transports both glucose (GLC) and *N*-acetylglucosamine (NAG), but with a strong specificity for NAG and a low specificity for GLC. Thus, we designated NagE to be an *N*-acetylglucosamine-specific transporter.

Keywords:
Faecalibacterium prausnitzii, Firmicutes, phosphotransferase system, general PTS, butyrate-producing bacteria

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I. Introduction

1. *Faecalibacterium prausnitzii*

1.1 Overview of *F. prausnitzii*

In healthy adults, the large intestine has the most dense and metabolically active microbial community, dominated by anaerobic bacteria belonging to the Firmicutes and Bacteroidetes phyla in addition to other bacteria. (Louis *et al.*, 2014). *F. prausnitz*ii, a member of the Firmicutes phylum, is a non-motile and non-spore forming Gram-positive bacterium. This bacterium has the shape of a long bacillus of around 2 μm with rounded ends (Miquel *et al.*, 2013). *F. prausnitzii* is known to represent more than 5% of the total fecal microbiota in healthy adults and is considered a biomarker of human health (Hold et al., 2003).

1.2 Clinical significance of *F. prausnitzii*

The abundance of *F. prausnitzii* within the microbiota has sparked interest in this bacterium and its implications in human health and disease. This bacterium is known to produce substantial quantities of butyrate as one of the major end products of glucose fermentation (Duncan *et al.*, 2004). Butyrate has been known to play numerous roles in health such as protection against pathogen invasion and modulation of the immune system (Macfarlane *et al.*, 2011). Furthermore, low levels of butyrate could be predictive for inflammatory bowel disease, ulcerative colitis, and Crohn's disease (Miquel *et al.*, 2013).

2. Phosphoenolpyruvate:sugar phosphotransferase system (PTS)

2.1 Overview of the PTS

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is a predominant system of carbohydrate uptake in many microbial species (Hayes *et al.*, 2017). This multicomponent system couples carbohydrate transport across the cytoplasmic membrane with their simultaneous phosphorylation (Reichenbach *et al.*, 2010) By examining the phosphorylation status of the PTS components, we can understand the availability of carbohydrates and the energy conditions of the cell (Kotrba *et al.*, 2001).

The PTS consists of two general cytoplasmic proteins, enzyme I (EI) and histidine-containing phosphocarrier protein (HPr), which lack sugar specificity, and various membranous carbohydrate-specific enzyme II complexes (EIIs), specific for one or a few sugars (Postma *et al.*, 1993). The EII complexes usually have three protein domains: cytosolic protein domains IIA and IIB, and a membranous domain IIC that forms the sugar translocation channel (Deutscher *et al.*, 2014). One exception is the mannose family, which has one additional membranous IID domain (Reizer *et al.*, 1997). The phosphorylation cascade initiates with the autophosphorylation of EI by the glycolytic intermediate PEP. Then, EI transfers its phosphoryl group to HPr and phosphoryl relay proceeds sequentially to the membrane-bound carbohydrate-specific EII domains (EIIA and EIIB), and finally to the incoming sugar, which is transported across the membrane concomitant with its phosphorylation (Deutscher *et al.*, 2006).

The PTS is ubiquitous in eubacteria but do not occur in eukaryotes and archaebacteria. The PTS proteins not only play an important role in the transport of numerous sugars but also participate in various regulatory functions such as metabolic and transcriptional regulation (Saier *et al.*, 2005), chemotaxis (Lux *et al.*, 1995), flagellar motility (O'Toole *et al.*, 1997), and cell division (Saier *et al.*, 1994).

2.2 Overview of the *F. prausnitzii* A2-165 PTS

The *F. prausnitzii* genome encodes 16 PTS transporters belonging to four different families including mannose, fructose, *N*-acetylglucosamine/glucose, and *N*-acetylgalactosamine (Fig. 1).

2.2.1 General components of the PTS

Unlike most bacteria, whose encoding genes *ptsI* and *ptsH* are encoded together in an operon, the PTS genes in *F. prausnitzii* are monocistronic. Another unique feature of the *F. prausnitzii* A2-165 genome is that it encodes two paralogues of both enzyme I (EI) and histidine-containing phosphocarrier protein, HPr.

A comparison of protein sequence identity with the general PTS components of *Bacillus subtilis*, also a member of the Firmicutes phylum indicate that both paralogues of EI and HPr exist. Protein sequence identity analysis shows EI-1 (536 aa, 62.2 kD) has 32% with EI of *B. subtilis* and EI-2 (548 aa, 61.7 kD) has 43%. *B. subtilis* EI has a phosphorylatable Histidine-189,

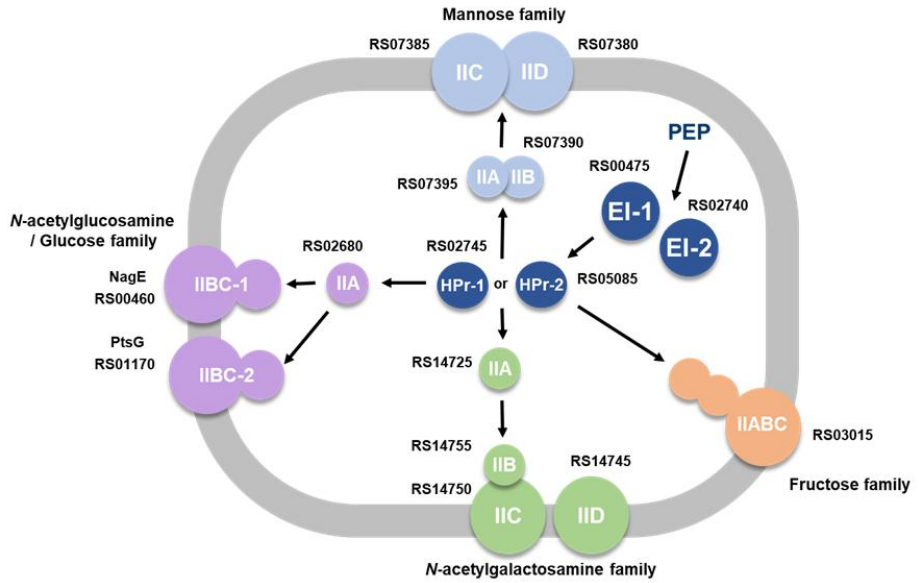

Figure 1. *F. prausnitzii* A2-165 PTS.

