β-Glucoside Kinase (BglK) from *Klebsiella pneumoniae*

PURIFICATION, PROPERTIES, AND PREPARATIVE SYNTHESIS OF 6-PHOSPHO-β-D-GLUCOSIDES

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ATP-dependent β-glucoside kinase (BglK) has been purified from cellobiose-grown cells of *Klebsiella pneumoniae*. In solution, the enzyme (EC 2.7.1.85) exists as a homotetramer composed of non-covalently linked subunits of *M*ₐ ~ 33,000. Determination of the first 28 residues from the N terminus of the protein allowed the identification and cloning of *bglK* from genomic DNA of *K. pneumoniae*. The open reading frame (ORF) of *bglK* encodes a 297-residue polypeptide of calculated *M*ₐ ~ 32,697. A motif of 7 amino acids (AFDYGIGGT) near the N terminus may constitute the ATP-binding site, and residue changes D7G and G9A yielded catalytically inactive proteins. BglK was progressively inactivated (*t½ ~ 19 min*) by *N*-ethylmaleimide, but ATP afforded considerable protection against the inhibitor. By the presence of a centrally located signature sequence, BglK can be assigned to the ROK (Repressor, ORF, Kinase) family of proteins.

Preparation of *His*<sup>6</sup>*BglK* by nickel-nitrilotriacetic acid-agarose chromatography provided high purity enzyme in quantity sufficient for the preparative synthesis (200–500 mg) of ten 6-phospho-β-D-glucosides, including cellobiose-6-P, gentiobiose-6-P, celllobitol-6-P, salicin-6-P, and arbutin-6-P. These (and other) derivatives are substrates for phospho-β-glucosidase(s) belonging to Families 1 and 4 of the glycosylhydrolase superfamilies.

Remarkably, and in contrast to other glycosylhydrolases described thus far, members of Family 4 require both a dinucleotide (NAD<sup>+</sup>) and a divalent metal ion (Mn<sup>2+</sup>, Fe<sup>2+</sup>, Co<sup>2+</sup>, or Ni<sup>2+</sup>) for activity. The one or more functions of these unique cofactors are presently unknown, and the catalytic mechanism for cleavage of the O-glycosyl linkage in their substrates has yet to be established.

The phosphoenolpyruvate-dependent sugar-phosphotransferase system (P-enolpyruvate:PTS),<sup>1</sup> was first described by Roseman and colleagues in *Escherichia coli* in 1964 (1). This multicomponent group translocation system is now recognized as the primary route for entry, and simultaneous phosphorylation, of carbohydrates by many bacterial species from both Gram-negative (2, 3) and Gram-positive genera (4, 5). Hexose monophosphates may immediately enter the central energy-generating pathways, but prior to their dissimilation, accumulated disaccharide phosphates must first be hydrolyzed by sugar-specific disaccharide-phosphate hydrolases. Of the latter inducible enzymes, phospho-β-galactosidase (EC 3.2.1.85) and phospho-β-glucosidase (EC 3.2.1.86) have received considerable attention (6, 7). By sequence-based alignment these enzymes are assigned to Family 1 of the 87-member superfamily of glycosylhydrolases (8).<sup>2</sup>

Until recently, microbial phosphoglycosylhydrolases were found only in Family 1 of glycosylhydrolases. However, studies begun in our laboratory (9) and, subsequently, in other institutions, have identified a novel group of phosphoglycosylhydrolases in an increasing number of bacterial species, including *Bacillus subtilis* (10, 11), *Fusobacterium mortiferum* (12), *E. coli* (13, 14), *Klebsiella pneumoniae* (15), and *Thermotoga maritina* (16). By their inherent instability, oligomeric structure, sequence homology, and conserved signature pattern, these enzymes are assigned to Family 4 of glycosylhydrolases.<sup>2</sup>

The carbohydrate-active enzymes, available at afmb.cnrs-mrs.fr/, encode a 297-residue polypeptide of calculated *M*ₐ ~ 33,000. Determination of the first 28 residues from the N terminus of the protein allowed the identification and cloning of *bglK* from genomic DNA of *K. pneumoniae* (15), and *Listeria monocytophagia* (16). By their inherent instability, oligomeric structure, sequence homology, and conserved signature pattern, these enzymes are assigned to Family 4 of glycosylhydrolases.<sup>2</sup>

The recently sequenced genomes of two *Listeria* species, *L. monocytogenes* EGD-e and *L. innocua* CLIP 11262, contain homologous genes (*lmo2764* and *lin2907*, respectively) that encode a 294-residue polypeptide (*M*ₐ ~ 32,200) that exhibits ~58% amino acid identity with BglK. The protein encoded by the two genes exhibits β-glucoside kinase activity and cross-reacts with polyclonal antibody to *His*<sup>6</sup>*BglK* from *K. pneumoniae*. The location of *lmo2764* and *lin2907* within a β-glucoside (cellobiose):phosphotransferase system operon may presage both enzymatic (kinase) and regulatory functions for the BglK homolog in *Listeria* species.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EBI Data Bank with accession number(s) AY093395.<sup>‡</sup> To whom correspondence should be addressed: NIDCR, National Institutes of Health, Bldg. 30, Rm. 528, 30 Convent Dr. MSC-4350, Bethesda, MD 20892. Tel.: 301-496-4083; Fax: 301-402-0396; E-mail: jthompson@dir.nidcr.nih.gov.

1 The abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; PTS, phosphotransferase system; BglK, β-glucoside kinase; P-β-glucoside, 6-phospho-β-D-glucoside; Ni<sup>2+</sup>-NTA, nickel-nitrilotriacetic acid; Hi/Tria, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ORF, open reading frame; G6PDH, glucose-6-phosphate dehydrogenase; PK, pyruvate kinase; LDH, L-lactate dehydrogenase; MES, 4-morpholinethanesulfonic acid; FAB, fast atom bombardment; Glec6P, glucose 6-phosphate; pgbA, phospho-glucosidase; HSS, high speed supernatant fluid; CelF, cellobiose-6-phosphate hydrolase; ROK, repressor, ORF, kinase; NEM, N-ethylmaleimide; GlK, glucokinase.

2 P. M. Coutinho and B. Henriantz (1999) Carbohydrate-Active Enzymes, available at afmb.cnrs-mrs.fr/~cazy/CAZYindex.html.
phoglycosylhydrolyase activities. The former chromogenic substrates (and structurally similar alkyl and aroyl glycoside phosphates) have usually been prepared by phosphorylation of the parent glycosides at the single primary -OH group with 2-cyanoethyl phosphate or phosphorous oxychloride (17-19). Unfortunately, these non-selective phosphorylating agents cannot be used for synthesis of the disaccharide-6'-P products of the P-enolpyruvate-PTS, because the presence of two primary -OH groups yields a tripartite mixture of the -6-P, -6'-P, and -6,6'-P2 derivatives. Regiospecific chemical syntheses of disaccharide 6'-phosphates would entail the addition and subsequent removal of protecting groups, together with potentially laborious methods for purification of the derivatives. It is perhaps in light of these difficulties that compounds such as cellobiose-6'-phosphate and maltose-6'-phosphate are not commercially available, and it was for these reasons that we sought enzymatic routes for the biosynthesis of the α- and β-conformers of disaccharide monophosphates. Fortuitously, during studies of PTS functions in permeabilized cells of K. pneumoniae, we discovered that, although low pH caused inactivation of intracellular phospho-α-glucosidase, the α-glucoside-specific PTS remained operative under the same conditions. This serendipitous finding permitted the synthesis and facile isolation of a wide variety of phospho-α-glucoside products of the PTS, including maltose-6'-P and the 6-phospho-derivatives of sucrose and its five linkage isomers (20).

