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Phospho-β-Glucosidase from *Fusobacterium mortiferum*: Purification, Cloning, and Inactivation by 6-Phosphoglucono-δ-Lactone

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6-Phosphoryl-β-D-glucopyranosyl:6-phosphoglucohydrolase (P-β-glucosidase, EC 3.2.1.86) has been purified from *Fusobacterium mortiferum*. Assays for enzyme activity and results from Western immunoblots showed that P-β-glucosidase (M_r, 53,000; pI, 4.5) was induced by growth of *F. mortiferum* on β-glucosides. The novel chromogenic and fluorogenic substrates, *p*-nitrophenyl-β-D-glucopyranoside-6-phosphate (pNPβGlc6P) and 4-methylumbelliferyl-β-D-glucopyranoside-6-phosphate (4MUβGlc6P), respectively, were used for the assay of P-β-glucosidase activity. The enzyme hydrolyzed several P-β-glucosides, including the isomeric disaccharide phosphates cellobiose-6-phosphate, gentiobiose-6-phosphate, sophorose-6-phosphate, and laminaribiose-6-phosphate, to yield glucose-6-phosphate and appropriate aglycons. The kinetic parameters for each substrate are reported. P-β-glucosidase from *F. mortiferum* was inactivated by 6-phosphoglucono-δ-lactone (P-glucono-δ-lactone) derived via oxidation of glucose 6-phosphate. The *pbgA* gene that encodes P-β-glucosidase from *F. mortiferum* has been cloned and sequenced. The first 42 residues deduced from the nucleotide sequence matched those determined for the N terminus by automated Edman degradation of the purified enzyme. From the predicted sequence of 466 amino acids, two catalytically important glutamyl residues have been identified. Comparative alignment of the amino acid sequences of P-β-glucosidase from *Escherichia coli* and *F. mortiferum* indicates potential binding sites for the inhibitory P-glucono-δ-lactone to the enzyme from *F. mortiferum*.

Fusobacteria are important human pathogens that collectively comprise a genus of the *Bacteroidaceae* family of microorganisms (14, 23). Most species of Fusobacteria rely upon amino acid fermentation to provide requisite energy for growth, but *F. mortiferum* has the additional capacity to utilize a wide variety of carbohydrates, including monosaccharides and α- and β-glucosides as fermentable energy sources. Our interest in the mechanisms of amino acid and sugar utilization by Fusobacteria stems from the fact that the end products of these metabolic pathways include lactic, acetic, propionic, and butyric acids. These organic acids are cytotoxic for epithelial and other tissue cells, and Fusobacteria are believed to be causative agents or a contributing factor in the etiology of oral and other diseases.

Studies in our laboratory (30, 31, 45) provided the first evidence for the operation of the phosphoenolpyruvate-dependent sugar:phosphotransferase system (PEP:PTS) in Fusobacteria. Although discovered serendipitously by Saul Roseman and his colleagues in *Escherichia coli* (16, 34), this multicomponent system (35) is now recognized as the primary mechanism for the simultaneous translocation and phosphorylation of sugars by bacteria from both gram-positive (11, 29, 43) and gram-negative (22, 28) genera. Catabolism of disaccharides that are accumulated by the PEP-PTS as phosphorylated derivatives requires the cleavage of these compounds by intracellular substrate-specific phospho-glycosylhydrolases, such as 6-phospho-β-glucosidase (P-β-glucosidase) (EC 3.2.1.86). Genes encoding putative P-β-glucosidase(s) have been cloned and sequenced from *E. coli* (10, 26, 36), *Bacillus subtilis* (53), and *Erwinia chrysanthemi* (6) but surprisingly the enzyme has been purified to homogeneity only from *E. coli* (49). (A partial purification of the enzyme from *Klebsiella pneumoniae* was reported some 25 years ago by Palmer and Anderson [25]). Purification of P-β-glucosidase from *F. mortiferum* is essential to our elucidation of the enzymes and mechanisms involved in the regulation of the β-glucoside fermentation pathway in this organism. Additionally, because this phospho-β-glucohydrolase has not previously been described in anaerobic bacteria, purification of this enzyme from *F. mortiferum* would also permit a comparative study with P-β-glucosidase from *E. coli*.

