Discovery of β-1,4-d-Mannosyl-N-acetyl-d-glucosamine Phosphorylase Involved in the Metabolism of N-Glycans*\(^1\)

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Background: N-Glycans are metabolized by sequential glycoside hydrolase-catalyzed reactions.

Results: A phosphorylase encoded in a gene cluster involved in N-glycan metabolism in the genome of Bacteroides thetaiotaomicron catalyzed reversible phosphorylisis of β-1,4-d-mannosyl-N-acetyl-d-glucosamine.

Conclusion: An N-glycan metabolic pathway containing a unique phosphorylase was discovered.

Significance: B. thetaiotaomicron efficiently utilizes the energy of ATP via a phosphorylase-dependent metabolic pathway.

A gene cluster involved in N-glycan metabolism was identified in the genome of Bacteroides thetaiotaomicron VPI-5482. This gene cluster encodes a major facilitator superfamily transporter, a starch utilization system-like transporter consisting of a TonB-dependent oligosaccharide transporter and an outer membrane lipoprotein, four glycoside hydrolases (α-mannosidase, β-N-acetylhexosaminidase, exo-α-sialidase, and endo-β-N-acetylglucosaminidase), and a phosphorylase (BT1033) with unknown function. It was demonstrated that BT1033 catalyzed the reversible phosphorylisis of β-1,4-d-mannosyl-N-acetyl-d-glucosamine in a typical sequential Bi Bi mechanism. These results indicate that BT1033 plays a crucial role as a key enzyme in the N-glycan catabolism where β-1,4-d-mannosyl-N-acetyl-d-glucosamine is liberated from N-glycans by sequential glycoside hydrolase-catalyzed reactions, transported into the cell, and intracellularly converted into α-d-mannose 1-phosphate and N-acetyl-d-glucosamine. In addition, intestinal anaerobic bacteria such as Bacteroides fragilis, Bacteroides helcogenes, Bacteroides salanitronis, Bacteroides vulgatus, Prevotella denticola, Prevotella dentalis, Prevotella melaninogenica, Parabacteroides distasonis, and Alistipes finegoldii were also suggested to possess the similar metabolic pathway for N-glycans. A notable feature of the new metabolic pathway for N-glycans is the more efficient use of ATP-stored energy, in comparison with the conventional pathway where β-mannosidase and ATP-dependent hexokinase participate, because it is possible to directly phosphorylate the α-mannose residue of β-1,4-d-mannosyl-N-acetyl-d-glucosamine to enter glycolysis. This is the first report of a metabolic pathway for N-glycans that includes a phosphorylase. We propose 4-O-β-d-mannopyranosyl-N-acetyl-d-glucosamine phosphate α-d-mannosyltransferase as a systematic name and β-1,4-d-mannosyl-N-acetyl-d-glucosamine phosphorylase as the short name for BT1033.

Bacteroides thetaiotaomicron is a Gram-negative anaerobic inhabitant of the human distal gut, an environment deficient in mono- and oligosaccharides as carbon sources. Because most of these saccharides are absorbed by the host and various gut microbiota in the upper intestinal tract (1), B. thetaiotaomicron possesses superior capacities to degrade a variety of host-derived glycans elaborated on the surfaces of intestinal epithelial cells, in addition to the dietary polysaccharides that are indigestible by the host (1–4). Recent whole genome transcriptional analyses of B. thetaiotaomicron revealed that B. thetaiotaomicron dedicates ~18% of its 6.26-Mb genome to 88 individual polysaccharide utilization loci (1, 3–6) for metabolism of various glycans such as N-glycans (7), mucin O-glycans (8), xyloglucan (9), and arabinogalactan (9). At least one of these loci is involved in the conventional metabolism for complex type N-glycans that contain N-acetyl-d-lactosamine (β-1,4-d-galactosyl-N-acetyl-d-glucosamine) units attached to the α-1,3- and/or α-1,6-mannosyl residues linked to the core saccharide β-1,4-d-mannosyl-N,N'-diacytethylchitobiose (ManGlcNAC₃) and commonly terminate with sialic acid residues (10). The complex type N-glycans are metabolized in this conventional pathway as follows (7). The oligosaccharide chain is cleaved from the glycoprotein by endo-β-N-acetylhexosaminidase (EC 3.2.1.96), belonging to glycoside hydrolase family (GH)18 (11). The oligosaccharide is transported into the periplasmic space by a starch utilization system (Sus)-like system (SusC and SusD) and is sequentially degraded by α-sialidase (EC 3.2.1.18), GH2 β-galactosidase (EC 3.2.1.23), GH20 β-N-acetylxosaminidase (EC 3.2.1.52), and GH92 α-mannosidase (EC 3.2.1.24) (7). The remaining β-1,4-d-mannosyl-N-acetyl-d-glucosamine (ManGlcNAC₃) is converted by GH2 β-mannosidase (EC 3.2.1.25) into d-mannose and GlcNac (7). The resultant monosaccharides are transported into the cytoplasm, are phosphorylated by ATP-dependent carbohydrate kinases, and enter glycolysis or amino sugar metabolism (12). The genes encoding the transporter system and glycoside hydrolases, except for endo-β-N-acetylhexosaminidase, constitute a gene cluster in the genome of B. thetaiotaomicron