As a potential route for the biosynthesis of phospho-β-glucosides, we turned our attention to the report some 30 years ago by Palmer and Anderson (21) of an ATP-dependent β-glucoside kinase present in K. pneumoniae. Although only partially purified, the enzyme preparation of Palmer and Anderson catalyzed the in vitro phosphorylation of several β-glucosides and, indeed, a small quantity of cellobiose-6'-phosphate was prepared by these investigators.

In the present communication we describe the purification, substrate specificity, and kinetic parameters of β-glucoside kinase (BglK, EC 2.7.1.185) from K. pneumoniae. The chromosomal gene bglK has been cloned, and catalytically active His6-BglK has been purified from a high expression system by NHEC-NTA-agarose chromatography. The availability of His6-BglK in quantity (and high purity) has allowed the first preparative synthesis of 10 phospho-β-glucosides. The biosynthesis, method of isolation, and some of the physicochemical properties of these novel phospho-β-glucosylhydrolyase substrates are presented herein. By sequence-based alignment we show that BglK of K. pneumoniae can be assigned to the ROK (Repressor, ORF, Kinase) family of proteins (22) and that a homolog of BglK is encoded within the -PTS operon in two species of Listeria.

**Experimental Procedures**

**Materials**

Salcin and gentiobiose were obtained from Pfannstiel Laboratories, and thio-D-glucose was purchased from Toronto Research Chemicals, Inc. Other carbohydrates and reagents, including dinucleotides, ATP, P-enolpyruvate, DEAE-TrisAcryl M, and ATP-agarose (4% cross-linked, nine spacer atoms) were purchased from Sigma Chemical Co. Other materials and suppliers, included Ni2+-NTA-agarose (Qiagen), Ultrogel-AAC (Sepracor), and Polygram cell 400 thin-layer microcrystalline cellulose (Amersham Biosciences Multiphor flat-bed electrophoresis unit, using precast AmphiPAG layers (pH range, 3.5–9.5) and broad range pI standards.

**Immunodetection of Native and Mutant Forms of BglK**

Cell extracts and SeeBlue (Invitrogen)-prestained markers were electrophoresed by SDS-PAGE, and proteins were electrophoretically transferred to nitrocellulose membranes in NuPage transfer buffer. Immunodetection of native and mutated forms of BglK was achieved by sequential incubation of the membranes with polyclonal antibody to His6-BglK and goat anti-rabbit horseradish peroxidase-conjugated antibody, as described previously (9).

**Physicochemical Methods**

Negative-ion FAB spectra of the P-β-glucosides were obtained on a JEOL SX102 mass spectrometer. The compounds were desorbed from a glycerol matrix with 6 Kev xenon atoms, and mass measurements [M-H]- in FAB mode were performed at 10,000 times resolution using electronic field scans and matrix ions as reference material. Low resolution analysis provided integer-mass information, and high resolving power was used for determination (and confirmation) of molecular formulae. Thin-layer chromatographic analyses were performed using 0.1-mm-thick layers of microcrystalline cellulose and a solvent containing 1-butanol/acetone/sodium acetate/water (5:2.3:3.5:12). The phosphate-containing derivatives were visualized by sequential dipping of the air-dried layers in solutions containing: (i) 50 mg of ferric chloride and 1 ml of 1 N HCl, dissolved in 100-ml of acetone, and (ii) 1.25 g of sulfosalicylic acid in 100 ml of distilled water (24). H and 13C NMR spectra of the phosphates and their parent sugars were recorded on a Bruker AVANCE 500 spectrometer. Signal assignments were confirmed by COSY, heteronuclear correlation, and total correlation spectroscopy experiments. Chemical shifts (listed below in Tables IV and V) are reported in D2O relative to sodium 2,2,3,3-tetradeutero-3-trimethylsilyl-propionate as internal standard.

**Bioisosteric Procedure for cellobiose-6'-P**

The procedure outlined for cellobiose-6'-P (with adjustment for the limited quantity of some substrates) was used for the preparation of other phosphorylated compounds. Cellobiose (2 mmol) was dissolved in 10 ml of 25 mm HEPES buffer (pH 7.5) containing 2 mmol MgSO4 and immediately added to 10 ml of water containing 1 mmol of ATP (adjusted to pH 7.5 with –0.6 ml of 3 M NH4OH). His6-BglK was added (40 units), and, throughout a 2-h incubation period at room temperature, the pH of the reaction mixture was maintained at 7.5 by addition of a 3 M NH4OH solution. Thereafter, the pH was adjusted to 8.2 with NH4OH, and 4 ml of barium acetate solution (3 mmol) was added with stirring. The heavy white precipitate of the barium salts of ADP and residual ATP was removed by centrifugation, and the supernatant fluid was clarified by filtration through a Millex (0.22-mum pore size) membrane. The filtrate (approximately 22 ml) was chilled on ice, 4 volumes of absolute ethanol (0 °C) was added, and the mixture was transferred to a cold room overnight. The flocculent precipitate of the Ba2+ salt of cellobiose-6'-P (together with trace amounts of nucleotide salts) was collected by centrifugation. The white pellet was dried at 37 °C for about 30 min, and Ba2+ ions were exchanged for H+ by addition of 2–3 ml of an aqueous suspension of Bio-Rad AG 50WX2 (H+ form) resin.

3 Welcome Trust Sanger Institute. ROK Family, available at www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00489.
**β-Glucoside Kinase from K. pneumoniae**

**TABLE I**

| Purification step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification | Yield (%) |
|-------------------|--------------------|------------------------|------------------------------|--------------|-----------|
| Dialyzed HSSb     | 900                | 79                     | 0.09                         | 1            | 100       |
| TrisAcyl-DEAE     | 143                | 43                     | 0.30                         | 3.4          | 54        |
| AcA-54            | 30                 | 38                     | 1.24                         | 14.2         | 48        |
| ATP-agarose       | 0.5                | 12                     | 24.0                         | 267          | 15        |

* Units expressed as micromoles of cellobiose-6-P formed min^-1.
* HSS, high-speed supernatant.

**FIG. 1. Determination of the M, and pl of native BglK by analytical PAGE.** A, purification and M, estimate of BglK. Samples from each stage of purification were denatured, resolved by SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. B, Western (immuno) blot of a duplicate gel of A showing the highly specific cross-reaction of BglK with polyclonal antibody prepared against His6-BglK. C, determination of the pl of BglK by analytical electrofocusing (lane 2).