Preliminary studies revealed high levels of P-β-glucosidase activity in cells of *F. mortiferum* grown on β-glucosides, and cell extracts rapidly hydrolyzed pNPβGlc6P or 4MUβGlc6P to yield Glc6P and either the yellow *p*-nitrophenolate ion or highly fluorescent 4-methylumbelliferone aglycons, respectively. In the earlier studies with *E. coli* and *K. pneumoniae*, P-β-glucosidase activities were measured by the rate of Glc6P formation from phosphorylated glucosides in the standard $NADP^+$-dependent glucose 6-phosphate dehydrogenase (G6PDH) assay. However, attempts to demonstrate P-β-glucosidase activity in cell extracts of *F. mortiferum* by this continuous spectrophotometric procedure were unsuccessful. It became apparent that the presence of $NADP^+$-dependent G6PDH was either directly or indirectly causing inhibition of the fusobacterial enzyme.

In this communication we first provide enzymatic and photographic documentation of the inhibitory action of $NADP^+$-dependent G6PDH upon P-β-glucosidase from *F. mortiferum*. Second, we report the purification and physicochemical properties of the P-β-glucosidase. Finally, we describe the cloning and nucleotide sequence of the gene, *pbgA*, that encodes P-β-glucosidase from *F. mortiferum*. From these data, we offer an explanation(s) for the unique mode of inactivation of fusobacterial P-β-glucosidase that occurs in the presence of $NADP^+$-dependent G6PDH.

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MATERIALS AND METHODS

Materials. Electrophoresis reagents, standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and protein assay reagents were obtained from Bio-Rad Laboratories. Protein calibration kits and PD-10 columns for gel filtration chromatography, isoelectric focusing (IEF) standards, Ampholine PAG plates, DEAE-Sephacel and phenyl-Sepharose-CL-4B were purchased from Pharmacia-LKB Biotechnology, Inc. Ultrogel AcA 54 and DEAE-TrisAcryl M were supplied by Sepracor. Highest purity carbohydrates were obtained from Pfanstiehl Laboratories. Sophorose was supplied by Adams Chemical Co. Enzymes, nucleotide cofactors, nitrophenyl glycosides, and other reagents were purchased from Sigma Chemical Co., and [U-^{14}C] Glc6P (specific activity, 208 mCi/mmol) was from ICN Pharmaceuticals. Trimethylphosphate, phosphorus oxychloride, pyridine, and cyclohexylamine were obtained from Aldrich Chemical Co., Inc. Centricon-10 concentration units, Diaflo PM-10 ultrafiltration membranes, and pressure cells were supplied by Amicon Corp., Danvers, Mass. Matrix-assisted laser desorption/ionization-mass spectra (MALDI-MS) were obtained on a Kratos MALDI 111 instrument operated at an accelerating voltage of 22 kV. Proteins were dissolved in 0.1% (vol/vol) trifluoroacetic acid and applied to the target. After coating with a sinapic acid matrix, the sample target was dried under vacuum. The singly and doubly charged ions of bovine serum albumin were used as internal calibration standards to provide greater accuracy in mass determinations.

Preparation of P-β-glucosides. The preparation of pNPβGlc6P, pNPα-D-mannopyranoside-6P, pNP-α-D-galactopyranoside-6P, and the novel 4MUβGlc6P was by the procedure described earlier for the synthesis of pNPαGlc6P (44, 49). Disaccharide phosphates were prepared enzymatically by use of ATP-dependent β-glucoside kinase from *K. pneumoniae* (24).

Preparation of P-glucono-δ-lactone. 6-Phospho-glucono-δ-lactone (P-glucono-δ-lactone) was prepared enzymatically by oxidation of Glc6P with $NADP^+$-dependent G6PDH (4, 37). The 2-ml reaction mixture contained 5 mM Glc6P, 5 mM $NADP^+$, 10 U of yeast G6PDH, and 0.1 M imidazole-HCl buffer (pH 7). After 15 min of incubation, the solution was centrifuged in a Centricon 10 filtration device to remove G6PDH. Analysis of the clarified filtrate revealed a decrease in Glc6P concentration of 56%, and it was assumed that this amount of hexose phosphate had been converted to P-glucono-δ-lactone. The compound was prepared fresh each time and, because of the high rate of spontaneous hydrolysis to 6P-gluconate, was used as soon as possible.