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† This article contains supplemental Table S1 and Figs. S1 and S2.

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and are well conserved in the genus *Bacteroides* (see Fig. 1A) (7, 13–17).

Several intestinal anaerobes such as *Bifidobacterium longum* (18) and *Clostridium perfringens* (19) possess a unique metabolic pathway that efficiently uses the energy of ATP, without an ATP-dependent carbohydrate kinase, to process host-derived O-glycans. In the metabolic pathway, a GH112 β-1,3-d-galactosyl-N-acetyl-d-hexosamine phosphorylase (EC 2.4.1.211) directly produces α-d-galactose 1-phosphate from galacto-N-biose (β-1,3-d-galactosyl-N-acetyl-d-galactosamine) and lacto-N-biose I (β-1,3-d-galactosyl-N-acetyl-d-glucosamine) as degradation products from mucin O-glycans and human milk oligosaccharides, respectively (18, 19). In addition, *Bacteroides fragilis* and *Ruminococcus albus*, a member of the human colon microbiota and a ruminal bacterium, respectively, possess metabolic pathways for plant polysaccharide breakdown (18, 19). In addition, *Bacteroides thetaiotaomicron* (20) and *B. thetaiotaomicron* (21). These pathways including the phosphorolysis that enables the anaerobes to produce phosphorylated sugars directly without consuming ATP are energetically efficient, in comparison with the conventional metabolic pathway containing sequential glycoside hydrolase-catalyzed reactions, because only three molecules of ATP are available via the glycolytic pathway from glucose 6-phosphate. However, there has been no report of energy-efficient N-glycan metabolism via phosphorylase.

In this study, we identified a gene cluster including a gene encoding a novel GH130 phosphorylase, BT1033, for the energy-efficient metabolism of complex type N-glycans in the genome of the human gut bacterium *B. thetaiotaomicron* VPI-5482. BT1033 phosphorylase is a key enzyme in a new metabolic pathway containing sequential glycoside hydrolase-catalyzed reactions, because only three molecules of ATP are available via the glycolytic pathway from glucose 6-phosphate. However, there has been no report of energy-efficient N-glycan metabolism via phosphorylase.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis**—Similarity searches were performed at the Swiss Institute of Bioinformatics using the basic local alignment search tool (BLAST) network service. The National Center for Biotechnology Information (NCBI) BLASTP tool was used to search the Swiss-Prot/TrEMBL database (22). Prediction of protein localization and signal peptide was conducted using PSORTb version 3.0.2 (23) and SignalP 4.1 server (24), and signal peptide was conducted at the Swiss Institute of Bioinformatics using the basic local alignment search tool (BLAST) network service. The National Center for Biotechnology Information (NCBI) BLASTP tool was used to search the Swiss-Prot/TrEMBL database (22). Prediction of protein localization and signal peptide was conducted using PSORTb version 3.0.2 (23) and SignalP 4.1 server (24), respectively.