Resin beads were removed by filtration, and the filtrate was adjusted to pH 7.2 by NH₄OH addition. The solution was frozen and lyophilized, to yield 300–500 mg of the white, crystalline ammonium salt of cellobiose-6-P. Except for thiocellobiose-6-P, all P-β-glucosides were quantitatively determined by enzymatic assay of Glc6P released by acid hydrolysis (1 N HCl for 2 h at 100 °C). Prior to mass spectrometry and NMR spectroscopy, trace contaminants of ADP and ATP were removed from ~50 mg of each derivative by paper chromatography (20).

**Cloning of the bglK Region of K. pneumoniae ATCC 23357**

Using sequence information from the unfinished genome project of *K. pneumoniae* strain MGH 78578 (Washington University Genome sequencing Center, St. Louis, MO), two primers, KPC808 (5′-TTGCCATCTGTGGCTTCCTGCGGAAAAATAC-3′) and KPC2116 (5′-TACAGTCTGGTGCTTGGGATCCC-3′, the sequence complimentary to the downstream region of bglK), were designed to amplify the DNA fragment encoding bglK and a portion of a putative phospho-β-glucoside (pbgA) gene of *K. pneumoniae* ATCC 23357. PCR amplification was carried out in a thermal cycler (PerkinElmer Life Sciences Model 9600) in a reaction mixture (100 µl) containing 100 ng of *K. pneumoniae* ATCC 23357 chromosomal DNA, 10 µl of 10× reaction buffer, 20 µl each of the four dNTPs, 250 ng of each primer, 5 units of Pfu DNA polymerase (Stratagene, La Jolla, CA), and 1% (v/v) Me₂SO. After an initial 2-min denaturation at 95 °C, the mixture was subjected to 30 cycles of amplification. Each cycle consisted of 1 min of denaturation at 95 °C, 1 min of annealing at 50 °C, and 2 min and 36 s of extension at 72 °C. These were followed by a 10-min runoff at 72 °C. The PCR product was purified (QIAquick PCR purification kit, Qiagen) and ligated into the pCR-Blunt vector (Invitrogen, Carlsbad, CA). The recombinant plasmid was transformed into *E. coli* TOP 10, and transformants were selected in LB agar plates containing 150 µg/ml ampicillin. A high level of expression of the histidine-tagged enzyme His6-BglK was initiated by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to logarithmic phase cultures (A600 nm ~ 0.3) of *E. coli* TOP 10 (pTrcHisB-BglK).

**Cloning and High Expression of His6-BglK in E. coli TOP 10**

For the amplification of bglK, two primers were synthesized from the sequence data presented in Fig. 2. Forward primer F1, 5′-CCCCGGATCCATGAAGAGATTCGGCATTGATCGG-3′ (the bglK sequence is in boldface, and the BamHI site is underlined); reverse primer R1, 5′-GGGGGTTAGCTTCTACGTAAATGTCGATCGTCGTCTGGC-3′ (the sequence complimentary to the downstream region of bglK is in boldface, and the HindIII site is underlined). After amplification with high fidelity Pfu DNA polymerase, the DNA fragment was digested with restriction endonucleases (BamHI and HindIII), electrophoresed (1% agarose), and purified (QiQuick gel extraction kit). The purified 0.9-kb DNA fragment was ligated into the similarly digested and purified high expression vector pTrcHisB. The recombinant plasmid (pTrcHisB-BglK) was transformed into competent cells of *E. coli* TOP 10, and transformants were selected in LB agar plates containing 150 µg/ml ampicillin. DNA Sequence Analysis

Sequencing was accomplished by the dideoxynucleotide chain-termination method using the Sequenase 7-deaza-dGTP sequencing kit (U.S. Biochemicals) and [α-35S]dATP for labeling. Both strands of the DNA insert were sequenced. Sequences were assembled, edited, and analyzed with MacVector sequence analysis package (version 7.0, Genetics Computer Group, Madison, WI).

**DNA Sequence Analysis**

Cloning and High Expression of the “BglK” Genes from *L. monocytogenes* EGD-e and *L. innocua* CLIP 11262 in *E. coli* TOP 10

From the complete genome sequence(s) of *L. monocytogenes* EGD-e and *L. innocua* CLIP 11262 (25), the following pairs of primers were designed for amplification of genes *lm02764* and *lin2907*, respectively. For amplification of *lm02764*, the forward primer EGD-eF1 was 5′-CCGGATCCATGAAAAATTGCAATTTTGATATCGG-3′ (the
**β-Glucoside Kinase from *K. pneumoniae***

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The nucleotide sequence of the BglK region of *K. pneumoniae*. This ~1.3-kilobase DNA fragment contains genes *bglK* and *pbgA* that encode the ATP-dependent β-glucoside kinase (BglK) and the incomplete coding sequence of a putative phospho-β-glucosidase (PbgA), respectively. The nucleotide sequence is numbered on the right, and the deduced amino acid sequences are shown below in single-letter code. A potential ribosomal binding site (RBS) preceding *bglK* is underlined (fine line). The N-terminal amino acid sequence (28 residues) obtained by microsequence analysis is underlined (bold line). Residues shown boxed represent the putative ATP-binding site. The *double underline* indicates those residues that comprise the signature sequence for proteins of the ROK family.

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**Site-directed Mutagenesis of BglK**

The method required the use of PfuTurbo DNA polymerase, a temperature cycler, and reagents that were obtained as a kit (QuickChange site-directed mutagenesis kit, Stratagene). *E. coli* TOP 10-competent cells were transformed with plasmids of pTrcHisB *bglK* containing the desired mutation. Base changes effected by site-directed mutagenesis were confirmed by sequence analysis.

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**Purification of Native BglK from *K. pneumoniae* ATCC 23357**

Washed cells (~16 g of wet wt) grown previously on cellobiose as the energy source were resuspended in 24 ml of TM buffer. Cells were disrupted at 0 °C, by 2× 1.5-min periods of sonic oscillation in a Branson (Model 350) instrument operated at ~75% of maximum power. BglK was purified in four stages by low pressure chromatography. Column flow rates were maintained by a P-1 peristaltic pump inter-
respectively.

The dialyzed HSS (containing 0.1 M sodium phosphate buffer (pH 7.2) containing 500 mM NaCl (designated as buffer). Fractions of 4 ml were collected, and the concentrate (100 µl) was dialyzed against 2 liters of TM buffer. The eluate was dialyzed against 2 liters of TM buffer, and the few contaminating proteins were removed by molecular filtration through a column of Ultrigel AcA-44 (1.6 × 87 cm) of ATP-linked agarose that had been washed extensively with TM buffer. Fractions (1 ml) were collected, and a large protein peak (devoid of BglK activity) was eluted by TM buffer (fractions 9–25). An attempt to elute BglK with a gradient of ATP (0–20 mM) was unsuccessful. However, the enzyme was readily eluted with 20 ml of TM buffer containing 1 mM NaCl. The elute was dialyzed against 2 liters of TM buffer, and concentrated to ~2 ml. This preparation contained 0.5 mg of electrophoretically pure BglK (specific activity, 24 units/mg).