Illustration of the PTS components of *F. prausnitzii* A2-165. There are a total of 16 PTS transporters belonging to 4 sugar-specific families.

while EI-1 and EI-2 have a phosphorylatable Histidine-183 and Histidine-188, respectively. EI transfers the phosphoryl group from PEP to HPr and consists of an N-terminal domain, which is responsible for phosphorylating HPr, and a C-terminal domain, which is important in PEP binding and dimerization (Garrett *et al.*, 1997; Seok *et al.*, 1996; Seok *et al.*, 1998).

HPr is a small monomeric thermostable protein that transfers the phosphoryl group from EI to the various sugar-specific EIIs (De Reuse *et al.*, 1985). Also, in Gram-positive bacteria, HPr is phosphorylated at a regulatory Serine-46 residue by ATP and HPr kinase (HprK) (Fig. 2). (Reizer *et al.*, 1985). This ATP-dependent phosphorylation of HPr has been known to regulate the induction and carbon catabolite repression (CCR) of several catabolic genes (Saier *et al.*, 1996). Thus, the primary function of the PEP-dependent phosphorylation of HPr was assumed to drive the concomitant sugar uptake and phosphorylation while the main function of the ATP-dependent phosphorylation of HPr was postulated to regulate sugar accumulation (Reizer *et al.*, 1985). In *F. prausnitzii*, HPr-1 (85aa, 11.1 kD) has 39% identity with *B. subtilis* HPr while HPr-2 (87aa, 9.0 kD) has 44% identity with *B. subtilis* Crh, an HPr-like protein. In the case of HPr-1, the sequence around residue Histidine-15 (His-15), which is phosphorylated by enzyme I, is highly conserved, whereas the sequence around Serine-46 (Ser-46), whih is phosphorylated by ATP and HprK is not (Tangney *et al.*, 2005). However, for HPr-2, the sequence around residue His-15 and Ser-46 are both conserved.

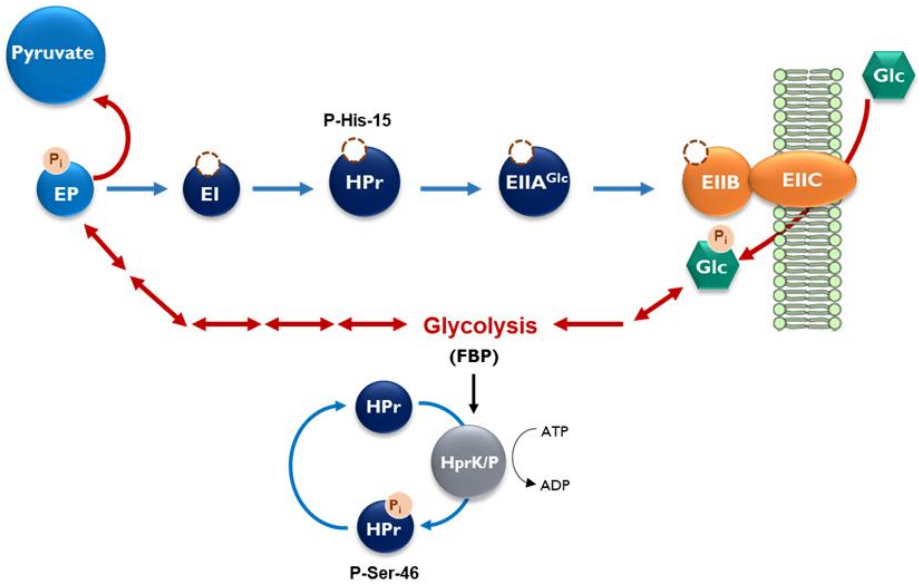

Figure 2. Gram-positive PTS.

Illustration of the Gram-positive PTS. HPr can be phosphorylated at two different sites: at Histidine-15 residue via phosphoryl group transfer from EI and at Serine-46 residue via HprK and ATP.

2.2.2 Sugar-specific components of the PTS

EIIs are the sugar-specific components in PTS (Fig. 1). They usually consist of three domains (EIIA, EIIB, and EIIC), which can be separate or fused together. For example, the EIIs of the *N*-acetylglucosamine and glucose family have a separate cytosolic $EIIA^{Glc}$ and a membrane-bound $EIIBC^{Glc}$ fused together, whereas all three domains (EIIA-C) of the fructose family are fused together in a single protein. Despite its different forms, all EIIs partake in the transfer of the phosphoryl group from PEP to the incoming carbohydrates.

Analysis of the *F. prausnitzii* genome sequence shows four different sugar families, including two EIIBC paralogues of the glucose-glucoside family (*N*-acetylglucosamine and glucose). Protein sequence comparison with *B. subtilis* identified EIIBC-1 as the *N*-acetylglucosamine transporter, NagE, and EIIBC-2 as the glucose transporter, PtsG.

3. The aims of this study

Despite its importance in human health, the physiological roles of *F. prausnitzii* are largely unknown. Thus, this study was a biochemical approach to better understand the physiology of *F. prausnitzii* A2-165 by studying its PTS.

Although the first complete genome of *F. prausnitzii* A2-165 was sequenced in 2010, its annotations are still incomplete (Miquel *et al.*, 2013). Especially regarding its PTS genes, many of them have been improperly named or have yet to be named. Therefore, through whole genome sequencing and by comparing

the protein sequence identities with the PTS components of bacteria whose PTS is well-known, the first aim of this study was to reannotate the PTS components within the *F. prausnitzii* A2-165 genome.

Moreover, *F. prausnitzii* possesses two paralogues of the general PTS components, EI and HPr, and EIIBC of the glucose-glucoside family. Through *in vitro* phosphorylation assays, we examined whether or not both paralogues of the general PTS components were capable of doing phosphorelay and identified the substrate specificity of the membrane-bound transporter of the glucose-glucoside family, EIIBC-1.