**Cloning, Expression, and Purification**—The gene encoding BT1033 (GenBank™ accession number AAO76410.1) was amplified by PCR from genomic DNA of *B. thetaiotaomicron* VPI-5482, using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with the following oligonucleotides based on the genome sequence (GenBank™ accession number AE015928 (2)): 5′-ggattccatatgaataagttacct-3′ as the forward primer containing an NdeI site (underlined) and 5′-ttctcagagata-gctgctgttgg-3′ as the reverse primer containing an XhoI site (underlined). The amplified gene was purified using a FastGene Gel/PCR extraction kit (Nippon Genetics Co., Tokyo, Japan), digested with NdeI and XhoI (New England Biolabs, Beverly, MA), and inserted into pET24a (+) (Novagen, Madison, WI) to encode a His6 tag fusion at the C terminus of the recombinant protein. The expression plasmid was propagated in *Escherichia coli* DH5α (Toyobo), purified by a FastGene Plasmid Mini Kit (Nippon Genetics Co.), and verified by sequencing (Operon Biotechnologies, Tokyo, Japan). An *E. coli* BL21 (DE3) (Novagen) transformant harboring the expression plasmid was grown at 37 °C in 200 ml of Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 50 µg/ml kanamycin, until the absorbance reached 0.6 at 600 nm. The expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside and continued at 18 °C for 24 h. The cells were harvested by centrifugation at 20,000 × g for 20 min and suspended in 50 mM HEPES-NaOH buffer (pH 7.0) containing 500 mM NaCl (buffer A). The suspended cells were disrupted by sonication (Branson sonifier 250A; Branson Ultrasonics, Emerson Japan, Kanagawa, Japan). The supernatant collected by centrifugation at 20,000 × g for 20 min was applied to a HisTrap HP column (GE Healthcare), equilibrated with buffer A containing 10 mM imidazole, using AKTA Prime (GE Healthcare). After washing with buffer A containing 22 mM imidazole and subsequent elution using a 22–400 mM imidazole linear gradient in buffer A, fractions containing recombinant protein (BT1033) were pooled, dialyzed against 10 mM HEPES-NaOH buffer (pH 7.0), and concentrated (AMICON Ultra-15 filter; Millipore, Billerica, MA). The protein concentration was determined spectrophotometrically at 280 nm using a theoretical extinction coefficient of ε = 37,823 M⁻¹ cm⁻¹, based on the amino acid sequence (25).

The molecular mass of purified BT1033 was estimated by SDS-PAGE (Mini-PROTEAN Tetra electrophoresis system; Bio-Rad) and by gel filtration (HiLoad 26/600 Superdex, 200 pg; GE Healthcare) equilibrated with 10 mM HEPES-NaOH buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 0.5 ml/min, using Marker Proteins for molecular Weight Determination on High Pressure Liquid Chromatography (Oriental Yeast Co., Tokyo, Japan) as standards.

**Measurement of Synthetic Activity**—The synthetic activity was routinely determined by measuring the increase in inorganic phosphate (Pi) using a reaction mixture containing 10 mM α-Man1P (α-Man1P bis(cyclohexylammonium) salt: Sigma-Aldrich) and 10 mM GlcNAc (Wako Pure Chemicals, Osaka, Japan) in 40 mM sodium acetate buffer (pH 5.5) at 30 °C following the method of Lowry and Lopez (26) as described previously (27). One unit of the synthetic activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of Pi per min under the above conditions.

**Acceptor Specificity Analysis**—To investigate the acceptor specificity of BT1033 (85 µM), the synthetic reaction was performed under the standard conditions described above, by substituting GlcNAc with putative carbohydrate acceptors (D-altrose, D-fructose, D-glucosamine, D-glucose, isomaltose, kojibiose, lactose, lactulose, maltose, D-mannose, melibiose, methyl-α-D-glucoside, methyl-β-D-glucoside, nigerose, L-rhamnose, D-ribose, sophorose, sucrose, D-talose, trehalose, xylobiose, D-xylene (Wako Pure Chemicals), N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, D-allohe, 1,5-anhydro-D-
glucitol, 2-deoxy-D-glucose, D-galactosamine, D-galactose, D-galacturonic acid, gentiobiose, α-D-glucose 1-phosphate, D-glucose 6-phosphate, D-lxosy, 3-O-methyl-D-glucose (Sigma-Aldrich), D-arabinose, L-arabinose, cellobiose, β-D-glucose 1-phosphate, D-glucuronic acid, gentiobiose (Tokyo Chemical Industry, Tokyo, Japan), β-1,4-mannobiose (Megazyme, Bray, Ireland), N,N′-diacetylchitobiose (β-1,4N-acetyl-d-glucosaminyl-N-acetyl-D-glucosamine (GlcNAc₂)), and laminaribiose (Seikagaku Bio-business, Tokyo, Japan) for 2 h. The reaction mixture was spotted on a TLC plate (Kieselgel 60 F254; Merck), and the plate was developed with a mobile phase of 80% acetonitrile in water. The TLC plates were soaked in 5% sulfuric acid-methanol solution and heated in an oven until the bands were visible.