Binding of [U-^{14}C]P-glucono-δ-lactone to P-β-glucosidase. The inhibitor, [U-^{14}C]P-glucono-δ-lactone, was prepared in a reaction mixture of 0.5 ml that contained 0.1 M imidazole-HCl buffer (pH 7.0), 100 nmol of $NADP^+$, 24 nmol (5 μCi) of [U-^{14}C]Glc6P, and 5 U of yeast G6PDH. After 10 min of incubation, the mixture was transferred to a Centricon 10 microconcentrator and centrifuged for 20 min to remove G6PDH. The filtrate (~450 μl), which contained ^{14}C-labeled P-glucono-δ-lactone, [^{14}C]6P-gluconate, and unreacted [U-^{14}C]Glc6P, was collected and mixed with 250 μg (4.7 nmol) of P-β-glucosidase from *F. mortiferum*. The mixture was incubated for 2 min at room temperature, and the volume was then adjusted to 2.5 ml with 50 mM imidazole-HCl buffer (pH 7.0). The solution was transferred to a PD-10 column and separated into high- and low-molecular-weight components by gel filtration. Fractions (0.6 ml) were collected, and those fractions (5 to 9, inclusive) that contained both P-β-glucosidase (A_{280}) and ^{14}C-labeled material were pooled and concentrated in a 10-ml Amicon concentrator. The preparation was twice reconstituted, and concentrated, from 5 ml of 50 mM imidazole-HCl buffer (pH 7.0) so as to remove nonadsorbed low-molecular-weight compounds. The concentrate (~0.5 ml) was adjusted to 2.5 ml with buffer, and the solution was passed through a PD-10 column previously equilibrated with 50 mM imidazole-HCl buffer (pH 7.0).

Organism and culture maintenance. *F. mortiferum* ATCC 25557 was maintained and grown under anaerobic conditions as described previously (32).

Purification of P-β-glucosidase. Cells of *F. mortiferum* were harvested by centrifugation (13,000 × *g* for 10 min at 4°C) from 12 liters of anaerobic culture containing cellobiose as energy source. The cells (50 g [wet weight]) were washed by resuspension and centrifugation from 25 mM HEPES buffer (pH 7.5). The cells were homogenized to a volume of about 95 ml with 25 mM HEPES buffer (pH 7.5), and the organisms were disrupted at 0°C by two 1.5-min periods of sonication in a Branson model 350 sonifier operated at 75% of maximum power. Unless otherwise stated, all procedures were performed in a cold room at 4 to 8°C. For column chromatography, flow rates were maintained by a P-1 peristaltic pump interfaced to a Frac-100 collector (Pharmacia-Biotech). Eluents were monitored at 280 nm by a UV-1 optical control unit connected to a single-channel chart recorder (REC-481, Pharmacia-Biotech). Purification of the enzyme was achieved in five stages: (i) preparation and dialysis of a high-speed supernatant (HSS) fluid, (ii) DEAE-TrisAcryl M ion-exchange chromatography, (iii) Ultrogel AcA 54 gel filtration chromatography, (iv) Phenyl-Sepharose CL-4B hydrophobic chromatography, and (v) DEAE-Sephacel ion-exchange chromatography. The final stage yielded approximately 4 mg of electrophoretically pure enzyme. P-β-glucosidase activity in column fractions was detected by the yellow color formed by the hydrolysis of pNPβGlc6P in a microtiter plate assay. Usually, 10 μl of the column fraction was added to wells containing 100 μl of a solution of 50 mM imidazole-HCl buffer (pH 7) containing 1 mM pNPβGlc6P.

Assay of P-β-glucosidase activity. The activity of P-β-glucosidase at the different stages of purification was determined by use of pNPβGlc6P as substrate. The 4-ml reaction mixture of 50 mM imidazole-HCl buffer (pH 7) containing 1 mM pNPβGlc6P was warmed to 37°C in a water bath, and the enzyme preparation was added. At intervals (usually) of 20, 40, 60, 90, and 120 s, 0.5-ml samples of the reaction were withdrawn and mixed with 0.5 ml of 0.5 M Na_2CO_3 solution. The absorbance of the yellow solution was measured at 400 nm, and the amount of pNP in the 1-ml samples was calculated by assuming a molar extinction for the *p*-nitrophenolate anion of $\varepsilon = 18{,}300\ M^{-1}\ cm^{-1}$ at pH 10.2. One unit of P-β-glucosidase is that amount of enzyme that catalyzes the formation of 1 μmol of pNP per min at 37°C.