II. Materials and Methods

1. Strains and plasmids

Genomic DNA of *F. prausnitzii* A2-165 was used as the template DNA for cloning. *E. coli* ER2566△pts (NEB; Nosworthy *et al*, 1998), carrying a chromosomal copy of the T7 RNA polymerase gene under the control of the lac promoter (Steen *et al*., 1986) as well as the deletion of the *pts* genes, was used for the overproduction of recombinant proteins. The expression vector pETDuet-1 (Novagen), which contain an ampicillin resistance gene and a T7 promoter/*lac* operator, was used to construct the overexpression vectors of His-tagged and untagged proteins.

2. Media and cell culture

Yeast extract-casein hydrolysate-fatty acids (YCFA) medium was used for the culture of *F. prausnitzii* (Duncan *et al*., 2003). The medium contained (per 200 ml): 2 g of Casitone, 0.5 g of yeast extract, 1 g of glucose, 9 mg of $MgSO_4 \cdot 7H_2O$, 18 mg of $CaCl_2 \cdot 2H_2O$, 90 mg of K_2HPO_4 and KH_2PO_4, 0.18 g of NaCl, 0.2 mg of resazurin, 0.8 g of $NaHCO_3$, 0.2 g of L-Cysteine-HCl, 2 mg of hemin, supplemented with a filter sterilized vitamin solution consisting of 2 μg of biotin and folic acid, 10 μg of pyridoxine-HCl, 5 μg of thiamine-$HCl \cdot 2H_2O$, D-Ca-pantothenate, riboflavin, and nicotinic acid, 0.1 μg of vitamin B_{12}, and 5 μg of p-Aminobenzoic acid and lipoid acid. Short-chain fatty acids (SCFA) were added to make final concentrations (vol/vol) of

0.19% acetic acid, 0.07% propionic acid, 0.009% isobutyric acid, 0.01% *n*-valeric and isovaleric acid.

Luria Bertani (LB) Broth consisting of 1% tryptone, 0.5% NaCl and 0.5% yeast extract was used for routine bacterial culture. The antibiotics ampicillin (100 μg/ml) and kanamycin (20 μg/ml) were added when required.

3. Recombinant DNA techniques

3.1 Preparation of genomic DNA

Genomic DNA (gDNA) from *F. prausnitzii* was extracted using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN). The eluted DNA was aliquoted in 1.5 ml microcentrifuge tubes to a final concentration of 350 ng/μl.

3.2 Construction of recombinant plasmids

PTS genes were identified using various databases (EcoCyc, NCBI, Chun Lab, etc.) and recombinant plasmids were constructed with either no tags or hexahistidine tags using pETDuet-1 vector. All gene open reading frames (ORF) were derived from *F. prausnitzii* A2-165. The strains and recombinant plasmids used in this study are listed in Table 1.

3.3 Site-directed mutagenesis

The Histidine-93 residue of HisEIIA^{Glc} was modified to alanine and aspartate to mimic the dephosphorylated and phosphorylated state of HisEIIA^{Glc} (H93A) and HisEIIA^{Glc} (H93D), respectively.

Table 1. Strains and plasmids used in this study.

Strains / Plasmid	Genotype and/or descriptions	Reference
Strains		
ER2566	F-*λ-fhuA2 [lon] ompT lacZ::T7p07 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73::miniTn10-TetS)2R(zgb-10::Tn10)(TetS)endA1 [dcm]*	New England Biolabs
ER2566Δ*pts*	ER2566 *ptsHIcrr*::Km^r	(Nosworthy *et al*, 1998)
Plasmid		
pETDuet-1	Expression vector under the control of the T7 promoter, Amp^r	Novagen
pET-EnzymeI-1	*ptsI-1* ORF cloned between NdeI and XhoI sites in MCS2 of pETDuet-1	This study
pET-EnzymeI-2	*ptsI-2* ORF cloned between NdeI and XhoI sites in MCS2 of pETDuet-1	This study
pET-HisHPr-1	*ptsH-1* ORF cloned between BamHI and SalI sites in MCS1 of pETDuet-1 with His_6 tag	This study
pET-HisHPr-2	*ptsH-2* ORF cloned between BamHI and HindIII sites in MCS1 of pETDuet-1 with His_6 tag	This study
pET-HisEIIA^{Glc}	*crr* ORF cloned between SacI and AflII sites in MCS1 of pETDuet-1 with His_6 tag	This study
pET-HisEIIA^{Glc} (H93A)	His93 mutated to Ala in pET-HisEIIA^{Glc}	This study
pET-HisEIIA^{Glc} (H93D)	His93 mutated to Asp in pET-HisEIIA^{Glc}	This study
pET-HisNagE	*nagE* ORF cloned between BamHI and SalI sites in MCS1 of pETDuet-1 with His_6 tag	This study
pET-HisHprK	*hprK* ORF cloned between SacI and AflII sites in MCS1 of pETDuet-1 with His_6 tag	This study

4. Protein expression and purification

4.1 Overexpression of proteins

E. coli ER2566△*pts* transformed with strains indicated in Table 1 was used for the overexpression of both cytoplasmic and membrane PTS proteins. Cells were grown in LB medium at 37°C until the culture reached A_{600} of 0.5, at which 1 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the culture medium. After 3-4 hours of induction, the cells were harvested at 9,300 x *g* for 5 minutes.

4.2 Purification of untagged proteins

Cytoplasmic untagged proteins were cloned into the multiple cloning site 2 (MCS2) of the pETDuet-1 vector and overexpressed in *E. coli* ER2566△*pts*. Harvested cells were resuspended in buffer A (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol) and disrupted by three passages through a French pressure cell at 8,000 psi. After centrifugation at 100,000 x *g* at 4°C for 40 min, the supernatant was applied to a Mono Q™ 10/100 GL column (GE Healthcare Life Sciences) equilibrated with buffer A. Proteins were eluted with buffer B (25 mM Tris-HCl, pH 8.0, 1 M NaCl, 5 mM β-mercaptoethanol, 5% glycerol). The fractions containing the desired protein were concentrated and chromatographed on a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Life Sciences) equilibrated with buffer C (20 mM sodium phosphate, pH 8.0, 10 mM dithiothreitol (DTT), 200 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol) to achieve a higher purity.