Structural Determination—Reaction products for structural determination were generated in 500 μl of reaction mixture (pH 5.5) containing BT1033 (1.5 and 3.7 μM GlcNAc and GlcNAc₂, respectively), 50 mM α-Man1P, and 50 mM GlcNAc or GlcNAc₂. The reaction mixtures were incubated at 30 °C for 24 h, followed by desalting using Amberlite MB-3 (Organo, Tokyo, Japan). The reaction products were purified using an HPLC system (Prominence; Shimadzu, Kyoto, Japan) equipped with a Shodex Asahipak NH2P-50 4E column (4.6-mm internal diameter × 25 cm; Showa Denko KK, Tokyo, Japan) at 30 °C under a constant flow (1.0 ml/min) of 75% acetonitrile in water as the mobile phase. Fractions containing the reaction products were collected, followed by lyophilization. The amounts of products obtained were 4 and 5 mg from GlcNAc and GlcNAc₂ as the acceptors, respectively. One-dimensional (1H and 13C) and two-dimensional (double-quantum filtered correlation spectroscopy, heteronuclear single-quadrature coherence, and heteronuclear multiple-bond correlation) NMR spectra of the product were acquired in D₂O with 2-methyl-2-propanol as an internal standard using a Bruker Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany). Proton signals were assigned based on the double-quantum filtered correlation spectra. 13C signals were assigned using the heteronuclear single-quadrature coherence spectra, based on the assignment of the proton signals. The linkage position of each disaccharide was determined by detecting the inter-ring cross-peaks in each heteronuclear multiple-bond correlation spectrum.

Measurement of Phosphorolytic Activity—The substrates for phosphorolysis of BT1033 were generated in 5 ml of reaction mixture (pH 5.5) containing 15 μM BT1033, 500 mM α-Man1P, and 500 mM GlcNAc or 500 mM GlcNAc₂. After incubation at 30 °C for 24 h, the reaction mixtures were desalted using Amberlite MB-3 and loaded onto a Toyopearl HW-40S column (50-mm internal diameter × 950 mm; Tosoh) equilibrated with 2-hydroxyethylaminotris(hydroxymethyl)methane-HCl (pH 7.0) containing 250 mM sucrose, 250 mM GlcNAc, 25 mM Pₐ, and 20 μg/ml D-glucose 1,6-bisphosphate (Sigma-Aldrich), 33 μg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose 1-phosphate, 0.34 mg/ml α-phosphoglucomutase (Sigma-Aldrich), 0.37 mg/ml D-glucose
6-phosphate isomerase, 0.23 mg/ml D-mannose-6-phosphate isomerase, 2.4 mg/ml D-mannose-6-phosphate isomerase, and 83 μg/ml β-phosphomannomutase were prepared as described previously (28, 29). The concentration of ManGlcNAc was monitored by a HPLC system (Prominence; Shimadzu) equipped with a Shodex Asahipak NH2P-50 4E column at 30 °C under a constant flow (1.0 ml/min) of 75% acetonitrile in water as the mobile phase. The reaction mixture was treated with 0.3 mg/ml invertase (Sigma-Aldrich) at 30 °C for 20 h to eliminate remaining sucrose, followed by desalting using Amberlite MB-3 (Organ). The synthesized ManGlcNAc was purified by a Toyopearl HW-40S column (50-mm internal diameter × 950 mm; Tosoh) equilibrated with distilled water at a flow rate of 1.0 ml/min. Fractions containing the reaction product were collected, followed by lyophilization. The amount of ManGlcNAc obtained was 22 mg.

RESULTS

Prediction of the Enzymatic Function of BT1033—A gene cluster involved in complex type N-glycan metabolism was identified in the genome of B. thetaiotaomicron of focus in this article. The cluster is involved in the conventional metabolism by sequential glycoside hydrolase-catalyzed reactions in the genomes of the genus Bacteroides. The gene cluster involves in the metabolic pathway where a phosphorylase (gray rectangle) participates. Dotted rectangle, the gene cluster of B. thetaiotaomicron of focus in this article. The cluster contains nine unidirectionally transcribed ORFs (Fig. 1B) that encode four glycoside hydrolases (GH92 -mannosidase (BT1032), GH20 -N-acetylgalactosaminidase (BT1035), exo-α-N-acetylhexosaminidase (BT1036), and GH18 -N-acetylhexosaminidase (BT1037)).
end-β-N-acetylglucosaminidase (BT1038)), a major facilitator superfamily transporter (BT1034), a hypothetical protein with unknown function (BT1037), an outer membrane lipoprotein (BT1039) and a TonB-dependent oligosaccharide transporter (BT1040) constituting a Sus-like protein, and a GH130 phosphorylase (BT1033). The sequence analysis including prediction of protein localization based on PSORTb version 3.0.2 (23) and signal peptide identification using version SignalP 4.1 (24) suggests that BT1033 plays a role in the intracellular phosphorylation of ManGlcNAc liberated from complex type N-glycans by sequential glycoside hydrolase-catalyzed reactions in the periplasmic space and transported into the cytoplasm. However, there have been no reports on the N-glycans metabolism that a phosphorylase participates. In this study, BT1033 was recombinantly expressed in E. coli BL21 (DE3) to investigate the detailed enzymatic properties, as described below.