Kinetic studies. A discontinuous end point assay was used for studies with the fusobacterial enzyme. For these experiments, 1-ml volumes of 0.1 M imidazole-HCl buffer (pH 7.0) containing increasing concentrations of appropriate P-β-glucosides were dispensed into 3-ml glass vials. The reactions were begun by addition of 10 μl (2.5 μg) of purified P-β-glucosidase from *F. mortiferum*. After 1 min of incubation, the vials were capped and transferred immediately to boiling water for 1 min to inactivate the hydrolase. This procedure was repeated for all samples in the series. When cooled to room temperature, $NADP^+$ (0.5 mM) and 2 U of G6PDH were added to each sample and the absorbance increase at 340 nm was monitored in a Beckman DU-70 spectrophotometer. The end point determination of NADPH (= Glc6P released) allowed calculation of *v*, the rate of substrate hydrolysis. Kinetic parameters (V_{max} and K_m) were calculated by fitting the data to the equation: $v = V_{max} \cdot S/(K_m + S)$ (Abelbeck Software) by means of the KaleidaGraph Program (S is the concentration [millimolar] of substrate in the assay). For studies of 4MUβGlc6P hydrolysis, the assay contained 1 ml of 0.1 M imidazole-HCl buffer (pH 7.0) and appropriate concentrations (0 to 0.5 mM) of 4MUβGlc6P. The reaction was started by addition of 0.5 μg of P-β-glucosidase, and after 30 s the reaction was stopped by addition of 1 ml of 0.5 M glycine-NaOH buffer (pH 10.3). The absorbance of the solution was recorded at 360 nm (19), and the amount of 4MU liberated was determined from a calibration curve of 4MU (0 to 0.05 μmol per ml) in 0.2 M glycine-NaOH buffer (pH 10.3).

Preparation of antibody. Polyclonal antiserum against *F. mortiferum* P-β-glucosidase was prepared in New Zealand White rabbits by Hazelton Research Laboratories, Inc., Denver, Pa.

Immunodetection of P-β-glucosidase. Proteins in cell extracts were separated by SDS-PAGE (Novex, Tris-glycine 4 to 20% gradient gels) and transferred electrophoretically to a nitrocellulose membrane. P-β-glucosidase was detected with polyclonal antibody as described in a previous communication (44).

Screening of a DNA library from *F. mortiferum*. Genomic DNA was prepared from *F. mortiferum* 25557 as described by Silhavy et al. (39). A partial *Sau*3A1 digestion was performed, and the resulting fragments were separated on a sucrose gradient (2). Fragments of 3 to 12 kb in size were pooled and ligated into the *Bam*HI-digested phage vector ZAP Express (Stratagene). The resulting library was plated for expression in *E. coli* XL1-Blue MRF′ as directed by the manufacturer. Phage-encoded proteins in the plaques were transferred to nitrocellulose filters and screened for reactivity with P-β-glucosidase polyclonal antisera by using goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (2). Plaques producing immunoreactive material were excised, amplified, and retested. A plasmid containing the *F. mortiferum* genomic DNA was then excised from the phage clone by employing helper phage as directed by the manufacturer (Stratagene).

PCR amplification of *pbgA*. The DNA fragment encoding the N-terminal 85 amino acids of P-β-glucosidase was amplified from *F. mortiferum* 25557 DNA by PCR (15). One primer was complementary to nucleotides 321 to 354 of *pbgA*. The other primer was a degenerate primer [5′TCATTTCC(A/T)AAA(A/G)A ATTTTTATGGGG-3′] whose sequence was derived by reverse translation of 22 residues from the N terminus of purified P-β-glucosidase. The codon usage for this primer was based upon that obtained from residues 86 to 466 of P-β-glucosidase, which were encoded by a phage clone. The PCR mixture contained the following components in 100 μl: 1× PCR buffer (Perkin-Elmer Cetus, Inc.), 0.5 mM $MgSO_4$, 40 mM nucleotide triphosphates, 25 pmol of each primer, 2.5 ng of *F. mortiferum* 25557 DNA, and 2.5 U of Ampli*Taq* polymerase (Perkin-Elmer Cetus, Inc.). The amplification reaction consisted of five cycles at a low annealing temperature (93°C, 30 s; 37°C, 1 min; 72°C, 1 min) followed by 25 cycles at a higher annealing temperature (93°C, 30 s; 45°C, 1 min; 72°C, 1 min). The PCR product was purified (Wizard PCR Preps, Promega, Inc.), ligated into the plasmid pCR-Script (Stratagene), and sequenced as described below.