Purified proteins were concentrated using Amicon Ultra-15 or Ultra-4 Centrifugal Filter Units (Merck) and the total protein concentration was determined using the Bradford protein assay at A595 nm using bovine serum albumin as the standard (Bradford, 1976).

4.3 Purification of His-tagged proteins

Cytoplasmic proteins with N-terminal His-tags were cloned into the multiple cloning site 1 (MCS1) of the pETDuet-1 vector and overexpressed in *E. coli* ER2566△*pts*. Harvested cells were resuspended in buffer D (20 mM sodium phosphate, pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol) and disrupted by three passages through a French pressure cell at 8,000 psi. After centrifugation at 9,300 x *g* at 4°C for 15 min to remove cell debris, the soluble fraction was purified using TALON metal-affinity resin (Takara Bio) according to the manufacturer's instructions. Proteins bound to the TALON resin were washed three times with wash buffer (buffer D containing 10 mM imidazole) and then eluted with elution buffer (buffer D containing 200 mM imidazole). The eluted proteins were further chromatographed on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences) equilibrated with buffer C to remove imidazole and increase purity.

Membrane proteins with N-terminal His-tags were purified using n-dodecyl-β-D-maltopyranoside (DDM). Harvested cells were resuspended in buffer D and disrupted by three passages through a French pressure cell at 8,000 psi and sonication. After

centrifugation at 9,300 x *g* at 4°C for 5 min to remove cell debris, the supernatant was centrifuged at 100,000 x *g* at 4°C for 60 min. The pellet was resuspended in buffer D containing 1% DDM and centrifuged again at 100,000 x *g* at 4°C for 30 min. After centrifugation, the supernatant was purified using TALON metal-affinity resin (Takara Bio) according to the manufacturer's instructions. Proteins bound to the TALON resin were washed three times with wash buffer (buffer D containing 10 mM imidazole and 0.1% DDM) and then eluted with elution buffer (buffer D containing 200 mM imidazole and 0.1% DDM). The eluted proteins were further chromatographed on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences) equilibrated with buffer C containing 0.05% DDM to remove imidazole and increase purity.

Purified His-tagged proteins were concentrated using Amicon Ultra-15 or Ultra-4 Centrifugal Filter Units (Merck) and the total protein concentration was determined using the Bradford protein assay at A595 nm using bovine serum albumin as the standard (Bradford, 1976).

5. *In vitro* phosphorylation assay

All phosphorylation assays were performed with purified proteins in the presence of 10 mM sodium phosphate, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, and 5 mM DTT in a total volume of 20 µl. All reactions were stopped by the addition of 4 µl of 6X SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (72 mM Tris-HCl, pH 6.8, 30%

glycerol, 2% SDS, 17.3 mM β-mercaptoethanol, 0.1% bromophenol blue) or 6X native-polyacrylamide gel electrophoresis (native-PAGE) sample buffer (72 mM Tris-HCl, pH 6.8, 30% glycerol, 17.3 mM β-mercaptoethanol, 0.1% bromophenol blue) and then analyzed by 4-20% or 16% SDS- or native-PAGE followed by staining with Coomassie Brilliant Blue. SDS-PAGE was used to detect the PDMS of HisEIIA^{Glc} and native-PAGE was used to detect either the PDMS of HisHPr.

PTS-dependent phosphorylation assays were done with 1 μg of untagged EI-1 or EI-2, 3 μg of HisHPr-1 or 2 μg of HisHPr-2 and 1 μg of HisEIIA^{Glc}. The reaction mixtures were incubated with 1 mM of PEP or pyruvate at 37°C for 10 minutes. Reactions were stopped as described previously.

PTS-dependent sugar phosphorylation assays were done with 1 μg of untagged EI-2, 3 μg of HisHPr-1 or 2 μg of HisHPr-2, 1 μg of HisEIIA^{Glc}, and 0.5 μg of HisNagE. In the phosphorylation assay using HisNagE, the reactions mixtures were incubated with 0.1 mM of PEP and 0.5 mM of glycerol (non-PTS sugar) and glucose, mannose, fructose, and *N*-acetylglucosamine (PTS sugars) at 37°C for 10 minutes. Reactions were stopped as described previously.

PEP-independent phosphorylation assays were done with 1 μg of untagged EI-2, 3 μg of HisHPr-1 or 2 μg of HisHPr-2, 1 μg of HisHprK, and 1 μg of HisEIIA^{Glc}. The reaction mixtures were incubated with or without 4 mM of ATP at 37°C for 10 minutes. For reaction mixtures including 1 mM of PEP or pyruvate and ATP, the mixture was incubated with 4 mM of ATP at 37°C for

10 minutes first, then 1 mM of PEP or pyruvate was added and the reaction mixture was further incubated at 37°C for 10 minutes. Reactions were stopped as described previously.

6. Determination of the phosphorylation state of $EIIA^{Glc}$

Quantification of the ratio of dephosphorylated:total $EIIA^{Glc}$ for the sugar phosphorylation test was done using NIH Image J software.

III. Results

1. *F. prausnitzii* A2-165 has 16 PTS components

Using various online databases and through whole genome sequencing, we identified a total of 16 PTS components in *F. prausnitzii* A2.-165 (Table 2). However, the names of the PTS genes varied among the databases and some were not classified into sugar families. Therefore, we compared the protein sequence of the PTS genes with that of *Escherichia coli* and *Bacillus subtilis*, whose PTS is well-known and designated new annotations for each PTS component.