Purification of His<sup>6</sup>-BglK from E. coli TOP 10 (pTrcHisBglK)

Washed cells (~22 g of wet weight) were resuspended with 45 ml of TM buffer, cells were sonicated, and HSS was prepared. His<sup>6</sup>-BglK was partially purified by DEAE-TrisAcryl M chromatography as described (above), and the concentrate (~14 ml) was dialyzed against 2 liters of 20 mM sodium phosphate buffer (pH 7.2) containing 500 mM NaCl (designated binding buffer). This preparation was transferred to a column of Ni<sup>2+</sup>-NTA-agarose (1.5 × 8 cm) equilibrated with binding buffer. Non-adsorbed proteins were eluted first with binding buffer and, subsequently, with a solution of 20 mM sodium phosphate buffer (pH 6.0) containing 500 mM NaCl (wash buffer). His<sup>6</sup>-BglK was eluted with 180 ml of wash buffer of increasing concentration of imidazole (0–150 mM). Appropriate fractions were pooled and concentrated to ~3 ml, and the few contaminating proteins were removed by molecular filtration through a column of Ultrogel AcA-44 (1.6 × 94 cm). These procedures yielded 12 mg of high purity His<sup>6</sup>-BglK (specific activity, ~87 units/mg).

### Table II

| Substrate                        | \(K_m\) (mM) | \(V_{max}\) (µmol/min/mg) |
|----------------------------------|--------------|--------------------------|
| n-Octyl                          | 0.11 ± 0.01  | 106.81 ± 1.65            |
| β-D-glucopyranoside              | 0.18 ± 0.01  | 102.42 ± 1.96            |
| Salicin<sup>a</sup>              | 0.21 ± 0.01  | 131.23 ± 1.89            |
| Arbutin<sup>a</sup>              | 0.47 ± 0.03  | 86.82 ± 1.90             |
| Cellobiose<sup>d</sup>           | 0.47 ± 0.05  | 94.21 ± 2.73             |
| Amygdalin<sup>e</sup>            | 0.56 ± 0.03  | 74.59 ± 1.40             |
| Laminaribiose<sup>e</sup>        | 0.63 ± 0.02  | 121.23 ± 1.07            |
| Phenyl                           | 0.65 ± 0.02  | 150.00 ± 1.98            |
| β-D-glucopyranoside              | 1.56 ± 0.07  | 85.72 ± 1.38             |
| Cellubiose<sup>f</sup>           | 2.03 ± 0.11  | 107.30 ± 2.25            |
| iso-Propyl                       | 2.23 ± 0.09  | 132.12 ± 1.78            |
| β-D-thioglucopyranoside          | 3.15 ± 0.31  | 105.46 ± 3.91            |
| Thioceholibiose<sup>f</sup>      | 10.54 ± 0.68 | 49.27 ± 1.92             |
| Glucose                          | 40.29 ± 3.89 | 86.29 ± 3.00             |

<sup>a</sup> Micromoles of substrate phosphorylated min<sup>-1</sup> mg<sup>-1</sup>

<sup>b</sup> 2-(Hydroxymethyl)phenyl β-D-glucopyranoside.

<sup>c</sup> Hydroquinone β-D-glucopyranoside.

<sup>d</sup> 4-O-β-D-Glucopyranosyl-β-D-glucopyranose.

<sup>e</sup> n-Mandelonitrile β-gentiobiose.

<sup>f</sup> β-D-β-D-Glucopyranosyl-β-D-glucopyranose.

<sup>g</sup> 3-O-β-D-Glucopyranosyl-β-D-glucopyranose.

<sup>h</sup> 4-O-β-D-Glucopyranosyl-β-D-glucopyranose.

<sup>i</sup> 2-O-β-D-Glucopyranosyl-β-D-glucopyranose.

<sup>j</sup> 4-S-β-D-Glucopyranosyl-β-D-glucopyranose.

FIG. 3. SDS-PAGE of samples obtained from various stages of purification of His<sup>6</sup>-BglK (Mr ~ 36 kDa). Lanes 1 and 2 show the results of heating His<sup>6</sup>-BglK in the presence and absence of dithiothreitol, respectively.

FIG. 4. Effect(s) of sulfhydryl-reactive agents on the activity of His<sup>6</sup>-BglK. In these experiments, the standard (Control) incubation mixture was 100 µl of 0.1 M HEPES buffer (pH 7.5) that contained 7.5 µg of His<sup>6</sup>-BglK. The mixture was maintained at room temperature (~23 °C) and at times indicated, 10-µl samples were removed and enzyme activity was measured in the PK/α-LDH coupled assay with cellobiose as the substrate. As required, the various compounds were included in the (100 µl) reaction mixture at the following concentrations: iodoacetate (IAA), NEM, and cellobiose, all 10 mM; and ATP, 2.5 mM. The inset shows the first-order rate of inactivation of the enzyme by NEM.

Step 3: Ultrogel AcA-54 (Molecular Sieve) Chromatography—5.5 ml of concentrate from step 2 was applied (flow rate of 0.3 ml/min) to a column of Ultrogel AcA-54 (2.6 × 94 cm) equilibrated with TM buffer containing 0.1 mM NaCl. Fractions of 4 ml were collected, and 20-ml samples were assayed for BglK activity. Fractions 49–53 were pooled, concentrated to ~7 ml, and dialyzed against 2 liters of TM buffer.

Step 4: ATP-linked Agarose (Affinity) Chromatography—The dialysate from step 3 was transferred (0.12 ml/min) to a small column (1 × 7 cm) of ATP-linked agarose that had been washed extensively with TM buffer. Fractions (1 ml) were collected, and a large protein peak (devoid of BglK activity) was eluted by TM buffer (fractions 9–25). An attempt to elute BglK with a gradient of ATP (0–20 mM) was unsuccessful. However, the enzyme was readily eluted with 20 ml of TM buffer containing 1 mM NaCl. The elute was dialyzed against 2 liters of TM buffer, and concentrated to ~2 ml. This preparation contained 0.5 mg of electrophoretically pure BglK (specific activity, 24 units/mg).

Washed cells (~22 g of wet weight) were resuspended with 45 ml of TM buffer, cells were sonicated, and HSS was prepared. His<sup>6</sup>-BglK was partially purified by DEAE-TrisAcryl M chromatography as described (above), and the concentrate (~14 ml) was dialyzed against 2 liters of 20 mM sodium phosphate buffer (pH 7.2) containing 500 mM NaCl (designated binding buffer). This preparation was transferred to a column of Ni<sup>2+</sup>-NTA-agarose (1.5 × 8 cm) equilibrated with binding buffer. Non-adsorbed proteins were eluted first with binding buffer and, subsequently, with a solution of 20 mM sodium phosphate buffer (pH 6.0) containing 500 mM NaCl (wash buffer). His<sup>6</sup>-BglK was eluted with 180 ml of wash buffer of increasing concentration of imidazole (0–150 mM). Appropriate fractions were pooled and concentrated to ~3 ml, and the few contaminating proteins were removed by molecular filtration through a column of Ultrogel AcA-44 (1.6 × 94 cm). These procedures yielded 12 mg of high purity His<sup>6</sup>-BglK (specific activity, ~87 units/mg).

