Genetic Requirements for Growth of *Escherichia coli* K12 on Methyl-α-D-glucopyranoside and the Five α-D-Glucosyl-D-fructose Isomers of Sucrose

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Strains of *Escherichia coli* K12, including MG-1655, accumulate methyl-α-D-glucopyranoside via the phosphoenolpyruvate-dependent glucose:phosphotransferase system (IICB/Glc/IIA/Glc). High concentrations of intracellular methyl-α-D-glucopyranoside 6-phosphate are toxic, and cell growth is prevented. However, transformation of *E. coli* MG-1655 with a plasmid (pAP1) encoding the gene aglB from *Klebsiella pneumoniae* resulted in excellent growth of the transformant MG-1655 (pAP1) on the glucose analog, AglB is an unusual NAD+/Mn2+-dependent phospho-α-glucosidase that promotes growth of MG-1655 (pAP1) by catalyzing the in vivo hydrolysis of methyl-α-D-glucopyranoside 6-phosphate to yield glucose 6-phosphate and methanol. When transformed with plasmid pAP2 encoding the *K. pneumoniae* genes aglB and aglA (an α-glucoside-specific transporter AglA (IICB/Agl)), strain MG-1655 (pAP2) metabolized a variety of other α-linked glycosides, including maltotriose, isomaltose, and the following five isomers of sucrose: trehalulose α(1→1), turanose α(1→3), maltulose α(1→4), leucrose α(1→5), and palatinose α(1→6). Remarkably, MG-1655 (pAP2) failed to metabolize sucrose α(1→2). The *E. coli* K12 strain ZSC112L (ptsG::cat manXYZ nagE gk glk lac) cannot grow on glucose nor transport methyl-α-D-glucopyranoside. However, when transformed with pTSGH11 (encoding ptsG) or pAP2, this organism provided membranes that contained either the PtsG or AglA transporters, respectively. In vitro complementation of transporter-specific membranes with purified general phosphotransferase components showed that although PtsG and AglA recognized glucose and methyl-α-D-glucopyranoside, only AglA accepted other α-D-glycosides as substrates. Complementation experiments also revealed that IIA/Glc was required for functional activity of both PtsG and AglA transporters. We conclude that AglA, AglB, and IIA/Glc are necessary and sufficient for growth of *E. coli* K12 on methyl-α-D-glucopyranoside and related α-D-glycopyranosides.

For almost 50 years, methyl-α-D-glucopyranoside (α-MGlc) has served as a model substrate for probing the mechanism(s) of sugar transport in bacteria. Fortuitously (but importantly), α-MGlc is not metabolized by *Escherichia coli* K12, and transport data were interpretable without the ambiguities caused by further catabolism of the accumulated sugar. Early studies of α-MGlc uptake by cells of *E. coli* introduced the "permease" concept to bacterial physiologists, and "active transport" was incorporated in the lexicon of microbial energetics (1–5). The toxicity of α-MGlc toward *E. coli* was soon recognized, and Rogers and Yu (2) unexpectedly found that the glucose analog was recoverable from cells in both free and phosphorylated form. An understanding of these observations awaited the serendipitous discovery, and subsequent biochemical dissection, of the bacterial phosphoenolpyruvate-dependent sugar:phosphotransferase system (PEP-PTS; EC 2.7.1.69) by Roseman and co-workers (6, 7). The multicomponent PEP-PTS includes membrane-localized, sugar-specific transporters (enzymes IICB) that are fused or associated with a third domain (IIA) and two general cytoplasmic proteins (enzyme I and HPr). Collectively, these interactive components constitute a phospho-relay that (in five sequential stages) transfers the high energy phosphoryl moiety from PEP to catalyze the simultaneous phosphorylation and translocation of sugars through the cytoplasmic membrane (for reviews of PTS functions and nomenclature see Refs. 8–14). Completion of the chromosomal DNA sequence of *E. coli* K12 strain MG-1655 in 1997 (15), confirmed that ptsG encodes one (IICB/Glc/ptsG) of two glucose-specific PTS transporters, whereas the genes for the general proteins and IIA/Glc reside within a separate ptsHilccr operon. After the discovery of other PEP-PTS systems, it became apparent that α-MGlc was a reasonably specific substrate for the PtsG transporter in *E. coli*. Subsequently (16–19), it was established that α-MGlc6P was accumulated via PtsG to toxic concentrations and that intracellular α-MGlc was formed via dephosphorylation of α-MGlc6P (Fig. 1, left).