2. Only EI-2 is capable of doing phosphorelay

As mentioned previously, two EIs exist within the *F. prausnitzii* A2-165 genome. Therefore, in order to see whether or not both of these proteins were functional, phosphorylation assays were performed as mentioned in the Materials and Method section and analyzed by native-PAGE. If EI phosphorylates HPr, HPr exhibits a phosphorylation-dependent mobility shift (PDMS) on native gel. In the case of EI-1, no band shift was observed when phosphorylation assays were performed with either HPr-1 or HPr-2 (Figure 3A). However, when the same reaction was conducted with EI-2, band shifts were detected in both HPrs (Figure 3B). Since HPr-1 appeared as a smeared band, it was hard to pinpoint a clear shift. Yet, the presence of a smeared

Table 2. PTS components in *F. prausnitzii* A2-165. A total of 16 PTS components were identified using various databases. Location within the genome is indicated in the far left lane. Various names are given in the second and third lanes. The far right lane indicates the newly annotated protein names.

Location	ChunLab Gene Name	EcoCyc / NCBI Blast Gene Name	Protein
RS00475	PTS-EI.PTSI|ptsI	Multiphosphoryl transfer protein mtp	**EI-1**
RS02740	PTS-EI.PTSI|ptsI	Phosphoenolpyruvate--protein phosphotransferase	**EI-2**
RS02745	Multiphosphoryl transfer protein	PTS galactitol transporter subunit IIC / phosphocarrier protein HPr	**HPr-1**
RS05085	Phosphocarrier protein HPr	PTS sugar transporter subunit IIA / HPr family phosphocarrier protein	**HPr-2**
RS02680	PTS-Glc-EIIA|crr	PTS glucose transporter subunit IIA	**EIIA^{Glc}**
RS00460	PTS-Nag-EIIB|nagE	PTS glucose transporter subunit IIBC; ptsG1, G2, NagE	**EIIBC1**
RS01170	PTS-Mal-EIIB|malX	PTS glucose transporter subunit IIBC; ptsG1	**EIIBC2**
RS03015	PTS-Fru-EIIA|fruB	PTS fructose transporter subunit IIC	**FruA**
RS07395	PTS-Man-EIIA|manX	PTS sugar transporter subunit IIA / PTS_IIA_man superfamily	**ManA**
RS07390	PTS-Man-EIIB|manX	PTS mannose transporter subunit IIB	**ManB**
RS07385	Putative *N*-acetylgalactosamine permease IIC component	PTS sugar transporter subunit IIC / EII_Sor_family	**ManC**
RS07380	hypothetical protein	PTS sugar transporter subunit IID / EIID_AGA_superfamily	**ManD**
RS14725	PTS-Man-EIIA|manX	PTS sugar transporter subunit IIA	**AgaA**
RS14755	PTS-Man-EIIB|manX	PTS IIB component	**AgaB**
RS14750	Putative *N*-acetylgalactosamine permease IIC component	PTS IIC component	**AgaC**
RS14745	Fructose permease IID component	PTS system, IID component	**AgaD**

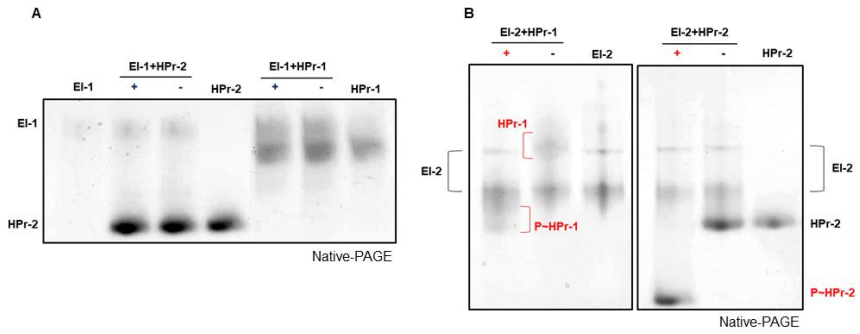

Figure 3. Only EI-2 phosphorylates both HPrs. *(A)* 1 μg of untagged EI-1 was mixed with either 2 μg of HisHPr-2 or 3 μg of HisHPr-1 and 1 mM of PEP (+) or pyruvate (-) in the presence of buffer P described in the Materials and Method section. After incubation at 37°C for 10 minutes, the reactions were stopped by adding 4 μl of 6X native-PAGE sample buffer and then analyzed by native-PAGE followed by staining with Coomassie Brilliant Blue. No band shift was observed. *(B)* 1 μg of untagged EI-2 was mixed with either 3 μg of HisHPr-1 or 2 μg of HisHPr-2 and phosphorylation assays were performed as described previously. Band shifts were observed for lanes with PEP.

band below the EI-2 band only in the lane with PEP, indicating that phosphorylation had occurred (Figure 3B, left). On the contrary, HPr-2 showed a very definite band shift in the lane with PEP (Figure 3B, right). Thus, based on these two results, we found that while two EIs exist within the *F. prausnitzii* A2-165 genome, only EI-2 is capable of phosphorylating both HPrs.

3. Both HPrs are capable of phosphorylating $EIIA^{Glc}$

Native-PAGE results from Figure 3 showed that only EI-2 was capable of doing phosphorelay to both HPrs. Next, phosphorylation assays were performed to see if both HPrs were capable of phosphorylating $EIIA^{Glc}$. The results were analyzed by SDS-PAGE to detect the PDMS of $EIIA^{Glc.}$ Theoretically, since both HPrs can be phosphorylated by EI-2, they should also be able to phosphorylate $EIIA^{Glc}$, whose shift can be seen on SDS-PAGE gel. As expected, no band shift was observed for $EIIA^{Glc}$ in the presence of PEP where EI-1 was used (Figure 4A), confirming once again that EI-1 is incapable of doing phosphorelay. Furthermore, $EIIA^{Glc}$ band shift was observed in lanes with EI-2 and HPr-1 or HPr-2 in the presence of PEP (Figure 4B and 4C), indicating that phosphorylation had occurred. However, one important observation was made. While both HPrs were able to phosphorylate $EIIA^{Glc}$, $EIIA^{Glc}$ was phosphorylated completely only when HPr-1 was the phosphoryl group donor (Figure 4B). While HPr-2 could phosphorylate $EIIA^{Glc}$, it was not