Step 2: DEAE-TrisAcryl M (Anion Exchange) Chromatography—The dialyzed HSS (~35 ml) was transferred at a flow rate of 0.8 ml/min to a column of DEAE-TrisAcryl M (2.6 × 10 cm) previously equilibrated with TM buffer. Non-adsorbed materials were removed by washing with TM buffer, and BglK was eluted with 400 ml of a linear, increasing concentration gradient of NaCl (0–300 mM) in TM buffer. Fractions of 6 ml were collected, and samples (30 µl) were assayed for enzymatic activity. Fractions (33–38) were pooled and concentrated to 6 ml in a pressure filtration unit (Amicon PM-10 membrane, 40 psi).

Step 1: Preparation of Dialyzed High Speed Supernatant Fluid—The sonicated extract was clarified by ultracentrifugation (180,000 × g for 2 h at 5 °C). The HSS was transferred to sacs and dialyzed overnight against 4 liters of TM buffer (at 4 °C).

Step 2: DEAE-TrisAcryl M (Anion Exchange) Chromatography—The dialyzed HSS (~35 ml) was transferred at a flow rate of 0.8 ml/min to a column of DEAE-TrisAcryl M (2.6 × 10 cm) previously equilibrated with TM buffer. Non-adsorbed materials were removed by washing with TM buffer, and BglK was eluted with 400 ml of a linear, increasing concentration gradient of NaCl (0–300 mM) in TM buffer. Fractions of 6 ml were collected, and samples (30 µl) were assayed for enzymatic activity. Fractions (33–38) were pooled and concentrated to 6 ml in a pressure filtration unit (Amicon PM-10 membrane, 40 psi). Fractions of 4 ml were collected, and 20-ml samples were assayed for BglK activity. Fractions 49–53 were pooled, concentrated to ~7 ml, and dialyzed against 2 liters of TM buffer.

Substrate specificity and kinetic parameters of His<sup>6</sup>-BglK from K. pneumoniae
Analytical Methods

Two procedures were used for the assay of enzyme activity. During purification of native and His6BglK, enzyme activity was detected by continuous spectrophotometric measurement of NADPH formation in a phospho-β-glucosidase (CelF)/G6PDH-coupled reaction. (The purification of NAD+/Mn2+-dependent CelF (EC 3.2.1.86) has been reported previously (14).) In this assay, cellobiose is first phosphorylated by ATP-dependent BglK to yield cellobiose-6-P, which is hydrolyzed (by CelF) to Glc6P and glucose. Oxidation of Glc6P is coupled to the reduction of NADP+ by G6PDH, and the increase in A340 nm is recorded in a Beckman DU 640 spectrophotometer. The standard 1-ml reaction contained 0.1M HEPES buffer (pH 7.5), 1 mM NADP+, 1 mM NAD+, 1 mM MgCl2, 1 mM MnCl2, 1 mM ATP, 2 mM cellobiose, 2 units of G6PDH, and non-limiting amounts of CelF. Reactions were initiated by addition of BglK preparation.

A separate assay was used to determine the substrate specificity and kinetic parameters of purified His6BglK. In this assay, the ATP-mediated phosphorylation of β-glucosides (by BglK) yields ADP that, in the presence of P-enolpyruvate and pyruvate kinase, forms ATP and pyruvic acid. Reduction of pyruvate to lactate by LDH is coupled to the oxidation of NADH. The 1-ml reaction mixture contained 0.1 M HEPES buffer (pH 7.5), 5 mM ATP, 10 mM MgCl2, 2 mM P-enolpyruvate, ~2 units each of PK/L-LDH, and 0.75 μg of purified His6BglK. In the two assays, initial rates of either NADP+ reduction or NADH oxidation (equivalent to rates of formation of β-glucoside-6-P) were determined using the kinetics program installed in the instrument. A molar extinction coefficient for the reduced forms of dinucleotides, ε = 6220 M⁻¹ cm⁻¹ was assumed in all calculations. One unit of BglK is the amount of enzyme that catalyzes the formation of 1 mol of β-glucoside-6-P/min. For most kinetic studies, the concentration range of the β-glucoside substrate exceeded the experimentally determined Kᵅ by 5- to 10-fold. Kinetic parameters were determined from Eadie-Hofstee plots generated by the dogStar software kinetics program, version 1.0c. Protein concentrations were measured either by the BCA protein assay (Pierce) or from the predicted molar absorption coefficients (26) for BglK (ε = 30,940 M⁻¹ cm⁻¹) and His6BglK (ε = 32,430 M⁻¹ cm⁻¹). The sequence of residues from the N terminus of BglK was determined with an ABI 477A protein sequencer (Applied Biosystems Inc.) with an on-line ABI phenylthiohydantoin analyzer.

RESULTS

Growth of K. pneumoniae on β-Glucosides—Palmer and Anderson (21) described induction of BglK during growth of K. pneumoniae on cellobiose, but whether other β-glucosides served as inducers of the enzyme, was not reported. Accordingly, the organism was grown on a variety of β-glucosides, and...
cell extracts were assayed for BglK activity (nanomoles of cellobiose-6-P formed min⁻¹ mg⁻¹): cellobiose (81), gentiobiose (46), cellobiitol (19), and methyl-β-glucoside (8). Extracts from cells grown previously on other β-glucosides, including arbutin, salicin, esculin, or phenyl-β-glucoside, contained no measurable BglK activity.

Purification of BglK—The enzyme was purified from cellobiose-grown cells of K. pneumoniae as described under “Experimental Procedures.” The four-step process yielded 0.5 mg of BglK (specific activity, ~24 units/mg) from 16 g of wet weight of cells (Table I). SDS-PAGE of the preparation of BglK eluted from ATP-agarose revealed a single polypeptide of Mr ~ 33,000 (Fig. 1A), whereas the molecular weight of the enzyme estimated from AcA-44 gel filtration chromatography was ~130,000 (data not shown). These results provide evidence that BglK is oligomeric and, in solution, most likely comprises four identical subunits. Microsequence analysis of BglK identified the first 28 residues from the N terminus: MKIAAFDIG-

### Table III

Some physical properties of enzymatically synthesized β-glucoside 6-phosphates

| Derivative | [M-H] - | Calculated Rf Value |
|------------|--------|---------------------|
| Arbutin-6-P | 351.2  | 0.47 |
| Cellobitol-6-P | 423.1 | 0.20 |
| Cellobiose-6-P | 421.1 | 0.20 |
| Gentiobiose-6-P | 421.1 | 0.16 |
| Isopropyl 1-thio-β-D-glucoside-6-P | 317.1 | 0.58 |
| Methyl β-D-glucoside-6-P | 273.1 | 0.37 |
| 4-Methylumbelliferyl β-D-glucoside-6-P | 417.2 | 0.54 |
| Phenyl β-D-glucoside-6-P | 335.1 | 0.60 |
| Salicin-6-P | 365.1 | 0.56 |
| Thiocebobiolose-6-P | 437.1 | 0.20 |

a Negative-ion mass was determined by FAB mass spectrometry.
b Rf values were determined by thin-layer chromatography (see Table III.).