Our interest in the transport and bactericidal effects of α-MGlc6P in *E. coli* K12 stems from the fact that *Klebsiella pneumoniae* ATCC 23357 (a taxonomically close relative) readily ferments α-MGlc as an energy source for growth. Furthermore, and again in contrast with *E. coli* K12 strains, *K. pneumoniae* also metabolizes the following five O-α-linked isomers of sucrose: trehalulose, turanose, maltulose, leucrose, and palatinose (20, 21). We presented evidence (21) for a tricis-tronic α-glycoside (Agl) operon comprising genes aglR, aglA, and aglB. We suggested that the three genes encode (in order), a transcriptional regulatory protein (AglR), an α-gluoside PTS transporter AglA (IICB/Agl Swiss-Prot accession number Q9AGA7), and an Mn2+/NAD+/Mn2+-dependent phospho-α-glycosidase AglB (EC 3.2.1.122; Swiss-Prot accession number Q9AGA6). A potential pathway for transport and metabolism of α-glycosides in *K. pneumoniae* is illustrated in Fig. 1.
were purchased from Pfannstiehl Laboratories, Inc. Maltitol (4-0-α-glucopyranosyl-β-maltobios) and isomaltose (6-0-α-β-glucopyranosyl-β-glucose) were from Sigma and TCI America, respectively. The five linkage isomers of sucrose were obtained from the following sources: trehalulose was a generous gift from Stühzicker, Germany; turanose was from Pfannstiehl Laboratories; maltulose was from TCI America; leucrose was from Fluka; and palatinose was purchased from Wako Chemicals. The chromogenic and fluorogenic substrates, p-nitrophenyl α-β-glucopyranoside 6-phosphate (pNPαGlcp6P) and 4-methylumbelliferyl-α-β-glucopyranoside 6-phosphate (4MUαGlcp6P), respectively, were prepared by phosphorylation of the parent compounds with phosphorus oxychloride in trimethyl phosphate containing small proportions of water (23). The preparations of phosphorylated sucrose isomers and other α-β-glucosides 6-phosphates have been described previously (20). Enzymes, including glucose-6-phosphate dehydrogenase/hexokinase (EC 1.1.1.49/EC 2.7.1.1) and phosphoglucoisomerase (EC 5.3.1.9), were obtained from Roche Applied Science. Phosphoenolpyruvate (NAD+ 3, NADP+ 3, pNPαGlcp, 4MUαGlcp, and other reagents were purchased from Sigma. Polyclonal rabbit antibody raised against purified phospho-α-glucosidase (MalH) from Fusobacterium mortiferum (23) was prepared by Covance Research Products.

### Bacterial Strains and Plasmids—K. pneumoniae (ATCC 23357) and E. coli K12 strain MG-1655 (ATCC 47006) were obtained from the American Type Culture Collection, Manassas, VA. E. coli K12 ZSC112L (ΔptsG::cat manXYZ nag ε-6-glk lac) was from the culture collection of the Erni laboratory. This strain (abbreviated ZSC112L in this study) carries a chromosomal deletion of ptsG, such that recombination between the plasmid-encoded ptsG gene and inactive chromosomal alleles is precluded. Plasmid pTSGH11 encodes lacI and, under the control of Ptcp, a ptsG allele for the IICBα−ε− transporter attached to a C-terminal His tag. Construction of plasmids pAP1 and pAP2, carrying genes aglB alone or both aglA and aglB, respectively, are described below.

**Growth of Organisms**—Batch cultures of organisms (10-ml or 1-liter volumes) were grown with vigorous aeration on a rotary shaker (200 rpm; 37°C) in a medium described previously by Sapico et al. (24) for growth of K. pneumoniae. This defined medium (designated SM), contained per liter: 7.1 g of Na2HPO4; 1.5 g of KH2PO4; 3 g of (NH4)2SO4; 0.1 g of MgSO4.7H2O, and 5 mg of FeSO4.7H2O. Filter-sterilized sugars were added to autoclaved medium (pH 7.4) to a final concentration of 0.4% (w/v). In some instances, LB broth and M9 minimal medium (25) were used for growth studies of different transformants. Ampicillin (100 μg/ml) was included in media as required.

**Construction of pAP1 and Expression of aglB in E. coli MG-1655—** Using information from the incomplete genome sequence of K. pneumoniae subsp. pneumoniae MGH 78578 (Washington University Genome Sequencing Center, St. Louis) and previously published data (21), the following pair of primers was designed to amplify aglB: forward primer KPBF, 5’-CCCCACACTGAGGAATTC-3’ (the aglB sequence is in boldface and the Ncol restriction site is underlined); reverse primer KPBR, 5’-CTTATGATTTGACATTCTTGAATG-3’ (the sequence complementary to the downstream region of aglB is in boldface, and the EcoRI restriction site is underlined). PCR amplification was performed using high fidelity Pfu DNA polymerase. The amplified 1.3-kbp DNA fragment was digested with restriction endonucleases Ncol and EcoRI, respectively, electrophoresed through 1% agarose gel, and purified (QiAquick gel extraction kit). The 1.3-kb fragment was ligated into the similarly digested and purified expression vector pSE800 (Invitrogen) to yield plasmid pAP1. In this construct, the aglB gene is also under control of the trc promoter, and expression of AglB is induced in the presence of isopropyl β-D-thiogalactopyranoside (IPTG). The recombinant plasmid was electroporated into the cells, and transformants

### EXPERIMENTAL PROCEDURES

**Materials**—High purity sugars, including glucose, sucrose (α-β-glucopyranosyl β-β-fructofuranoside, α(1→2) linkage), maltose (4-0-α-β-glucopyranosyl-β-glucosepyranosyl-β-glucosepyranosyl), and 1-O-methyl-α-β-glucopyranoside,

![FIGURE 1. Schematic representation of the IIC α−ε− (PtsG)-mediated transport and phosphorylation of α-MGlc in E. coli K12 (left) and functional complementation of the α-glucoside-PTS transporter (AglA) from K. pneumoniae by IIAα−ε− of E. coli K12 (right).](image)
Transport and Metabolism of α-D-Glucosides by E. coli K12

MG-1655 (pAP1) were selected on LB agar plates containing 100 μg/ml ampicillin.