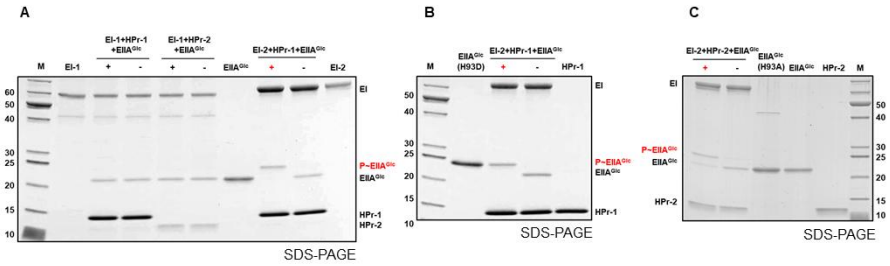

Figure 4. Both HPrs can phosphorylate EIIA^{Glc}. *(A)* 1 μg of untagged EI-1 was mixed with either 3 μg of HisHPr-1 or 2 μg of HisHPr-2, 1 μg of HisEIIA^{Glc}, and 1 mM of PEP (+) or pyruvate (-) in the presence of buffer P described in the Materials and Method section. After incubation at 37°C for 10 minutes, the reactions were stopped by adding 4 μl of 6X SDS-PAGE sample buffer and then analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. No band shift was observed in reactions with untagged EI-1. The last three lanes were included as control. *(B)* 1 μg of untagged EI-2 was mixed with 3 μg of HisHPr-1 and 1 μg of HisEIIA^{Glc}. Phosphorylation assays were performed as described previously. Band shift was observed for the lane with PEP. The phosphomimetic mutant, HisEIIA^{Glc} (H93D) was included as control to mimic the phosphorylated state of HisEIIA^{Glc}. *(C)* 1 μg of untagged EI-2 was mixed with 2 μg of HisHPr-2 and 1 μg of HisEIIA^{Glc}. Phosphorylation assays were performed as described previously. Partial band shift was observed for the lane with PEP. The phosphomimetic mutant, HisEIIA^{Glc} (H93A) was included to mimic the dephosphorylated state of HisEIIA^{Glc}.

as efficient as HPr-1 in phosphorylating $EIIA^{Glc}$ (Figure 4C).

4. EIIBC-1 is a transporter of *N*-acetylglucosamine

To determine the identity of the EIIBC-1 membrane-bound transporter, phosphorylation assays were performed as mentioned in the Materials and Method section and analyzed by SDS-PAGE. Band intensities of the dephosphorylated $EIIA^{Glc}$ were analyzed using NIH Image J software, and the percentage of dephosphorylated $EIIA^{Glc}$ over total $EIIA^{Glc}$ was indicated below the gel. If a membrane-bound transporter transports a particular sugar, then $EIIA^{Glc}$ becomes dephosphorylated in the presence of that particular sugar since the phosphoryl group is transferred to the sugar. SDS-PAGE results showed that $EIIA^{Glc}$ becomes dephosphorylated in the presence of both glucose (GLC) and *N*-acetylglucosamine (NAG) (Figure 5). In the presence of HPr-1, only a partial dephosphorylation of $EIIA^{Glc}$ (33%) occurs in the presence of glucose (Figure 5A), whereas in the presence of HPr-2, $EIIA^{Glc}$ is almost fully dephosphorylated (85%) in the presence of glucose (Figure 5B). However, since $EIIA^{Glc}$ becomes completely dephosphorylated only in the presence of NAG, we identified this membrane-bound transporter as NagE.

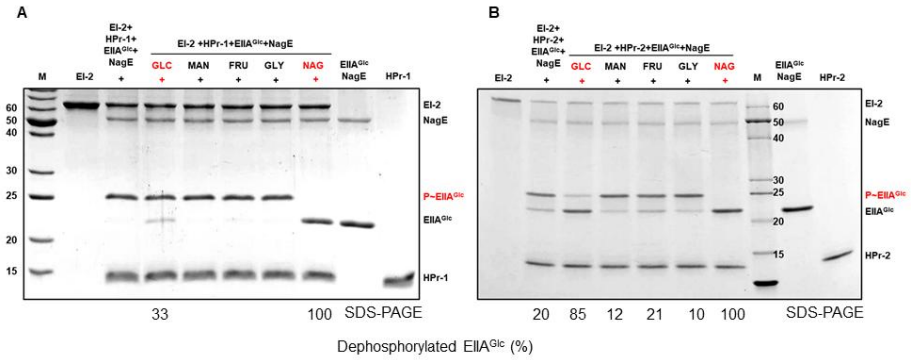

Figure 5. EIIBC-1 has a stronger preference for NAG than GLC. *(A)* 1 μg of untagged EI-2, 3 μg of HisHPr-1, 1 μg of HisEIIA^{Glc}, and 0.5 μg of HisNagE was mixed with 0.1 mM of PEP (+) and 0.5 mM of non-PTS and PTS sugars in the presence of buffer P described in the Materials and Method section. The same reaction mixture without sugar was included as control. After incubation at 37°C for 10 minutes, the reactions were stopped by adding 4 μl of 6X SDS-PAGE sample buffer and then analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Only lanes with GLC and NAG exhibited the dephosphorylated form of EIIA^{Glc}, with EIIA^{Glc} being slightly dephosphorylated in the presence of GLC and fully dephosphorylated in the presence of NAG. *(B)* The same sugar phosphorylation was performed as described in Figure 5A, with 2 μg of HisHPr-2 instead of HisHPr-1. Only lanes with GLC and NAG exhibited the dephosphorylated form of EIIA^{Glc}, with EIIA^{Glc} being mostly dephosphorylated in the presence of GLC and completely dephosphorylated in the presence of NAG.