### Table IV

1H NMR data (500 MHz in D2O, 25 °C, ppm from internal TSP) of β-D-glucosyl-glucoses, cellobiitol, and β-D-glucosides compared with their monophosphates, uniformly carrying the phosphate group at the terminal primary OH

| Compound          | β-D-Glucopyranoside residue | Reducing glucose residue |
|-------------------|-----------------------------|--------------------------|
|                   | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-2 | H-3 | H-4 | H-5 | H-6 | H-2 | H-3 | H-4 | H-5 | H-6 | H-2 | H-3 | H-4 | H-5 | H-6 |
| β-D-Cellobiose    | 4.51 | 3.33 | 3.51 | 3.43 | 3.49 | 3.74 | 3.92 | 4.67 | 3.29 | 3.63 | 3.63 | 3.63 | 3.81 | 3.96 |
| β-D-Cellobiose-6-P | 4.52 | 3.35 | 3.53 | 3.57 | 3.57 | 4.02 | 4.07 | 4.67 | 3.29 | 3.63 | 3.63 | 3.63 | 3.82 | 3.96 |
| β-D-Thioellobiose | 4.65 | 3.36 | 3.52 | 3.43 | 3.47 | 3.71 | 3.89 | 4.64 | 3.29 | 3.58 | 2.89 | 3.69 | 3.95 | 4.09 |
| β-D-Thioellobiose-6-P | 4.67 | 3.37 | 3.54 | 3.59 | 3.54 | 4.01 |      | 4.67 | 3.29 | 3.61 | 2.97 | 3.72 | 3.92 | 4.11 |
| β-D-Gentiobiose   | 4.52 | 3.33 | 3.51 | 3.40 | 3.47 | 3.73 | 3.93 | 4.66 | 3.26 | 3.48 | 3.47 | 3.63 | 3.85 | 4.21 |
| β-D-Gentiobiose-6-P | 5.47 | 3.35 | 3.51 | 3.60 | 3.49 | 4.03 |      | 4.67 | 3.27 | 3.49 | 3.44 | 3.65 | 3.85 | 4.21 |
| Cellobital        | 4.58 | 3.34 | 3.51 | 3.44 | 3.44 | 3.76 | 3.91 | 3.60 | 3.78 | 3.97 | 3.85 | 3.88 | 3.94 | 3.75 | 3.88 |
| Cellobiose-6-P    | 4.57 | 3.37 | 3.53 | 3.58 | 3.51 | 4.03 |      | 3.63 | 3.82 | 3.98 | 3.84 | 3.89 | 3.95 | 3.74 | 3.89 |
| Methyl β-D-glucoside | 4.37 | 3.27 | 3.49 | 3.38 | 3.46 | 3.73 | 3.93 |      | 4.08 |
| Methyl β-D-glucoside-6-P | 4.39 | 3.29 | 3.52 | 3.57 | 3.52 | 4.05 |      | 4.08 |
| Phenyl β-D-glucoside | 5.14 | 3.35 | 3.63 | 3.51 | 3.63 | 3.76 | 3.94 | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |
| Phenyl β-D-glucoside-6-P | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |      | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |
| Isopropyl 1-thio-β-D-glucoside | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |      | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |
| Isopropyl 1-thio-β-D-glucoside-6-P | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |      | 5.14 | 3.63 | 3.66 | 3.71 | 3.66 | 4.04 | 4.10 |
| Salicin-6-P       | 5.14 | 3.69 | 3.69 | 3.69 | 3.69 | 4.08 |      | 5.14 | 3.69 | 3.69 | 3.69 | 3.69 | 4.08 |      | 5.14 | 3.69 | 3.69 | 3.69 | 3.69 | 4.08 |
| Arbutin-6-P       | 4.95 | 3.59 | 3.64 | 3.55 | 3.55 | 3.80 | 3.95 | 4.97 | 3.57 | 3.62 | 3.71 | 3.62 | 4.06 |
| Arbutin-6-P       | 5.20 | 3.60 | 3.65 | 3.56 | 3.71 | 3.81 | 3.98 | 5.17 | 3.69 | 3.74 | 3.77 | 3.74 | 4.10 | 4.18 |

a TSP, sodium 2,2,3,3-tetradeutero-3-trimethylsilyl-propionate.
TABLE V

| Compound                      | β-β-Glucopyranoside residue | Reducing glucose residue |
|-------------------------------|-----------------------------|-------------------------|
|                               | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| β-p-Celllobiose               | 105.3 | 75.9 | 78.3 | 72.8 | 73.8 | 68.3 | 98.5 | 76.7 | 78.9 | 77.6 | 81.5 | 62.9 |
| β-p-Celllobiose-6'-P          | 105.7 | 76.2 | 78.1 | 71.9 | 78.2 | 65.9 | 98.6 | 76.7 | 77.2 | 77.6 | 82.2 | 63.1 |
| β-p-Thiocebiose               | 86.7 | 75.5 | 79.9 | 72.2 | 82.6 | 63.6 | 98.5 | 78.2 | 75.8 | 50.0 | 79.3 | 64.3 |
| β-p-Thiocebiose-6'-P          | 86.8 | 75.4 | 79.5 | 71.7 | 82.0 | 66.0 | 98.4 | 78.3 | 75.8 | 50.4 | 79.0 | 64.3 |
| β-p-Gentiocebiose             | 105.5 | 75.9 | 78.5 | 72.5 | 78.9 | 63.6 | 98.0 | 76.9 | 78.5 | 72.4 | 77.7 | 71.7 |
| β-p-Gentiocebiose-6'-P        | 105.8 | 78.2 | 78.0 | 71.8 | 78.2 | 65.5 | 98.8 | 78.9 | 78.5 | 72.5 | 77.7 | 72.9 |

| Compound                      | d-Glucoside residue         |
|-------------------------------|-----------------------------|
|                               | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| Celllobitol                   | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Celllobiose-6-P               | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| Methyl β-0-glucoside          | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Methyl β-0-glucoside-6'-P     | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| Phenyl β-0-glucoside          | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Phenyl β-0-glucoside-6'-P     | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| iPropyl 1-thio-β-0-glucoside  | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| iPropyl 1-thio-β-0-glucoside-6'-P | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| Salicin                       | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Salicin-6-P                   | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| Arbutin                       | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Arbutin-6-P                   | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| 4-Methylumbelliferyl          | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| β-0-glucoside                 | 65.3 | 74.9 | 72.3 | 82.4 | 74.2 | 65.0 |
| 4-Methylumbelliferyl-6'P      | 65.5 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |

| Compound                      | p-Glucoside residue         |
|-------------------------------|-----------------------------|
|                               | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| Cellobiose                    | 104.4 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Cellobiose-6-P                | 104.4 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Methyl β-0-glucoside          | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Methyl β-0-glucoside-6'-P     | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Phenyl β-0-glucoside          | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Phenyl β-0-glucoside-6'-P     | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| iPropyl 1-thio-β-0-glucoside  | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| iPropyl 1-thio-β-0-glucoside-6'-P | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Salicin                       | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Salicin-6-P                   | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Arbutin                       | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| Arbutin-6-P                   | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| 4-Methylumbelliferyl          | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| β-0-glucoside                 | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |
| 4-Methylumbelliferyl-6'P      | 104.0 | 75.1 | 72.3 | 82.1 | 74.0 | 64.9 |

a TSP, sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate.