Construction of pAP2 and Expression of aglA and aglB in MG-1655—The following primer pairs were designed to amplify a fragment of DNA encoding adjacent genes aglA and aglB from chromosomal DNA of *K. pneumoniae* 23357: forward primer KP3, 5′-CCGACCTGAGCATGCTAGTCAAATACACGGC-3′ (aglA sequence is in boldface, and the XhoI restriction site is underlined); reverse primer KP4, 5′-CCCGAGATTCAACATCGCTAGCA-3′ (sequence complementary to the downstream region of aglB is in boldface, and the EcoRI site is underlined). Amplification was carried out using high fidelity *Pfu* DNA polymerase from Stratagene. The components of the amplification mixture were as follows: 100 ng of DNA, 1× reaction buffer provided by the manufacturer, 20 mM each of the four deoxyoligonucleotide triphosphates, 250 ng of each primer, 5 units of *Pfu* DNA polymerase, 25 mM Tris-HCl, and 1% (v/v) Me2SO in a total volume of 100 μl. Amplification was performed in a thermal cycler (GeneAmp PCR System 9700, PE Applied Biosystems). After a 2-min period of incubation at 95 °C, the mixture was subjected to five cycles under the following conditions: denaturation at 95 °C, 1 min; annealing at 50 °C, 1 min, and extension at 62 °C for 2 min/kb of insert. These five cycles were followed by 30 cycles under the following conditions: denaturation at 95 °C, 1 min; annealing at 58 °C, 1 min, and extension at 72 °C for 2 min/kb of insert. This procedure was followed by a 10-min runoff at 72 °C. The amplicon was digested with restriction endonucleases (XhoI and EcoRI), electrophoresed under nonreducing conditions (at 10 °C) in precast Tris-glycine (8%) gels. The running buffer for these experiments (Tris-glycine, 0.1 M, pH 8.3) was supplemented with 1 mM MnCl2, 0.1 mM DTT to maintain enzyme activity. Immediately after electrophoresis, and transformants MG-1655 (pAP2) were selected on Luria-Bertani agar plates containing 150 μg/ml ampicillin. It should be noted that in the pTrcHis2B vector the fusion peptide is located at the C terminus, rather than the N terminus as is the case in pTrcHis vectors.

The recombinant plasmid (pTrcHis2B aglAaglB) was transferred by electroporation, and transformants MG-1655 (pAP2) were selected on Luria-Bertani agar plates containing 150 μg/ml ampicillin. The following primer pairs were designed to amplify a fragment of DNA from progress plots, assuming a molar extinction coefficient of ε = 18,300 M⁻¹ cm⁻¹ for the (yellow) p-nitrophenolate anion. AglB activity was expressed as nanomoles of pNFP formed (pNPaGlc6P hydrolyzed) min⁻¹ mg⁻¹ protein⁻¹.

Electrophoresis Procedures and in Situ Detection of AglB Activity—Native (nondenaturing) electrophoresis and SDS-PAGE experiments were carried out in the Novex X-Cell mini system as described previously (26, 27). When polypeptide-containing slices were to be excised for tryptic digestion and LC-MS/MS, the SDS-polyacrylamide gels were fixed, stained with Colloidal Blue staining kit (Invitrogen), and destained in distilled water. Western (immunoblot) detection of AglB was achieved by sequential incubation of the membrane with (a) polyclonal antibody to phospho-α-glucosidase (MalH; EC 3.2.1.122) from *F. mortiferum*, and (b) goat anti-rabbit horseradish peroxidase-conjugated antibody (23). For *in situ* detection of AglB activity, cell extracts were electrophoresed under nonreducing conditions (at 10 °C) in precast Tris-glucone (8%) gels. The running buffer for these experiments (Tris-glucone buffer (pH 8.3)) was supplemented with 1 mM MnCl2, 0.1 mM NAD⁺, and 1 mM DTT to maintain enzyme activity. Immediately after electrophoresis, the gel was immersed in 30 ml of 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM MnCl2, 0.1 mM NAD⁺, 1 mM DTT, and 0.1 mM of glucogenic substrate (4MUαGlc6P). After ~5 min of incubation at room temperature, the gel was photographed under UV light (366 nm, 20s exposure) using a Nikon D70 digital camera equipped with a green filter.

Preparation of Cell Extracts—Stationary phase cultures were harvested by centrifugation (13,000 × g for 10 min at 5 °C), and the cells were washed by resuspension and centrifugation from 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM MnCl2, 1 mM NAD⁺, and 1 mM dithiothreitol (designated TMND buffer). The various cell pellets (3–4 g wet weight/liter) were homogenized in 2.5 volumes of TMND buffer, and organisms were disrupted (twice for 1.5-min periods of oscillation at 0 °C) in a Branson model 350 sonifier operating at ~75% of maximum power. The preparations were centrifuged (180,000 × g for 2 h at 5 °C), and the clarified high speed supernatant fluids were transferred to small tubes, frozen in dry ice, and stored at ~20 °C until required for enzymatic analysis and electrophoresis experiments.

Assays of NAD⁺/Mn2⁺-dependent AglB Activity—The activity of AglB (phospho-α-glucosidase) in extracts from cells of MG-1655, MG-1655 (pAP1), and MG-1655 (pAP2) was measured using chromogenic pNPaGlc6P as substrate. Enzyme activity was determined in a discontinuous assay that contained (in 2 ml) 50 mM Tris-HCl buffer (pH 7.5), 1 mM MnCl2, 1 mM NAD⁺, 1 mM DTT, and 0.5 mM pNPaGlc6P. After equilibration to temperature (37 °C), the reactions were started by addition of 15 μl of cell extract (350–400 μg of protein), and enzyme activity was followed as described previously (26). Absorbance was measured at 400mm, and the rates of pNP formation were calculated from progress plots, assuming a molar extinction ε = 18,300 M⁻¹ cm⁻¹ for the (yellow) p-nitrophenolate anion. AglB activity was expressed as nanomoles of pNP formed (pNPaGlc6P hydrolyzed) min⁻¹ mg⁻¹ protein⁻¹.