Preparation of Recombinant BT1033—Recombinant BT1033 was purified by nickel chelate affinity chromatography with a yield of 24 mg from the cell lysate of a 200-ml culture. Purified BT1033 migrated in SDS-PAGE as a single protein band with an estimated size of 35 kDa in agreement with the theoretical molecular mass of 37,823. However, the molecular mass was estimated by gel filtration to be 147 kDa, indicating that BT1033 is a molecular mass of 37,823. However, the molecular mass was estimated size of 35 kDa in agreement with the theoretical yield of 24 mg from the cell lysate of a 200-ml culture. Purified was purified by nickel chelate affinity chromatography with a

Preparation of Recombinant BT1033—Recombinant BT1033 was purified by nickel chelate affinity chromatography with a yield of 24 mg from the cell lysate of a 200-ml culture. Purified BT1033 migrated in SDS-PAGE as a single protein band with an estimated size of 35 kDa in agreement with the theoretical molecular mass of 37,823. However, the molecular mass was estimated by gel filtration to be 147 kDa, indicating that BT1033 is a homotetramer in solution, whereas R. albus GH130 β-1,4-d-mannosyl-d-glucose phosphorylase (EC 2.4.1.281) and β-1,4-d-mannooligosaccharide phosphorylase (EC 2.4.1.-) showed 39 and 70% sequence similarities with BT1033, respectively, have been reported to be homodimeric and homoheaxeric, respectively (21).

Synthetic Reaction Catalyzed by BT1033—The acceptor specificity in the synthetic reaction was examined using various carbohydrate acceptor candidates (see “Experimental Procedures”) together with α-Man1P as the donor. BT1033 utilized GlcNAC and GlcNAC2 as the suitable acceptors, with specific activities of 41 and 8 units/mg, respectively. Each synthetic reaction gave a single product from GlcNAC and GlcNAC2. The products from GlcNAC and GlcNAC2 were identified by 1H and 13C NMR spectroscopic analysis to be the corresponding β-1,4-d-mannopyranosyl-β-1,4-N-acetyl-d-glucosamine (ManGlcNAc) (supplemental Fig. S1) and β-1,4-d-mannopyranosyl-β-1,4-N-acetyl-d-glucosaminyl-β-1,4-N-acetyl-d-glucosamine (ManGlcNAc2) (supplemental Fig. S2), respectively. In addition BT1033 showed weak synthetic activities with d-glucose and d-mannose. Therefore, the kinetic parameters for the four acceptors (GlcNAC, GlcNAC2, d-glucose, and d-mannose) were determined to investigate the acceptor preference of BT1033 in the presence of α-Man1P as the donor (Table 1). The $K_m$ value for GlcNAC was in the millimolar range and was 12 times lower than that for GlcNAC2. However, the $k_{cat}$ value for GlcNAC was in the same range with that for GlcNAC2. These results indicate that the catalytic efficiency ($k_{cat}/K_m$) values for GlcNAC and GlcNAC2, mainly depend on the $K_m$ values. In addition, the fact that the $k_{cat}/K_m$ value for GlcNAC was 140–200 times greater than those for the other acceptors indicates that GlcNAC is the most effective acceptor for BT1033. Based on the acceptor specificity and considering that the kinetic parameters for α-Man1P are in the same ranges as those of the other invertible phosphorylases for their specific donors (20, 21, 27, 30–36), we here propose 4-O-β-d-mannopyranosyl-N-acetyl-d-glucosamine-phosphatase α-d-mannosyltransferase as the systematic name and β-1,4-d-mannosyl-N-acetyl-d-glucosamine phosphorylase as the short name for BT1033 (Fig. 2).