5. ATP-dependent phosphorylation of HPr does not inhibit its PEP-dependent phosphorylation

As mentioned previously, in Gram-positive bacteria, HPr can be phosphorylated at two different sites, Histidine-15 and Serine-46 (Deutscher *et al.*, 1989). The phosphorylation at Histidine-15 occurs through a PEP-dependent mechanism on receiving a phosphoryl group from EI. However, the phosphorylation at Serine-46 occurs through a PEP-independent mechanism, via HprK and ATP. This ATP-dependent phosphorylation of HPr is known to inhibit PEP-dependent phosphorylation of HPr (Deutscher *et al.*, 1984). Therefore, experiments were done to see whether or not the same was true for *F. prausnitzii* A2-165.

The native-PAGE result showed that HPr-1 was incapable of being phosphorylated by HprK and ATP (Figure 6A), while HPr-2 was able to be phosphorylated by HprK and ATP (Figure 6B). As shown in the native-PAGE gel result, the PDMS is different for HPr phosphorylated via the PEP-dependent mechanism and HPr phosphorylated via the ATP-dependent mechanism (Figure 6B). The band shift in HPr phosphorylated at His-15 is between the band shift in HPr phosphorylated at Ser-46. To determine the effect of ATP-dependent phosphorylation of HPr on its PEP-dependent phosphorylation, phosphorylation assays were performed as mentioned in the Materials and Method section and analyzed by native-PAGE. The reaction mixture containing both PEP, ATP, and HprK had the same HPr band shift as the reaction mixture containing just

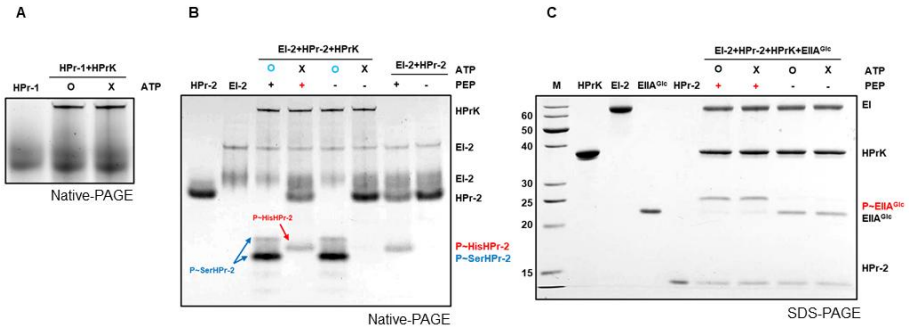

Figure 6. ATP-dependent phosphorylation of HPr-2 does not inhibit the PEP-dependent phosphorylation of HPr-2. *(A)* 1 μg of untagged EI-1 was mixed with 3 μg of HisHPr-1 and 1 μg of HisHprK, with (O) and without (X) 4 mM of ATP in the presence of buffer P described in the Materials and Method section. After incubation at 37°C for 10 minutes, the reactions were stopped by adding 4 μl of 6X native-PAGE sample buffer and then analyzed by native-PAGE followed by staining with Coomassie Brilliant Blue. No band shift was observed. (*B*) 1 μg of untagged EI-2 was mixed with 3 μg of HisHPr-1 and 1 μg of HisHprK, with 1 mM of PEP (+) or pyruvate (-) and with (O) or without (X) 4 mM of ATP. Phosphorylation assays were performed as described previously. No band shift was observed in the lane containing the reaction mixture incubated with both ATP and PEP. *(C)* The same phosphorylation assay as Figure 6B was done except with the addition of 1 μg of HisEIIA^{Glc}. SDS-PAGE analysis was done to detect the PDMS of HisEIIA^{Glc}. HisEIIA^{Glc} was phosphorylated even in the presence of HprK and ATP.

ATP and HprK (Figure 6B). Furthermore, the same reaction was performed on SDS-PAGE to observe the PDMS of $EIIA^{Glc}$ (Figure 6C). Interestingly, the reaction mixture containing both PEP, ATP, and HprK had the same $EIIA^{Glc}$ band shift as the reaction mixture containing just PEP.

IV. Discussion

1. Characterization of the PTS components and their phosphorelay

Living organisms have the ability to sense environmental conditions surrounding them and to respond to them. Carbon source supply is crucial to all organisms and in many bacteria, the PTS functions as the central processing unit for the modulation of carbohydrate utilization (Deutscher *et al.*, 2006). The PTS is not only responsible for the transport of numerous sugar substrates, but also for their phosphorylation in both Gram-negative and Gram-positive prokaryotes (Reizer *et al.*, 1988). The phosphorylation state of the PTS proteins reflects the sugar supply and links the availability of sugars and the physiological state of the cell to the activity of transport proteins, metabolic enzymes, and transcriptional regulators (Stülke *et al.*, 2004).

In both types of bacteria, the PTS is made up of two general components, EI and HPr, and the sugar-specific proteins that are collectively termed EIIs (Postma *et al.*, 1993). However, the genome of *F. prausnitzii* A2-165 is unique in that it possesses two copies of the general components, EI and HPr, and the EIIBC component of the glucose-glucoside family. Therefore, we conducted protein sequence comparisons with other bacteria whose PTS is well-known and gave new annotations. We also performed *in vitro* phosphorylation assays with untagged or

His-tagged purified proteins to see whether or not both paralogues of each PTS protein could do phosphorelay.

The results of our research indicated that only one of the two enzyme Is, EI-2, was functional. We designated EI-1 to be a pseudogene, because while it has similarities to EI in other bacteria, it is incapable of doing phosphorelay to both HPrs. An explanation for this occurrence is possible when examining the phosphorylation site of each of these two proteins. In *B. subtilis* and *E. coli*, phosphorylation occurs at the His-189 residue during autophosphorylation with PEP. However, EI-1 has a His-183 residue, not to mention the fact that its identity with *B. subtilis* EI-1 (32%) is significantly lower than that of EI-2. which has a His-188 residue and 43% identity with *B. subtilis* EI. Based on these comparisons, it is only fitting that we designate EI-2 to be the sole functional EI protein of the PTS. Likewise, the same measures were taken to identify the two HPrs, but more is to be discussed in the next section.