A continuous spectrophotometric method (27) was used to follow hydrolysis of α-glucoside-6-P substrates. This glucose-6-phosphate dehydrogenase/NADP⁺-coupled assay monitors the release of Glc6P, and the 1-ml assay contained the following: 0.1 M HEPES buffer (pH 7.5), 1 mM MgCl2, 1 mM MnCl2, 1 mM NAD⁺, 10 mM DTT, 10 μM Glc6P as substrate, and 3 units of glucose-6-phosphate dehydrogenase/hexokinase. Reactions were initiated by addition of 50 μl of cell extract (1–1.2 mg of total protein), and the increase in A340 nm was followed in a Beckman DU 640 recording spectrophotometer. Initial rates of Glc6P formation were determined using the kinetics program of the instrument. A molar extinction coefficient ε = 0.220 M⁻¹ cm⁻¹ was assumed for calculations of NADPH formed (Glc6P released). AglB activity was expressed as nanomoles of substrate hydrolyzed min⁻¹ mg⁻¹ protein⁻¹.

Electrophoresis Procedures and in Situ Detection of AglB Activity—Native (nondenaturing) electrophoresis and SDS-PAGE experiments were carried out in the Novex X-Cell mini system as described previously (26, 27). When polypeptide-containing slices were to be excised for tryptic digestion and LC-MS/MS, the SDS-polyacrylamide gels were fixed, stained with Colloidal Blue staining kit (Invitrogen), and destained in distilled water. Western (immunoblot) detection of AglB was achieved by sequential incubation of the membrane with (a) polyclonal antibody to phospho-α-glucosidase (MalH; EC 3.2.1.122) from *F. mortiferum*, and (b) goat anti-rabbit horseradish peroxidase-conjugated antibody (23). For *in situ* detection of AglB activity, cell extracts were electrophoresed under nonreducing conditions (at 10 °C) in precast Tris-glucone (8%) gels. The running buffer for these experiments (Tris-glucone buffer (pH 8.3)) was supplemented with 1 mM MnCl2, 0.1 mM NAD⁺, and 1 mM DTT to maintain enzyme activity. Immediately after electrophoresis, the gel was immersed in 30 ml of 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM MnCl2, 0.1 mM NAD⁺, 1 mM DTT, and 0.1 mM of glucogenic substrate (4MUαGlc6P). After ~5 min of incubation at room temperature, the gel was photographed under UV light (366 nm, 20s exposure) using a Nikon D70 digital camera equipped with a green filter.
were retained for analysis by SDS-PAGE and two-dimensional electrophoresis.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)—Coomassie-stained bands were excised from SDS-polyacrylamide gels, and proteins were digested robotically with trypsin (Mass PREP™ Station, Waters, Milford, MA). An aliquot of the tryptic peptides (1 pmol) was separated by LC-MS/MS. A micromass CapLC QTOF2 system (Micromass/Waters, Manchester, UK) consisting of a CapLC, an autosampler, and a QTOF2 mass spectrometer were used. MassLynx software was used for data acquisition and processing. The instrument was calibrated with a [Glu]fibrinopeptide standard. Tryptic peptides were concentrated online on a Waters Symmetry 300 C18 5-mum trap column and, after a 10-min delay, were directed onto a Vydac C18 reversed phase column (100 × 0.15 mm inner diameter). Peptides were separated (65 min; flow rate 1 ml min⁻¹) with a gradient comprising solvent A (98.8% water, 1% acetonitrile, 0.2% formic acid) and solvent B (98.8% acetonitrile, 1% water, 0.2% formic acid). Argon was used as the collision gas, and nitrogen gas was used to assist nebulization and desolvation. Acetonitrile, water of high pressure liquid chromatography gradient grade quality, and formic acid (96%) ACS grade were supplied by Sigma.

Preparation of Membranes from ZSC112L (pTSGH11) and ZSC112L (pAP2)—Transformants were grown in 250 ml of LB with requisite antibiotics on a rotary shaker at 37 °C. At A₆₀₀nm = 0.9, protein expression was elicited by addition of 0.5 mM IPTG, and growth was continued for 4 h. Cells were harvested by centrifugation (5800 × g; 5°C; 15 min), and the cell pellet was resuspended to a volume of 4 ml in buffer E (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 10 mM β-mercaptoethanol). Cells were disrupted by two passages through a French pressure cell. Intact cells and debris were removed by low speed centrifugation (12,000 × g; 4°C; 10 min), and membrane fractions were collected by ultracentrifugation (350,000 × g; 4°C; 10 min). The membrane pellets were homogenized in 500 µl of buffer E, and protein concentrations were determined by the Lowry procedure. The protein concentrations of the pAP2 and pTSGH11 membrane preparations were 25 and 32 mg/ml, respectively.

Assay and In Vitro Complementation of PEP-PTS Activities—PEP-catalyzed in vitro phosphorylation of sugars was determined by ion exchange, using [U-¹⁴C]glucose, α-[U-¹⁴C]Glc, or [U-¹⁴C]maltoolose as substrates. The “mastermix” used for the Pts assays contained the following: 50 mM potassium phosphate buffer (pH 7.5), 2.5 mM dithiothreitol, 2.5 mM NaF, 5 mM MgCl₂, 1 mM PEP, 0.5 mM ¹⁴C-labeled sugar (1000 dpm/nmol) and the following purified PTS components: EII (0.5 µl; 34.6 µM); HPr (2.4 µl; 277 µM); and IIA ⁴⁺ (5 µl; 4.7 µM). The reaction was started by the addition of (20–100-fold prediluted) membrane suspension to 95 µl of mastermix. After 30 min of incubation at 37°C, free and phosphorylated sugars were separated by anion exchange chromatography (AG-1-X2, 50–100 mesh; Bio-Rad), and radioactivity was determined by liquid scintillation counting. To demonstrate the dependence of AglA and IICB-Glu in E. coli MG-1655 on either PEP or phosphoenolpyruvate (PEP), a protein of comparable size and intensity was not present for functional activity of AglA or AglB in transformants.)