Phosphorolytic Reaction Catalyzed by BT1033—BT1033 catalyzed the phosphorylisis of ManGlcNAc and ManGlcNAc2 with inversion of the anomeric configuration to release α-Man1P and GlcNAC or GlcNAC2, respectively. The specific activity values on ManGlcNAc and ManGlcNAc2 were 258 and 30 units/mg of protein, respectively. In addition, BT1033 did not cleave the substrates in the absence of P$i$. Double reciprocal plots of the initial velocities against various initial concentrations of ManGlcNAc and P$i$ gave a series of lines intersecting at a single point (Fig. 3). These results indicate that the phosphorolytic reactions on ManGlcNAc follow a sequential Bi Bi mechanism, similar to inverting phosphorylases (21, 27, 30–35). In addition, BT1033 did not phosphorolyze β-1,4-d-mannosyl-d-glucose and β-1,4-mannooligosaccharides (degree of polymerization of 2–6), known substrates of the GH130 β-1,4-d-mannosyl-d-glucose phosphorylase (20, 21), and β-1,4-mannooligosaccharide phosphorylase (21), respectively. The kinetic parameters for ManGlcNAc and ManGlcNAc2 are summarized in Table 2. The $K_m$ value for ManGlcNAc was 58 times greater than that for ManGlcNAc2. In addition, the parameters for ManGlcNAc are in the same ranges with those of the other inverting phosphorylases (21, 27, 30–35), indicating that ManGlcNAc is the true substrate of BT1033.

Basic Properties of BT1033—BT1033 was stable up to 55 °C during 15 min of incubation (Fig. 4A) and in the range of pH 4.5–10.5 at 4 °C for 24 h (Fig. 4B). The optimum pH for both the phosphorolytic and synthetic reactions was pH 5.5 (Fig. 4C).

Synthesis of ManGlcNAc from Sucrose and GlcNAC—A one-pot enzymatic reaction by concomitant actions of sucrose phosphorylase (EC 2.4.1.7), α-phosphoglucomutase (EC 5.4.2.2), d-glucose 6-phosphate isomerase (EC 5.3.1.9), d-mannose-6-phosphate isomerase (EC 5.3.1.8), α-phosphomannomutase (EC 5.4.2.8), and BT1033 was demonstrated to synthesize ManGlcNAc from sucrose and GlcNAC as inexpensive starting materials (Fig. 5A). As shown in Fig. 5B, synthesis of ManGlcNAc was observed along with a decrease in GlcNAC at
the early stage of the reaction. After 276 h of reaction, the product formation reached a plateau, and 58 mM ManGlcNAc was produced in the reaction mixture.

**DISCUSSION**

*Enzymatic Function of BT1033—*BT1033 has unique substrate specificity in the phosphorolytic reaction; it recognizes ManGlcNAc and ManGlcNAc₂ as the substructures of the N-glycans. The amino acid sequence of BT1033 shows 42, 39, and 79% similarities with those of *B. fragilis* and *R. albus* β-1,4-mannosyl-β-glucose phosphorolyses and *R. albus* β-1,4-mannooligosaccharide phosphorolase belonging to GH130 (supplemental Table S1), respectively, which are involved in the catabolism for plant polysaccharide β-1,4-mannan (20, 21). To date, no three-dimensional structures of reported GH130 phosphorolyses have been solved. In addition, there has been no report of enzymatic activities of four putative GH130 glycosidases from *Bacteroides ovatus*, *B. thetaiotaomicron*, *Parabacteroides distasonis*, and *Thermotoga maritima*, of which the three-dimensional structures are available (Protein Data Bank codes 3QC2, 3R67, 3TAW, and 1VKD, respectively). Furthermore, information regarding the amino acid residues involved in the substrate recognition or catalysis is not available because the substrate–enzyme complex structure has not been solved for GH130. However, together with the fact that BT1033 showed no phosphorolytic activity toward β-1,4-mannosyl-β-glucose, the substrate for the reported GH130 β-1,4-mannosyl-β-glucose phosphorolysis (20, 21), the much lower *k₅/Kₐ* values of β-glucose and β-mannose substituted at C2 position of GlcNAc as an suitable acceptor in the synthetic reaction with strict β-1,4-regioselectivity clearly suggest that BT1033 recognizes the C2 N-acetyl group at subsite +1. Regarding the recognition of the C2 N-acetyl group of the acceptor molecule at subsite +1, a GH94 N,N’-diacetylchitobiose phosphorylase (EC 2.4.1.280) from *Vibrio proteolyticus* has been reported to recognize the C2 N-acetyl group of GlcNAc at subsite +1 by interaction with the methyl group in a small hydrophobic pocket formed by Cys-493 and Val-631. In contrast, the space is occupied by a well conserved bulky Tyr residue in case of GH94 cellobiose phosphorylase (EC 2.4.1.20) (37, 38). In addition, BT1033 did not utilize N-acetyl-β-galactosamine with an axial hydroxyl group at the C4 position as the acceptor in the synthetic reaction, clearly indicating that the equatorial C4 hydroxyl group of GlcNAc is essential for acceptor binding at subsite +1 in the enzyme. GlcNAc₂ acted as the acceptor in the synthetic reaction, suggesting that BT1033 possesses a moderately large binding space to accommodate the oligosaccharide acceptor. Therefore, addition of an excess of the enzyme was confirmed to cause elongation of successive β-mannose residue at
**β-1,4-β-Mannosyl-N-acetyl-β-glucosamine Phosphorylase**