DNA sequencing and analysis. *F. mortiferum* DNA fragments present in plasmids pCelH-1 and pCelH-NT were sequenced by the dideoxy chain-termination method with Sequenase 2.0 T7 polymerase (U.S. Biochemicals, Inc.). Nucleotides 1144 to 2654 were sequenced by PCR-based automated sequencing (DNA Technologies, Inc.). Nucleotide sequence analysis was carried out with the Genetics Computer Group suite of programs (Version 8, Genetics Computer Group, Madison, Wis.). The FASTA (27) and BLAST (1) algorithms were used to search the Swiss-Protein (Release 33) and PIR-Protein (Release 48) databases for homologous sequences. Amino acid sequences were aligned with the Genetics Computer Group Gap program.

Nucleotide sequence accession number. The GenBank accession number for the sequence reported here is U81184.

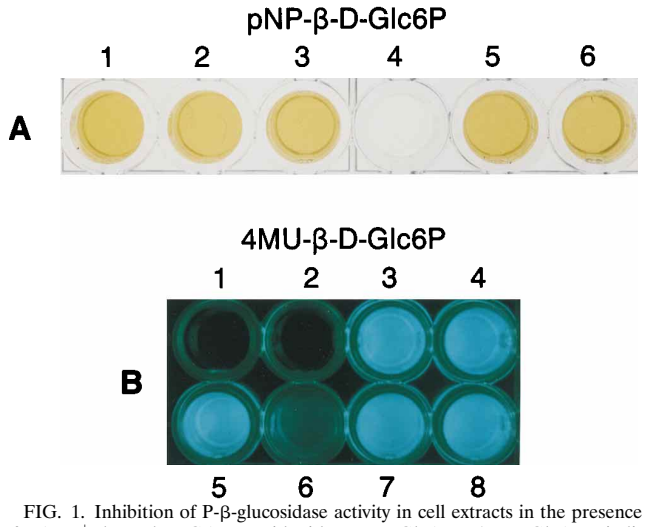

FIG. 1. Inhibition of P-β-glucosidase activity in cell extracts in the presence of $NADP^+$-dependent G6PDH with either pNPβGlc6P or 4MUβGlc6P as indicated as substrate for the enzyme. Each well contained 200 μl of complete assay solution comprising 50 mM HEPES buffer (pH 7.5), 0.5 mM P-β-glucoside, and 5 μl (~100 μg protein) of HSS extract from cellobiose-grown cells of *F. mortiferum*. Additions of either 0.5 U of yeast G6PDH and/or 1 mM $NAD(P)^+$ were made to the appropriate wells. (A) pNPβGlc6P. Wells: 1, control (no addition); 2, $NADP^+$; 3, G6PDH; 4, $NADP^+$ and G6PDH; 5, NAD^+; 6, NAD^+ and G6PDH. (B) 4MUβGlc6P. Wells: 1, no extract; 2, no substrate; 3, control (no additions); 4, $NADP^+$; 5, G6PDH; 6, $NADP^+$ and G6PDH; 7, NAD^+; 8, NAD^+ and G6PDH.

RESULTS

P-β-glucosidase activity in *F. mortiferum*. An extract prepared from cellobiose-grown cells of *F. mortiferum* rapidly hydrolyzed the chromogenic substrate pNPβGlc6P to yield Glc6P and the yellow *p*-nitrophenolate ion. (Rate = 2.42 μmol of pNPβGlc6P cleaved per min per mg of protein.) The isomeric analogs pNPαGlc6P and oNP-β-galactopyranoside-6P were cleaved at 2.5% and 5.4%, respectively, of the rate of pNPβGlc6P. There was no detectable hydrolysis of pNP-α-galactopyranoside or pNP-α-mannopyranoside in either phosphorylated or nonphosphorylated forms (data not shown).

Inhibition of P-β-glucosidase in the presence of $NADP^+$-dependent G6PDH. In addition to the chromogenic analog pNPβGlc6P, we also prepared a variety of other P-β-glucosides for use as substrates for the continuous spectrophotometric determination of P-β-glucosidase activity. The following reactions formed the basis for the assay:

$$\text{P-β-glucoside} \xrightarrow{\text{P-β-}glucosidase} \text{Glc6P} + \text{aglycon} \qquad \text{(reaction 1)}$$

$$\text{Glc6P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{P-glucono-δ-lactone} + \text{NADPH} \qquad \text{(reaction 2)}$$

$$\text{P-glucono-δ-lactone} \xrightarrow{H_2O} \text{6P-gluconate} \qquad \text{(reaction 3)}$$

However, in repeated attempts with cell extracts, we failed to generate the NADPH expected upon inclusion of cellobiose-6P, gentiobiose-6P, or any other P-β-glucosides in this assay. Separately, reactions 1 and 2 were shown to be operative, but no P-β-glucosidase activity was detectable when the two steps were coupled. Insight into this problem was provided by the results of microtiter assays in which chromogenic (pNPβGlc6P) and fluorogenic (4MUβGlc6P) analogs served as substrates for the enzyme (Fig. 1A and B, respectively). The addition of $NADP^+$ or G6PDH to separate wells had qualitatively little effect upon P-β-glucosidase activity as revealed by the immediate formation of the pNP (yellow) and 4MU (fluorescent) aglycons. However, there was no discernible hydrolysis of either substrate when $NADP^+$ and G6PDH were present in the same reaction mixture (Fig. 1A, well 4, and Fig. 1B, well 6).