**TABLE 1**

| Sugar addition | MG-1655 | MG-1655 (pAP1) | MG-1655 (pAP2) |
|---------------|---------|----------------|----------------|
| Glucose       | 1.18    | 1.19           | 1.11           |
| α-MGlu        | 0.01    | 0.93           | 0.90           |
| Sucrose       | NDG     | 0.03           | 0.02           |
| Maltose       | 1.03    | 1.10           | 0.92           |
| Isomaltose    | 0.03    | 0.02           | 0.94           |
| Maltotriose   | 1.19    | 1.19           | 0.81           |
| Maltitol      | NDG     | 0.03           | 0.78           |
| Trehalose     | 1.10    | 0.98           | 1.01           |
| Trehalulose   | 0.03    | 0.01           | 1.12           |
| Turanose      | 0.04    | 0.01           | 0.87           |
| Maltulose     | 0.01    | NDG            | 0.95           |
| Leucrose      | 0.02    | 0.01           | 0.92           |
| Palatinose    | NDG     | NDG            | 0.90           |

*NDG indicates no detectable growth.

IPTG was added to cultures of MG-1655 (pAP1) and MG-1655 (pAP2), respectively. The organisms were harvested at the end of exponential growth, and both cytoplasmic and membrane fractions were prepared from the three strains (see “Experimental Procedures”). Analyses of cytoplasmic preparations from MG-1655 (pAP1) and MG-1655 (pAP2) by SDS-PAGE (Fig. 2, left) revealed high level expression of a polypeptide of the molecular weight expected (49 kDa) for phospho-α-glucosidase, AglB. A protein of comparable size and intensity was not present in a similarly prepared extract of MG-1655. Spectrophotometric assays, using pNPαGlc6P as the chromogenic substrate, confirmed AglB activity in the two former extracts (specific activity 25–30 nmol pNP formed min⁻¹ mg protein⁻¹), but no phospho-α-glucosidase activity was detectable in cell extracts of MG-1655.
TABLE 2

Hydrolysis of α-α-D-glucoside 6-phosphates by extracts from cells of MG-1655 and MG-1655 (pAP2) grown previously on different sugars

Rates expressed as nanomoles of substrate hydrolyzed (Glc6P formed), min⁻¹ mg protein⁻¹, are from two separate assays.

| Strain    | Growth sugar | pNPaGlc6P α-MGlc6P Maltose-6-P Sucrose-6-P Trehalulose-6-P Trehalose-6-P Maltulose-6-P Leucrose-6-P Palatinose-6-P |
|-----------|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| MG-1655   | Glucose      | NDH*            | 1.9             | 1.9             | 8.8             | 2.5             | 4.1             | 2.2             | 2.1             |
|           | Maltose      | NDH             | 1.8             | 2.5             | 7.8             | 1.9             | 3.4             | 3.2             | 2.5             |
| MG-1655   | Glucose      | 27.0            | 19.8            | 35.5            | 7.2             | 41.8            | 124.0           | 66.4            | 20.3            |
| (pAP2)    | Maltose      | 46.6            | 28.9            | 53.8            | 7.8             | 99.7            | 157.5           | 101.9           | 34.0            |
| α-MGlc    |              | 24.6            | 22.5            | 45.3            | 6.9             | 63.0            | 118.7           | 80.0            | 25.9            |
| Leucrose  |              | 64.2            | 36.1            | 64.3            | 7.5             | 88.0            | 165.6           | 104.5           | 37.5            |
| Palatinose|              | 51.3            | 27.6            | 56.5            | 6.5             | 72.5            | 136.3           | 82.3            | 31.8            |

* NDH indicates no detectable hydrolysis.

Growth of MG-1655 and Its Transformants on Selected Carbohydrates—The capacity of MG-1655, MG-1655 (pAP1), and MG-1655 (pAP2) cells to utilize carbohydrates as energy source(s) for growth was determined in SM supplemented with the appropriate sugar. Of 13 carbohydrates tested, glucose, maltose, maltotriose, and trehalose supported growth of MG-1655 mediated by the chromosomally encoded transporters PtsG, MalKFG, and TreB (Table 1, column 2). The transformant MG-1655 (pAP1) showed a similar sugar preference, but importantly this strain (containing AglB) exhibited excellent growth on α-MGlc (Table 1, column 3). With the exception of sucrose, MG-1655 (pAP2) grew well on all sugars tested, including α-MGlc, maltitol, and the five α-linked isomers of sucrose (Table 1, column 4). Cells were harvested from these cultures, and AglB activity was determined in cell extracts using chromogenic pNPaGlc6P as substrate. There was only background hydrolysis of pNPaGlc6P by extracts of nontransformed MG-1655 cells grown previously on either glucose or maltose (Table 2, column 3). However, similarly prepared extracts of MG-1655 (pAP2) cells grown previously on glucose, maltose, α-MGlc, leucrose, and palatinose contained high levels of AglB activity (specific activity 25–65 nmol of pNPaGlc6P hydrolyzed min⁻¹ mg protein⁻¹) (Table 2, column 3). Furthermore, E. coli MG-1655 (pAP2) grown on other α-glucosides including maltitol, isomaltose, trehalulose, turanose, and maltulose, also contained comparably high levels of phospho-α-glucosidase activity (data not shown).