β-Glucoside Kinase from K. pneumoniae

The presence of these extra residues (MGGSHHHHHHG-MASMTGQGQMGRLDYYDZZD) was reflected in the increased size of the monomer, whose estimated molecular mass...
(−36 kDa) was similar to that calculated from the amino acid composition of His<sub>6</sub>BglK (M<sub>r</sub> = 36,338). Like the native enzyme, His<sub>6</sub>BglK is also tetrameric, and from studies with dithiothreitol (Fig. 3, lanes 1 and 2, respectively) formation of inter-subunit disulfide linkage(s) appears not to be a prerequisite for oligomerization. A polyclonal antibody against His<sub>6</sub>BglK was prepared for use in site-directed mutagenesis studies (see below). As shown in the immunoblot (Fig. 1B), this antibody also cross-reacted specifically with native BglK.

**Substrate Specificity and Kinetic Properties of His<sub>6</sub>BglK**

Using various buffer systems, Palmer and Anderson (21) reported a pH optimum of 7–8 for activity of BglK. For our kinetics experiments, Tris-HCl (pH 7.5) was the buffer of choice in the PK/L-LDH coupled assay described under “Experimental Procedures.” A variety of β-glucosides were phosphorylated by His<sub>6</sub>BglK, and the pertinent kinetic parameters are presented in Table II. The importance of both the glucopyranosyl moiety and β-O-linkage for enzyme recognition is evident from the findings that glucose per se is an extremely poor substrate (K<sub>m</sub>(Glc) ~ 40 μM) and at 50 μM final concentration, there was no detectable phosphorylation of the glucose epimers (mannose C-2, allose C-3, or galactose C-4) or of glucose analogs, including 2-deoxy-β-D-glucose, 2-deoxy-2-fluoro-β-D-glucose, and 5-thio-D-glucoside. Of two cellobiose analogs tested (at 25 μM), N<sub>1</sub>, N<sub>3</sub>-diacetylchitobiose was a substrate for BglK (~ 62 μmol phosphorylated min<sup>−1</sup> mg<sup>−1</sup>), but there was no detectable phosphorylation of thioglucoside (D-glucopyranosyl-β-D-1-thioglucopyranoside). Eadie-Hofstee transformation of kinetic data (obtained in assays containing cellobiose and Mg<sup>2+</sup> at 5 and 10 mM, respectively) provided an estimated K<sub>m</sub>(ATP) of 0.24 ± 0.02 for BglK. The deduced amino acid sequence for BglK predicts 5 cysteine residues (per monomer), and the effects of sulfhydryl-reactive agents were accordingly investigated (Fig. 4). Exposure of His<sub>6</sub>BglK to N-ethylmaleimide (10 mM) resulted in a gradual inactivation of the enzyme (t<sub>1/2</sub> ~ 19 min), but surprisingly other -SH reagents, including iodoacetate and iodoacetamide, caused little or no inactivation. The presence of ATP (and to a lesser extent the substrate cellobiose) resulted in partial protection of His<sub>6</sub>BglK from the inhibitory effect of NEM. The effect of ADP was comparable to that of ATP, but AMP afforded no protection from the inhibitor.

**Biobiosynthesis and Proof of Structure of the 6-P-β-D-Glucones**

The availability of His<sub>6</sub>BglK in milligram quantities, allowed the preparative synthesis of the ten phosphorylated compounds illustrated in Fig. 5. Except for thiocellobiose-6-P, all P-glucosides were confirmed by negative-ion FAB mass spectrometry (Table III). Analytical thin-layer chromatography provided evidence for purity of the 10 derivatives, by revealing a single phosphate-containing spot for each preparation (Fig. 6). The estimated R<sub>p</sub> values of the derivatives, under the prescribed conditions, are listed in Table III. Proof of structure and verification of the exclusive phosphorylation at the primary glucosyl-0-6 were provided by comparison of the <sup>1</sup>H and <sup>13</sup>C NMR data of the mono- and disaccharide phosphates with their respective parent (non-phosphorylated) glucoses. It is well
established (20, 32, 33) that phosphorylation of a hydroxyl group in a mono- or disaccharide usually results in a small but distinctive downfield shift of 0.2–0.5 ppm for the protons attached to the phosphate-carrying carbon (here the 6-CH2), whereas hydrogens situated vicinal thereto (H-5) or in the β-position (H-4) exhibit only minor deshielding, with all other signals being essentially identical to those of the parent sugar. As borne out by the juxtaposition of 1H NMR data for the parent sugar versus monophosphate in Table IV, all ten glycosides (Fig. 5) were phosphorylated at O-6, because there is a uniform 0.2- to 0.3-ppm downfield shift of the respective 6-CH2 protons relative to their parent sugars (cf. column of data under "6-H2" in Table IV). Similarly, the 0.15- to 0.20-ppm shift of all H-4 signals to lower magnetic field can be attributed to the spatial proximity of the phosphate ester group at O-6 and the axially oriented H-4 proton. All other resonances, including that of H-5 vicinal to the phosphate group, remain essentially unchanged.

Substantiation of these assignments was provided by comparative data obtained from compounds that contain two glucose residues (cellobiose, thioceolobiose, and gentiobiose). Of the two 6-CH2 groups present in these disaccharides, only the terminal one (situated in the non-reducing portion of the molecule) was shifted in the phosphorylated derivative, whereas signals from CH2 in the reducing moiety remained unchanged. Finally, the signals from the aglycon moieties of salicin (primary benzylc OH group) and arbutin (phenolic OH group) also remained constant and indicative of the absence of a phosphate ester at these positions. The 13C NMR data presented in Table V confirmed the findings from 1H spectroscopy. In accord with previous analyses of various sugar phosphates (20, 32, 33), the most pronounced 13C downfield shift was observed for the carbon carrying the phosphate ester. The marked downfield shift of 1.7–2.4 ppm recorded in Table V (column of data under "C-6") confirmed the terminal C-6 as the site of phosphorylation in all derivatives. The differences found for the β- and γ-carbons (C-5 and C-4, respectively) were comparatively small and have not been relied upon for structural assignments. Additional evidence for phosphorylation at O-6 in celllobitol and the six β-D-glucosides is provided by the doublet splitting of the C-6 resonance with a coupling constant, 3J(C,P), of about 4–5 Hz that is, however, not detectable in the more complex 13C data of the disaccharides.