TABLE 1. Summary of the purification of P-β-glucosidase from *F. mortiferum* ATCC 25557

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
1. Dialyzed HSS	2,320	6,184	2.7	1	100
2. DEAE-TrisAcryl M	860	4,348	5.1	2	70
3. Ultrogel AcA 54	73	2,776	38	14	45
4. Phenyl-Sepharose-CL-4B	17	782	46	17	13
5. DEAE-Sephacel	4.2	256	61	23	10

Purification of P-β-glucosidase. To understand the nature of this unexpected inhibition, it was necessary to purify the P-β-glucosidase from *F. mortiferum* (Table 1). This was achieved by conventional chromatographic procedures with pNPβGlc6P as the substrate for the assay of enzyme activity. Salt gradient elution of protein from a column of DEAE-Sephacel (Table 1, step 5) yielded ~4 mg of electrophoretically pure P-β-glucosidase. The enzyme was purified 23-fold to a specific activity of 61 U/mg, and the recovery was about 10%. The visual demon-

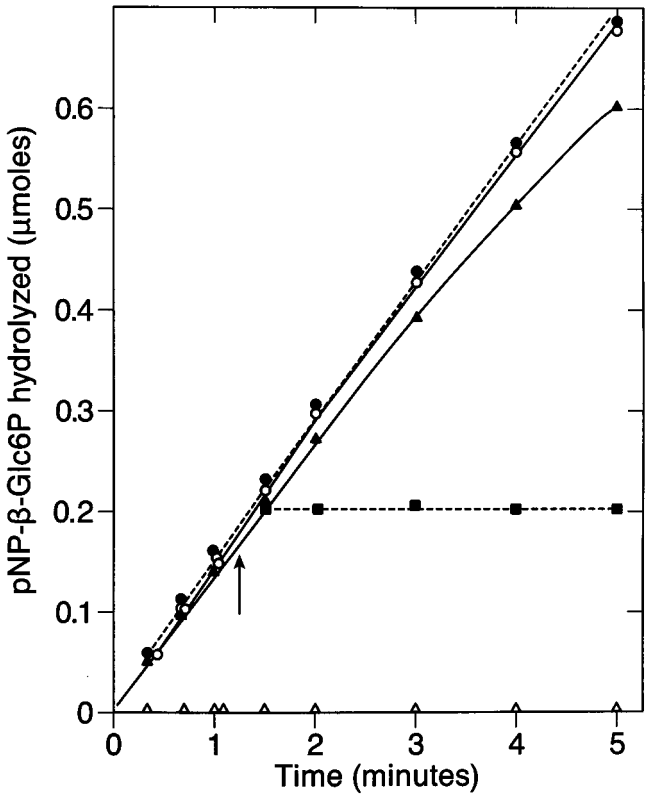

FIG. 2. Inhibitory effect of $NADP^+$ plus G6PDH upon purified P-β-glucosidase from *F. mortiferum*. The 5-ml assay contained 0.1 M imidazole-HCl buffer (pH 7) and 1 mM pNPβGlc6P and, as appropriate, the following supplements: no addition (control) (●), 2 U of G6PDH (○), 0.1 mM $NADP^+$ (▲), 2 U of G6PDH and 0.1 mM $NADP^+$ (△), and 2 U of G6PDH followed by the addition at t = 1.5 min (arrow) of 0.1 mM $NADP^+$ (■). Reactions were initiated by addition of 2.5 μg of purified P-β-glucosidase, and enzyme activity was measured as described in Materials and Methods.