Detection and Substrate Specificity of AglB in Extracts of MG-1655 (pAP2)—Results from enzymatic analyses (Table 2, column 3) were confirmed by experiments illustrated in Fig. 3. Standard SDS-PAGE and Coomassie Blue staining procedures (Fig. 3A) revealed high level expression of the ~49-kDa polypeptide (AglB) only in extracts from cells of MG-1655 (pAP2). A Western blot of a duplicate gel (Fig. 3B) showed that this polypeptide cross-reacted strongly with polyclonal rabbit antibody prepared against the NAD⁺/Mn²⁺-dependent phospho-α-glucosidase (MalH) from F. mortiferum (23). By sequence alignment, MalH and AglB exhibit 75% residue identity. Finally, non-denaturing PAGE and in situ hydrolysis of the fluorogenic substrate 4MUαGlc6P (Fig. 3C) revealed a single phospho-α-glucosidase activity in extracts of MG-1655 (pAP2). The "natural" substrates for AglB are formed by PEP-dependent phosphorylation via AgLA (IICβαβ) during growth on α-α-D-glucosides. Although not commercially available, a number of these PTS products have been prepared in our laboratory (20), and their availability permitted studies of substrate specificity of AglB to be conducted. From the results of these experiments (Table 2, columns 4–11), it is apparent that extracts of MG-1655 cells grown previously on glucose or maltose hydrolyzed the eight 6-phospho-α-glucosides at comparatively low rates (~2–8 nmol min⁻¹ mg protein⁻¹). In contrast, extracts prepared from cells of MG-1655 (pAP2) grown on a variety of sugars catalyzed the cleavage of these compounds at rates 10–40-fold greater than extracts of MG-1655. It is noteworthy that although the five phosphorylated isomers of sucrose were rapidly hydrolyzed by extracts of MG-1655 (pAP2), the rate of cleavage of sucrose-6-P was essentially that determined in extracts of MG-1655. These data provide confirmation (21) that sucrose-6-P per se is not a substrate for AglB.

Detection of the AgLA (IICβαβ) Transporter in Membranes of MG-1655 (pAP2)—Visual inspection of SDS-polyacrylamide gels and rapid formation of the yellow p-nitrophenolate anion from pNPaGlc6P con-
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For these studies, E. coli ZSC112L (ΔptsG::cat manXYZ nagE gik lac) was chosen as the host organism. This strain is unable to grow on glucose because of mutations and deletions in all systems that facilitate the transport and/or phosphorylation of glucose and α-MGlc (see “Experimental Procedures”). For unknown reasons, this strain grows poorly on maltose as the sole carbon source (28, 29). Transformation of this glucose-defective strain with pAP1 and pTSGH11, encoding AglA and PtsG, respectively, allowed unambiguous comparison of the substrate specificities of the two transporters. Membranes of the two ZSC112L transformants were prepared as described under “Experimental Procedures” and assayed for PEP-dependent sugar:phosphotransferase activity with radiolabeled glucose, α-MGlc, and maltose in the presence of the purified PTS components EI, HPr, and IIAGlc (see “Experimental Procedures”). The results of these complementation experiments are presented in Fig. 4. As anticipated, membranes containing PtsG readily phosphorylated both glucose and α-MGlc, and product formation was proportional to the amount of membrane included in the assay (Fig. 4A). Membranes prepared from the pAP2 transformants also catalyzed the PEP-dependent phosphorylation of glucose and α-MGlc (Fig. 4B). Importantly, however, the latter membranes also catalyzed the phosphorylation of maltose, whereas the PtsG transporter exhibited little, if any, affinity for the O-α-linked glucose disaccharide (Fig. 5). These findings established the following: (i) AglA was not only present but was also fully functional in membranes of ZSC112L (pTSGH11) and ZSC112L (pAP2), expressing transporters PtsG and AglA, respectively. After 30 min of incubation at 37 °C, sugar phosphorylation was determined as described under “Experimental Procedures.”

![FIG. 4. PEP-dependent in vitro phosphorylation of Glc (○) and α-MGlc (■) catalyzed by membrane-localized transporters, PtsG and AglA. The assay mixture (100 μl) contained PEP, 14C-labeled sugar, and purified PTS components EI, HPr, and IIAGlc. Reactions were initiated by addition of increasing amounts (μg) of cell membranes prepared from transformants ZSC112L (pTSGH11) and ZSC112L (pAP2), expressing transporters PtsG and AglA, respectively. After 30 min of incubation at 37 °C, sugar phosphorylation was determined as described under “Experimental Procedures.”](image)

### TABLE 3

Identification of AglA in membranes of MG-1655 (pAP2) by LC-MS/MS analyses provided physicochemical evidence for expression of AglA in cell membranes, but it was deemed essential to demonstrate functional activity of this transporter by *in vitro* and *in vivo* experiments. For these studies, *E. coli* ZSC112L (ΔptsG::cat manXYZ nagE gik lac) was chosen as the host organism. This strain is unable to grow on glucose because of mutations and deletions in all systems that facilitate the transport and/or phosphorylation of glucose and α-MGlc (see “Experimental Procedures”). For unknown reasons, this strain grows poorly on maltose as the sole carbon source (28, 29). Transformation of this glucose-defective strain with pAP1 and pTSGH11, encoding AglA and PtsG, respectively, allowed unambiguous comparison of the substrate specificities of the two transporters. Membranes of the two ZSC112L transformants were prepared as described under “Experimental Procedures.”

| Tryptic fragment (residue number) | M<sub>e</sub> (expected) | M<sub>c</sub> (calculated) | Amino acid sequence | M<sub>r</sub> (expected) | M<sub>r</sub> (calculated) | Amino acid sequence |
|-----------------------------------|--------------------------|---------------------------|--------------------|--------------------------|--------------------------|--------------------|
| T5 (72–87)                        | 1653.02                  | 1652.97                   | WPLGVNPLPGLPK      | 58.2 kDa                 | 58.2 kDa                 | WPLGVNPLPGLPK      |
| T6 (133–144)                      | 2263.29                  | 2263.36                   | TLDSPFGAIVSGLVALHNR | 57.9 kDa                 | 57.9 kDa                 | TLDSPFGAIVSGLVALHNR |
| T10 (203–215)                     | 1472.88                  | 1472.84                   | VQLGISLQAFLR       | 48.1 kDa                 | 48.1 kDa                 | VQLGISLQAFLR       |
| T28 (490–497)                     | 938.47                   | 938.43                    | TQGDLDFVK          | 60.3 kDa                 | 60.3 kDa                 | TQGDLDFVK          |