> FIGURE 4. Effect of pH and temperature on the activity and stability of BT1033. A, stability of 7 μM BT1033 at the temperature range of 30–90 °C for 15 min. B, pH stability of 11 μM BT1033 at 4 °C for 24 h. C, pH activity dependence of the phosphorolysis and synthesis of ManGlcNAc by 212 nM BT1033 in 40 mM sodium citrate (pH 3.0–5.5), bis(2-hydroxyethyl)aminomethan(methoxymethyl) methanol-HCl (pH 5.5–7.0), HEPES-NaOH (pH 7.0–8.5), and glycine-NaOH (pH 8.5–10.5). The closed and open symbols represent synthetic and phosphorolytic activities, respectively.

> FIGURE 5. One-pot enzymatic synthesis of ManGlcNAc from sucrose and GlcNAc. A, reaction scheme for the synthesis of ManGlcNAc. Sugars used as the starting materials are boxed. Enzymes used are represented using gray shading. SP, sucrose phosphorylase; PGM, α-phosphoglucomutase; G6PI, β-glucose 6-phosphate isomerase; M6PI, β-mannose 6-phosphate isomerase; PMM, α-phosphomannomutase; ManGlcNAcP, β-1,4-β-mannosyl-N-acetyl-β-glucosamine phosphorylase. B, HPLC monitoring of the one-pot enzymatic reaction from sucrose and GlcNAc as the starting materials. The remaining sucrose in the reaction mixture was removed by incubating with invertase, allowing the quantification of ManGlcNAc. Inset, time course of the ManGlcNAc concentration calculated from the peak areas.

the nonreducing end of the acceptor molecule, although β-mannose was not a suitable acceptor at the subsite +1.

It should be noted that the enzymatic properties of BT1033, such as strict regioselectivity and acceptor specificity, enable the enzymatic syntheses of ManGlcNAc and ManGlcNAc P, which are common core structures shared by all N-glycans such as high mannose, complex, and hybrid types (10). Stereoase formation of the β-mannosidic linkages is a considerable challenge in synthetic glycochemistry because the vicinal C2 hydroxyl group blocks access to the β-face because of its steric and polar effects (39). Therefore, the new synthetic method for core structure of N-glycans employing the synthetic reaction of this unique mannoside phosphorylase would be a strong tool for the efficient preparation of various N-glycans and glycomucojatures used in the research field of glycobiology and glycotherapy. In addition, ManGlcNAc was synthesized by a one-pot enzymatic reaction from sucrose and GlcNAc as the starting materials, which are industrially prepared and are available at reasonable cost (Fig. 5A). The details of ManGlcNAc synthesis are as follows: (i) sucrose is phosphorolyzed into α-D-glucose 1-phosphate and fructose by sucrose phosphorylase; (ii) α-D-glucose 1-phosphate is converted into α-Man1P via β-D-fructose 6-phosphate, β-D-fructose 6-phosphate, and β-mannose 6-phosphate by the sequential reactions of α-phosphoglucomutase, β-D-glucose 6-phosphate isomerase, α-phosphomannomutase, and α-phosphomannomutase, and (iii) ManGlcNAc is generated from α-Man1P and GlcNAc by BT1033 together with the release of P γ. Because P γ is recycled in the reaction, the overall reaction can be described as the transformation of sucrose and GlcNAc to ManGlcNAc and fructose by the concomitant action of the six enzymes in the presence of catalytic amounts of P γ. Notably, this strategy circumvents the addition of costly α-Man1P and is compatible with large scale production. Moreover, starch and cellulose/cellobiose/cellobextrin biomass are available as the starting materials by substituting sucrose phosphorylase with starch phosphorylase (EC 2.4.1.1) and cellobiose/cellobextrin phosphorylase (EC 2.4.1.20/EC 2.4.1.49), respectively, which catalyze the release of α-D-glucose 1-phosphate by the phosphorylase. This one pot enzymatic approach using the inexpensive starting materials can be extended to include the production of a variety of valuable β-mannosides using β-1,4-β-mannosyl-β-glucose and β-1,4-mannooligosaccharide phosphorylases belonging to GH130 with known acceptor specificities in the synthetic reaction (21).