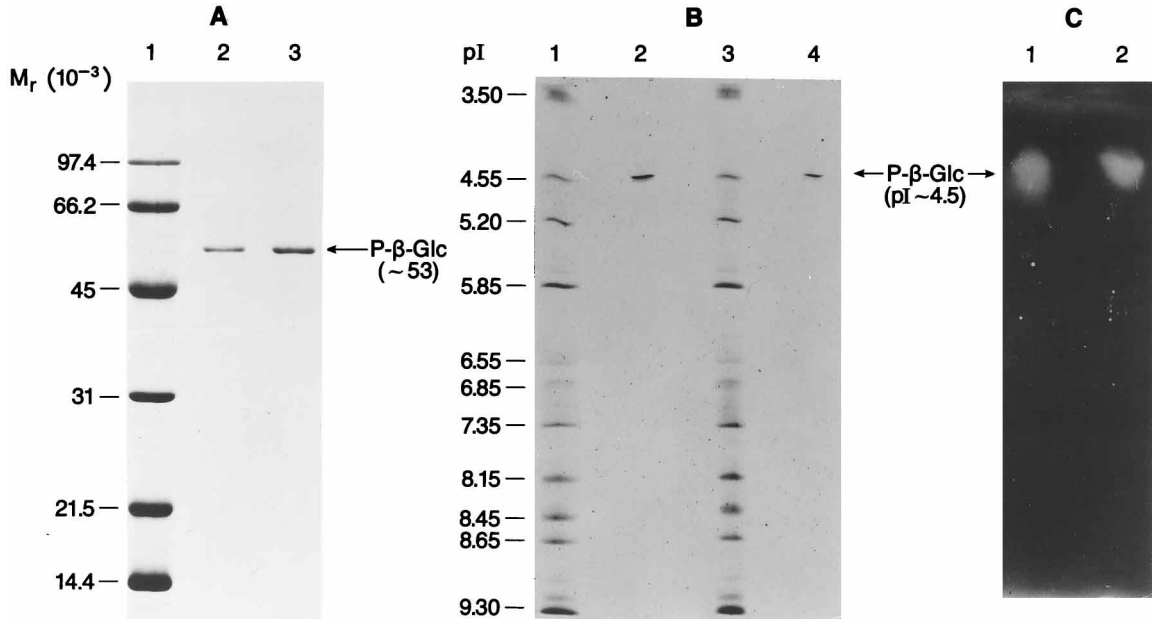

FIG. 3. Determination by PAGE of the molecular weight (M_r) and isoelectric point (pI) of P-β-glucosidase from *F. mortiferum*. (A) SDS-PAGE gel. Lane 1, molecular weight standards (Bio-Rad); lane 2 ~3 μg of P-β-glucosidase (M_r, ~53,000); lane 3, 5 μg of P-β-glucosidase. (B) IEF gel. Lanes 1 and 3, protein standards (Pharmacia-Biotech) (pI range, 3.5 to 9.5); lanes 2 and 4, 2 and 1 μg, respectively, of P-β-glucosidase (pI, ~4.5). (C) In situ activity of P-β-glucosidase after IEF, with fluorogenic 4MUβGlc6P as substrate. Approximately 1 and 2 μg of protein were applied to the gel in lanes 1 and 2, respectively.

strations of $NADP^+$ plus G6PDH-mediated inhibition of P-β-glucosidase in cell extracts (Fig. 1) were substantiated by the results obtained with purified enzyme (Fig. 2). In a discontinuous assay, the rate of pNPβGlc6P hydrolysis was not significantly affected by the separate addition of $NADP^+$ or G6PDH to the reaction mixture. However, there was no detectable cleavage of the chromogenic substrate when both $NADP^+$ and G6PDH were present in the assay. Furthermore, the addition of $NADP^+$ to a reaction mixture that already contained G6PDH resulted in immediate inhibition of P-β-glucosidase activity (Fig. 2).

Properties of P-β-glucosidase. The relative molecular weight (M_r) of P-β-glucosidase was estimated by several methods. Conventional gel filtration chromatography (Ultrogel AcA 54) yielded an M_r of ~54,000 for the native enzyme, and SDS-PAGE of enzyme denatured with β-mercaptoethanol revealed a single polypeptide with a M_r of ~53,000 (Fig. 3A). The molecular weight determined by MALDI-MS was 53,564 ± 42. The pI of P-β-glucosidase was determined by analytical electrofocusing (Fig. 3B, lanes 2 and 4). Staining of the IEF gel with Coomassie blue dye revealed a single polypeptide (pI ~4.5). Incubation of a duplicate IEF gel in a solution of 4MUβGlc6P resulted in the coincident liberation of an intensely fluorescent zone of 4MU (Fig. 3C, lanes 1 and 2). The sequence of the first 42 amino acids from the NH_2 terminus of P-β-glucosidase was determined by automated Edman degradation to be SFPKNFLWGSATAAYQVEGAWNQDGKG PSIWDL(X)SKLPGT(X)F. Purified P-β-glucosidase from *F. mortiferum* remained stable after repeated (at least five times) freezing and thawing from −20°C. Enzyme activity did not decline significantly during storage for 6 months at −20°C. The optimum rate of P-β-glucosidase activity occurred within a pH range of 6.5 to 7.0, and the optimum temperature was 35 to 40°C (data not shown).