*Note:* The high expression of AglA (59 kDa) was not detected in membranes of MG-1655 (pAP1) but was confirmed high expression of AglB in cytoplasmic preparations from this transformant. However, evidence for expression of the membrane transporter AglA (IICB<sup>abcd</sup>) in cells of MG-1655 (pAP2) was more difficult to obtain. From the amino acid composition of AglA, theoretical molecular mass and pI values of ~58.3 kDa and 6.9, respectively, are predicted. Unfortunately, computerized comparisons of polypeptides present in two-dimensional electrophoretograms of the three membrane preparations failed to unequivocally identify AglA expected in MG-1655 (pAP2). However, results obtained by one-dimensional SDS-PAGE (Fig. 2, right) were encouraging, and the polypeptide(s) band at ~59 kDa in the membrane preparation of MG-1655 (pAP2) was more intense than the comparable band in the other two membrane preparations. The ~59-kDa polypeptide(s) present in membranes of MG-1655 (pAP2) was excised from the gel, and after incubation with trypsin, the digest was analyzed by LC-MS/MS. Four peptides were identified by LC-MS/MS that are unique to AglA (Table 3), thereby confirming the presence of IICB<sup>abcd</sup> in the membrane fraction of MG-1655 (pAP2). Although the sequence coverage for AglA was low (~9% of total), the probability based MOWSE score of 107 was high and confirmed the statistical significance of the data. A number of peptides derived from other MG-1655 proteins were also identified in the 59-kDa digest, including the tetradecameric chaperonin GroEL (subunit ~57.3 kDa; GenBank<sup>TM</sup> accession number AAA97042) and the dipeptide-binding/transport protein (~60.3 kDa; Swiss-Prot accession number P23847). It is of interest that membranes prepared from both MG-1655 (pAP1) and MG-1655 (pAP2) also contained significant amounts of AglB (Fig. 2, right). Indeed, tryptic digestion of this ~49-kDa band and subsequent LC-MS/MS yielded 20 peptides that encompassed ~80% of the complete sequence of the AglB. Host proteins, identified by their unique peptide fragments in this digest, included Trigger Factor (~48.1 kDa; Swiss-Prot accession number P22257) and the β-chain of ATP synthase (EC 3.6.3.14; ~50.2 kDa; Swiss-Prot accession number P00824).
activities of AglA and PtsG for α-MGlc are the same, the difference for maltose would be not only 30 but ~150-fold. Membranes prepared from the plasmid-free host strain failed to catalyze the \emph{in vitro} phosphorylation of the two sugars (data not shown).

Sugar PEP-PTS Activities of Both PtsG and AglA Transporters Are Dependent on the Presence of IIA^{Glc}—The rate of phosphorylation of α-MGlc by membrane fractions containing PtsG and AglA transporters increased in a saturable manner with respect to the amount of IIA^{Glc} present in the assay (Fig. 6). Complementation by IIA^{Glc} (from \textit{E. coli} K12) is clearly prerequisite for PEP-dependent phosphorylation of sugars by the IIC^{Glc} transporter.

Phenotypic Traits of pTSGH11 and pAP2 Transformants—The fermentative characteristics of the host strain ZSC112L, and its transformants containing pTSGH11 or pAP2, were determined by streaking of the organisms onto MacConkey indicator plates containing different sugars (25). The salient results from these \emph{in vivo} experiments (Table 4) include the following findings: (i) expression of PtsG (from pTSGH11) and AglA (from pAP2) permits fermentation and growth on glucose, and (ii) because expression of AglA is linked to that of AglB (phospho-α-glucosidase), the pAP2 transformants grew well on α-MGlc and formed large red colonies. By contrast, PtsG transformants although able to transport α-MGlc lack phospho-α-glucosidase activity, and the toxicity of accumulated α-MGlc6P was evident from the yellow color and very small size of the colonies. Confirmation of these qualitative findings was obtained by transfer of the transformants to liquid medium containing α-MGlc as the sole carbon source. Although ZSC112L containing pAP2 grew exponentially, the same strain transformed with pTSGH11 failed to grow at all (data not shown).

**DISCUSSION**

The aims of this investigation were to test the three hypotheses presented in the Introduction. Results presented in this study have validated the proposed hypotheses. Proof of hypothesis I was achieved by transformation of MG-1655 with a plasmid (pAP1) encoding the \textit{aglB} gene from \textit{K. pneumoniae}. Inspection of Fig. 1, left, shows that in MG-1655 (pAP1) the intracellular α-MGlc6P now becomes a substrate for the NAD^+/Mn^{2+}-dependent AglB. This unusual phospho-α-glucosidase catalyzes the hydrolysis of α-MGlc6P, via a series of redox and elimination reactions, to yield Glc6P and methanol. Toxicity is overcome, and intracellular Glc6P is fermented via the glycolytic pathway to generate the ATP necessary for growth of the cells. It is axiomatic that the hydrolytic activity of AglB (Fig. 1, left) must be considerably greater than that of the endogenous "phosphatase" to provide Glc6P at a rate commensurate with growth of the organism.