Site-directed Mutagenesis of BglK—Several residues of BglK, including Asp7, Gly8, Asp103, Gly131 and Gly133 are positionally conserved in microbial glucokinases that belong to the ROK family of proteins (24). Although it is reasonable to assume that these particular amino acids are structurally or catalytically important, the issue was addressed by site-directed mutagenesis of bglK. Residue changes effected were D7G, G9A, D103G, G131A, and G133A. After transformation of E. coli TOP 10 with plasmids (pTrcHis-BglK) containing the desired mutation, the cells were grown and extracts were prepared. High level expression of the five mutant forms of His6-BglK was confirmed by Western blot analyses using polyclonal antibody against this protein (Fig. 7). All extracts contained a single immunoreactive polypeptide of the size (~36 kDa) expected for His6-BglK, but remarkably, none of the extracts contained measurable activity of β-glucoside kinase.

Homologs of BglK—Presently, BglK activity has been described only in K. pneumoniae, and (except for two species of Listeria) a BLAST (26) search of non-redundant protein data bases revealed no entries with significant homology to this protein. Surprisingly, the recently sequenced genomes (25) of L. monocytogenes EGD-e (serovar 1/2a) and L. innocua CLIP 11262 (serovar 6a) contain a gene (lmo2764 and lin2907, respectively) whose ORF encodes a predicted polypeptide of 294 amino acids (Mw ~ 32,200) that exhibits ~59% residue identity (~72% similarity) with BglK (Fig. 8). Both lin2907 and lmo2764 were cloned into pTrcHis2B, and the gene products were expressed in E. coli TOP 10. SDS-PAGE and Western blot analyses of cell extracts of E. coli TOP 10 (pTrcHis2B lmo2764) and E. coli TOP 10 (pTrcHis2B lin2907) revealed high level expression of a polypeptide of Mw ~ 32 kDa, which also cross-reacted with antibody prepared against His6-BglK from K. pneumoniae (Fig. 9, A and B, respectively). In both cases, the expressed protein catalyzed the ATP-dependent phosphorylation of cellobiose (specific activity (avg.) ~ 2.8 μmol of cellobiose-6'-P formed min⁻¹ mg⁻¹) and was thus identified as a homolog of BglK.

**DISCUSSION**

Experiments reported in this communication have allowed the high expression, purification, and use of His6-BglK, for the first preparative synthesis of a wide variety of P-β-glucosides. The ATP-dependent procedure provides a facile and stereospecific synthesis of sophorose-6'-P (β,1−2), laminaroibiose-6'-P (β,1−3), cellobiose-6'-P (β,1−4), and gentiobiose-6'-P (β,1−6). The four disaccharide-6'-Ps and related P-β-glucoside analogs are substrates for Family 4 P-β-glucosylhydrolases. This large family of NAD⁺- and Me₂⁺-dependent bacterial enzymes includes P-β-glucosidasises, P-α-glucosidasises, and α-glucosylhydrolases (11–16). A preliminary x-ray analysis of one P-α-glucosidase (GlvA) from Bacillus subtilis has been reported (10), but the complete structure of a Family 4 member has yet to be solved. The availability of the P-β-glucosides described here will assist in the crystallization, structural analysis, and elucidation of the catalytic mechanism (inversion or retention (35–37)) of Family 4 enzymes.

**Properties of BglK**—The enzyme is exacting with respect to β-glucoside substrates, and NMR data establish that phosphorylation occurs exclusively at the C-6 position of the glucopy-
anoyl moeity. BglK tolerates wide variation in both size and structure of the aglycon component, and substrates include molecules such as hexose linked via C-2, C-3, C-4, or C-6 positions (sophorose, laminaribiose, cellobiose, and gentiobiose, respectively); hexitol (cellobitot); aliphatic group (methyl β-glucoside); and aryl groups (salicin, arbutin, phenyl β-glucoside, and 4-methylumbelliferyl β-glucoside). The importance of the β-O-linked aglycon in substrate recognition is evidenced by the fact that BglK does not phosphorylate maltose (α-isomer of cellobiose) and has remarkably little affinity for glucose itself.

Indeed, the monosaccharide is phosphorylated only when present at high concentration (K_m ~ 40 mM). These findings affirm the classification of BglK as a β-glucoside kinase (EC 2.7.1.85) that is separate and distinct from glucokinase (EC 2.7.1.2) found in many species of bacteria, including *K. pneumoniae*. Interestingly, the two ATP-dependent kinases contain the common signature sequence by which they are assigned to the ROK family of proteins. Site-directed mutagenesis of the individual residues Asp^7, Gly^9, Asp^103, Gly^131, and Gly^133 of BglK, yields proteins that are catalytically inactive. It is noteworthy that residues Asp^7 and Gly^9 reside in a motif X^2D^X^2G^GT that is conserved in many microbial glucokinases and that may represent the ATP-binding site in these enzymes (31). The loss of activity of BglK, attendant upon the amino acid changes D^7^G and G^9^A, would be consistent with an inability of the two mutant proteins to bind the phosphoryl donor. Whether residues Asp^103, Gly^131, and Gly^133 fulfill catalytic or structural functions cannot be discerned from our results.

BglK contains 5 cysteine residues, but the resistance of the enzyme to high concentrations of sulphhydryl-reactive agents (iodoacetate and iodoacetamide) suggests that Cys residues are not prerequisite for catalysis. Although the time-dependent inactivation of BglK by NEM appears to contradict this statement, an earlier discussion of NEM reactivity by Means and Feeney (38) may provide a satisfactory accommodation of the data. Means and Feeney point out that, although NEM is fairly specific in its action, it will modify the thiol group of sulphydryl-containing proteins at high concentration (i.e., 100 mM). These findings affirm the potential of ATP-dependent glucokinase (Glk) to serve as substrate for EII^β^G of the PTS. Whether BglK functions in conjunction with (or can substitute for) EII^β^G of the PTS remains to be determined. However, it is pertinent that Erni and his colleagues (39) have investigated the potential of ATP-dependent glucokinase (Glk) to catalyze the phosphorylation of β-glucosides using different phosphoryl donors: (i) ATP-dependent phosphorylation catalyzed by BglK and (ii) P-enolpyruvate-dependent phosphorylation mediated via EIIB^β^G of the PTS. Whether BglK functions in conjunction with (or can substitute for) EII^β^G of the PTS remains to be determined. However, it is pertinent that Erni and his colleagues (39) have investigated the potential of ATP-dependent glucokinase (Glk) to serve as substrate for EII^β^G of the PTS. Whether BglK functions in conjunction with (or can substitute for) EII^β^G of the PTS remains to be determined. However, it is pertinent that Erni and his colleagues (39) have investigated the potential of ATP-dependent glucokinase (Glk) to serve as substrate for EII^β^G of the PTS.

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β-Glucoside Kinase from K. pneumoniae

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β-Glucoside Kinase (BglK) from *Klebsiella pneumoniae*: PURIFICATION, PROPERTIES, AND PREPARATIVE SYNTHESIS OF 6-PHOSPHO-β-d-GLUCOSIDES

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