**Physiological Role of BT1033**—The complex type N-glycans had been considered to be metabolized in a conventional pathway where β-mannosidase and ATP-dependent hexokinase participate. In this study, a gene cluster including a gene encoding a β-1,4-β-mannosyl-N-acetyl-β-glucosamine phosphorylase (BT1033) for the efficient preparation of various N-glycans and glycomucojatures used in the research field of glycobiology and glycotherapy. In addition, ManGlcNAc was synthesized by a one-pot enzymatic reaction from sucrose and GlcNAc as the starting materials, which are industrially prepared and are available at reasonable cost (Fig. 5A). The details of ManGlcNAc synthesis are as follows: (i) sucrose is phosphorolyzed into α-D-glucose 1-phosphate and fructose by sucrose phosphorylase; (ii) α-D-glucose 1-phosphate is converted into α-Man1P via β-D-fructose 6-phosphate, β-D-fructose 6-phosphate, and β-mannose 6-phosphate by the sequential reactions of α-phosphoglucomutase, β-D-glucose 6-phosphate isomerase, α-phosphomannomutase, and α-phosphomannomutase, and (iii) ManGlcNAc is generated from α-Man1P and GlcNAc by BT1033 together with the release of P γ. Because P γ is recycled in the reaction, the overall reaction can be described as the transformation of sucrose and GlcNAc to ManGlcNAc and fructose by the concomitant action of the six enzymes in the presence of catalytic amounts of P γ. Notably, this strategy circumvents the addition of costly α-Man1P and is compatible with large scale production. Moreover, starch and cellulose/cellobiose/cellobextrin biomass are available as the starting materials by substituting sucrose phosphorylase with starch phosphorylase (EC 2.4.1.1) and cellobiose/cellobextrin phosphorylase (EC 2.4.1.20/EC 2.4.1.49), respectively, which catalyze the release of α-D-glucose 1-phosphate by the phosphorylase. This one pot enzymatic approach using the inexpensive starting materials can be extended to include the production of a variety of valuable β-mannosides using β-1,4-β-mannosyl-β-glucose and β-1,4-mannooligosaccharide phosphorylases belonging to GH130 with known acceptor specificities in the synthetic reaction (21).
complex type N-glycans. One notable feature of the new N-glycan metabolic pathway is that these intestinal anaerobes efficiently use the energy of ATP, because the D-mannose residue of ManGlcNAc can be directly phosphorylated without consuming ATP by ATP-dependent carbohydrate kinase. Hence, the energy-efficient strategy for N-glycan metabolism in which a unique β,1,4-D-mannosyl-N-acetyl-D-glucosamine phosphorylase participates would have provided intestinal anaerobic bacteria with evolutionary advantages because anaerobic respiration is in general less energy-efficient than aerobic respiration.

Conclusions—A gene cluster involved in complex type N-glycan metabolism was identified in the genome of *B. thetaiotaomicron* VPI-5482. It was demonstrated that BT1033 encoded in the gene cluster catalyzed the reversible phosphorylisis of ManGlcNAc in a typical sequential Bi Bi mechanism. This is the first report of a metabolic pathway for complex type N-glycans in which a unique β,1,4-D-mannosyl-N-acetyl-D-glucosamine phosphorylase participates. In addition, several intestinal anaerobes were also identified to possess the similar metabolic pathway for the N-glycans. One notable feature of the new metabolic pathway for N-glycans is that these intestinal anaerobes efficiently use the energy of ATP, in comparison with a conventional pathway in which β-mannosidase and ATP-dependent hexokinase participate, because it is possible to directly phosphorylate the D-mannose residue of ManGlcNAc to enter glycolysis.

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