Unequivocal proof of hypothesis II was obtained from growth studies conducted with transformants of \textit{E. coli} MG-1655 (Table 1). From these data, it is clear that when transformed with plasmid pAP2 (encoding \textit{aglA} and \textit{aglB}), MG-1655 acquires the additional capacity to metabolize not only α-MGlc but also maltool, isomaltose, and all five isomers of sucrose. Importantly, the detection of AglA-specific peptides by LC-
MS/MS and in vitro complementation of functional PTS<sup>Agl</sup> activity provided unequivocal evidence that AglA is the membrane transporter for α-glucosides in <i>K. pneumoniae</i> and K12 transformants. Although technically simple in execution, the transference and expression of the product of <i>aglA</i> in <i>E. coli</i> K12 were not trivial exercises nor was the experimental outcome predictable. Failure of AglA to be incorporated into the membrane of K12 was a distinct possibility, particularly if host-specific chaperones or other unknown proteins were required for membrane insertion. This crucial experiment would also have failed if AglA-associated porins present in <i>K. pneumoniae</i> were absent from the membranes of the <i>E. coli</i> K12 recipient. Finally, the possibility that the presence of a "foreign" transporter might prove lethal to K12 was of some concern to us. Clearly, any of these factors would have precluded growth of K12 transformants and rendered impossible the assignment of function and sugar specificity to AglA.

As mentioned previously, the <i>agl</i> operon of <i>K. pneumoniae</i> lacks a gene for the third phospho-transfer protein (IIA<sup>Agl</sup>) required for functional complementation of the membrane-localized IICB<sup>Agl</sup> transporter. The absence of IIA genes has also been described for other PTS operons, including those for trehalose (<i>tre</i>) and sucrose (<i>scr</i>) in <i>Bacillus subtilis</i> (30, 31) and in the plasmid-encoded PTS<sup>Agl</sup> operon of <i>E. coli</i> K12 (32, 33). In these instances it has been found that IIA<sup>Glc</sup> of the host can substitute for the "missing" protein to yield a functional sugar-specific PTS. With these reports as precedent, it seemed reasonable that in <i>K. pneumoniae</i> IIA<sup>Glc</sup> might also fulfill a complementary role for IICB<sup>Agl</sup> activity. The proof of this proposal, hypothesis III, was obtained by in vitro complementation experiments, using purified, individual PTS components, including IIA<sup>Glc</sup> and membrane fractions derived from <i>E. coli</i> ZSC112L. Because IIA<sup>Glc</sup> of <i>E. coli</i> and <i>K. pneumoniae</i> share >90% sequence identity, it is likely that complementation between IIA<sup>Glc</sup> and AglA is also operative in <i>K. pneumoniae</i>. Transformation of the glucose-defective <i>E. coli</i> strain, with plasmids expressing PtsG and AglA, allowed comparison of the substrate specificities of the two transporters. Although both PtsG and AglA accept glucose and α-MGlc, only AglA catalyzed the phosphorylative translocation and permitted growth of the transformed cells on other α-glucosides. It is of interest to note that GvC and GvB of <i>E. coli</i> K12 exhibit 86 and 68% amino acid sequence identity, respectively, with the C and B domains of AglA. However, the former domains must be nonfunctional, as judged from the low background activity of the nontransformed <i>E. coli</i> K12 host (Tables 1, 2, and 4). These observations support the earlier proposal by Reizer et al. (34) for crypticity of the giv operon in <i>E. coli</i>. In summary, we propose that three proteins, AglA, AglB, and IIA<sup>Glc</sup>, are necessary and sufficient for growth of enteric microorganisms on α-MGlc and related O-α-linked glucosides by the pathway illustrated in Fig. 1, right.

Substrate Discrimination by AglB—Transformant MG-1655 (pAP2) grew readily on the isomers of sucrose but, surprisingly, failed to metabolize sucrose itself as an energy source for growth (Table 1). Furthermore, whereas the NAD<sup>+</sup>/Mn<sup>2+</sup>-dependent phospho-α-glucosidase (AglB) readily hydrolyzed the five phosphorylated isomers, the enzyme failed to accept sucrose-6-P per se as substrate (20, 21) (Table 2, column 6). Several factors, including the solution state conformation of substrates and the active site architecture of the enzyme, must contribute to the stringent specificity of AglB. Previous molecular dynamics simulations provide evidence that the molecular geometry of sucrose-6-P in solution is quite different from the conformations assumed by the five phosphorylated isomers (21, 35). It is likely that a water bridge between Glc-2-O—H<sub>2</sub>O—O-1-Fru serves to "lock" the molecule of sucrose-6-P into a globular and energetically more favorable structure (Fig. 7). Intramolecular water bridges cannot be formed between —OH groups of the phosphorylated isomers, and these compounds assume an extended, more linear conformation in solution (Fig. 7). Furthermore, and in contrast with sucrose-6-P, the Glc6P and fructosyl moieties of the isomeric 6-phosphates. Conformational analyses of the compounds in the solution state were accomplished by molecular dynamics simulations (21, 35).
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a series of oxidation, elimination, addition, and reduction steps (42–45), in a reaction mechanism unanticipated during the past 50 years of research on glycoside hydrolysis. Amino acids at the active site of GlvA (shown in boldface) are positionally conserved in AglB as follows: Ser-15 (13); Asp-41 (39); Arg-95 (93); Glu-111 (109); Asn-149 (147); Cys-171 (169); Asp-172 (170); His-202 (200); Tyr-265 (263); Arg-285 (283); and Glu-359 (357). Furthermore, comparative modeling of AglB with 1U8X as template shows that the structures of the two phospho-α-glucosidases are virtually identical. Future attempts to “fit” the solution-state models of the phosphosucro-derivatives (Fig. 7) into the active site(s) of GlvA and AglB should provide insight to the molecular basis for substrate discrimination by these catalytically unique hydrolases.

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