In addition to the general PTS components, four sugar families of the membrane-bound transporters are present within the *F. prausnitzii* genome. Of the four sugar families, there are two paralogues of the EIIBC protein of the glucose-glucoside family. Again, through sequence comparison, we identified EIIBC-1 to be the *N*-acetylglucosamine-specific transporter, NagE, and EIIBC-2 to be the glucose-specific transporter, PtsG. For further confirmation, we performed *in vitro* sugar phosphorylation assays and identified NagE as the main transporter responsible for transporting *N*-acetylglucosamine.

2. Additional role of HPr

As mentioned previously, HPr is phosphorylated at two different sites in Gram-positive bacteria: on His-15 via phosphoryl group transfer from EI by a PEP-dependent mechanism and on Ser-46 by a PEP-independent mechanism via HprK and ATP (Reizer *et al.*, 1985). The ATP-dependent phosphorylation of HPr plays a role in CCR (Saier *et al.*, 1996).

Sequence comparison with *B. subtilis* identified HPr-1 as HPr with His-15 and Ser-41 residues, but HPr-2 as HPr-like protein Crh (catabolite repression HPr) with His-15 and Ser-46 residues. In *B. subtilis*, the active-site His-15 of HPr is replaced with glutamine in Crh, meaning it can only be phosphorylated by ATP and HprK at Ser-46 (Galinier *et al.*, 1997). However, since HPr-2 possesses a His-15 residue, it is unfitting to say that it is Crh. *In vitro* phosphorylation assays showed that both HPrs were phosphorylated at the histidine residue, with HPr-1 showing greater efficiency than HPr-2, but only HPr-2 was phosphorylated at the serine residue.

Therefore, it seems reasonable to conclude that HPr-1 and HPr-2 have divided functions of HPr: HPr-1 functions mainly as HPr in Gram-negative bacteria in that it is phosphorylated on His-15 via PEP and EI, while HPr-2 functions mainly as HPr in Gram-positive bacteria in that ATP-dependent phosphorylation is dominant over PEP-dependent phosphorylation of HPr.

Furthermore, the ATP-dependent phosphorylation of HPr is known to inhibit the PEP-dependent phosphorylation of HPr by slowing it down by a factor of 5000 (Deutscher *et al.*, 1984).

Thus, we speculated that if HPr was incubated with PEP, HprK, and ATP, the effect of ATP-dependent phosphorylation would be dominant. Based on our phosphorylation assay results, we can say the same is true for HPr-2 of *F. prausnitzii* A2-165 (Fig. 6B and 6C). In the lane with both PEP and ATP, and EI, HPr, and HprK, only the seryl phosphorylated HPr band appeared, showing that ATP-dependent phosphorylation of HPr by ATP and HprK inhibits its PEP-dependent phosphorylation (Fig. 6B). However, when the same experiment was conducted with the addition of $EIIA^{Glc}$, SDS-PAGE result showed otherwise. $EIIA^{Glc}$ exhibited a PDMS even in the presence of ATP and HprK (Fig. 6C). Based on this result, we concluded that while the effect of ATP-dependent phosphorylation of HPr is dominant, it does not inhibit the PEP-dependent phosphorylation of HPr.

3. Significance of study

In this study, we characterized the PTS components of *F. prausnitzii* A2-165. By performing phosphorylation assays, we were able to uncover the functional phosphorelay of the general PTS components as well as the *N*-acetylglucosamine transporter, NagE. The results of this study can be used to further study the interaction of other proteins with the PTS proteins in different signal transduction strategies linking the presence of sugars and the physiological state of the cell to the activity of transcriptional regulators, transport proteins, and metabolic enzymes.

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국문 초록

피칼리박테리움 프로스니치는 절대혐기성 그람양성균이며, 건강한 성인 장내 세균 총을 구성하는 세균들 중에서 가장 풍부하게 존재한다. 이 균은 다양한 염증성 장질환에 효과가 있다고 알려진 부티르산을 생산하는 대표 장내 유익 세균이다. 당수송 인산전달계(phosphoenolpyruvate:sugar phosphotransferase system, PTS)는 박테리아가 사용하는 가장 효율적인 당 수송 시스템으로써, 세포 외부의 영양분의 변화를 감지하여 당 수송 뿐만 아니라 다양한 생리활성을 조절한다고 알려져 있다. 이 균에 대한 면역학적 연구는 진행되었으나, 아직 생리학적 연구는 전무한 상태이다.

PTS는 모든 당 수송방식에서 공통으로 사용되는 일반 PTS 단백질인 EI과 HPr, 그리고 당 특이적으로 사용되는 EII 단백질로 구성되어 있다. 하지만, 피칼리박테리움 프로스니치 PTS는 특이적으로 EI과 HPr, 그리고 glucose-glucoside family에 속해 있는 당 특이적인 EII 단백질 모두 두 개의 파라로그 (paralog)를 가지고 있다는 것을 확인할 수 있었다.

본 연구를 통해, 피칼리박테리움 프로스니치 A2-165의 모든 PTS 구성요소들을 규명하고, 일반적인 PTS 단백질인 EI과 HPr, 그리고 glucose-glucoside family에 속해 있는 EII 단백질들 간의 인산 전달 과정을 확인하는 연구를 수행하였다. 단백질 서열 분석과 *in vitro* phosphorylation assay를 통해, EI의 파라로그들 중에 오직 하나만이 정상적으로 당수송 기능을 수행하는 것을 확인하였고, 모든 HPr은 PEP에 의한 인산화가 일어나지만 HPr의 하나의 파라로그인 HPr-2만 ATP와 HprK에 의한 인산화가 일어나는 것을 확인하였다. 마지막으로, EIIBC-1는 *N*-아세틸글루코사민을 수송한다는 것을 확인하였고 NagE로 새로 명명하였다.

주요어:
피칼리박테리움 프로스니치, 후벽세균, 당수송 인산전달계, 일반 PTS, 부티르산 생성균

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