Substrate specificity of P-β-glucosidase. Purified P-β-glucosidase from *F. mortiferum* hydrolyzed all of the P-β-glucosides tested (Table 2). The experimentally determined K_m values were less than 0.4 mM for all substrates, and V_{max} values varied within a fourfold range. Enzymatic cleavage of the P-β-glucosides produced 1 mol each of Glc6P and aglycon per mol of substrate. There was no detectable hydrolysis of the corresponding nonphosphorylated β-glucosides (data not shown).

Expression of P-β-glucosidase. Except for cells grown on melibiose (an α-galactoside), high levels of P-β-glucosidase activity were found only in extracts from organisms grown on β-glucosides (Table 3). Western blot experiments with polyclonal antibody raised against P-β-glucosidase revealed an immunoreactive polypeptide (M_r, ~65,000) of unknown function that was common to all extracts (Fig. 4A). However, expression of high levels of P-β-glucosidase (M_r, ~53,000) was found only in cells grown previously on β-glucosides. The latter extracts

TABLE 2. Substrate specificity and kinetic parameters of purified P-β-glucosidase from *F. mortiferum* ATCC 25557

Phospho-β-glucoside	K_m (μM)	V_{max}[a]
4MUβGlc6P	9.2 ± 0.7	42.6 ± 0.4
pNPβGlc6P	29.1 ± 2.8	42.0 ± 0.4
Laminaribiose-6P[b]	87.5 ± 3.6	67.9 ± 0.6
Salicin-6P	88.6 ± 3.8	46.6 ± 0.5
Arbutin-6P	97.7 ± 8.1	29.4 ± 0.6
Sophorose-6P[c]	121.2 ± 9.6	40.8 ± 1.0
Methyl-β-glucoside-6P	138.4 ± 28.6	20.6 ± 1.1
Cellobiose-6P	182.3 ± 18.6	43.1 ± 1.2
Gentiobiose-6P	335.5 ± 15.9	21.1 ± 0.3
Esculin-6P[d]	Not determined	78.1 ± 2.0[e]

[a] Micromoles of P-β-glucoside hydrolyzed per minute per milligram of protein.
[b] 3-*O*-β-D-glucopyranosyl-D-glucopyranose-6P.
[c] 2-*O*-β-D-glucopyranosyl-α-D-glucopyranose-6P.
[d] 6-*O*-β-D-glucopyranosyl-6,7-dihydroxycoumarin-6P.
[e] Rate with 1 mM esculin-6P in the assay.

TABLE 3. Phospho-β-glucosidase activities in cell extracts of *F. mortiferum* ATCC 25557 after growth of the organism on different carbohydrates

Carbohydrate	Systematic name	P-β-glucosidase activity[a]
Methyl-β-glucoside		3.48 (144)
Gentiobiose	6-*O*-β-D-glucopyranosyl-D-glucopyranose	2.70 (112)
Cellobiose	4-*O*-β-D-glucopyranosyl-D-glucopyranose	2.42 (100)
Salicin	*O*-hydroxy-methylphenyl-β-D-glucopyranoside	1.88 (78)
Arbutin	4-hydroxyphenyl-β-D-glucopyranoside	1.27 (52)
Melibiose	6-*O*-α-D-galactopyranosyl-D-glucopyranose	1.14 (47)
Methyl-α-glucoside		0.18 (7)
Galactose		0.14 (6)
Maltose	4-*O*-α-D-glucopyranosyl-D-glucopyranose	0.12 (5)
Turanose	3-*O*-α-D-glucopyranosyl-D-fructose	0.10 (4)
Trehalose	1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside	0.10 (4)
Palatinose	6-*O*-α-D-glucopyranosyl-D-fructofuranose	0.08 (3)
Lactulose	4-*O*-β-D-galactopyranosyl-D-fructofuranose	0.06 (2)
Glucose		0.04 (2)
Lactose	4-*O*-β-D-galactopyranosyl-D-glucopyranose	0.02 (<1)
Sucrose	1-*O*-α-D-glucopyranosyl-β-D-fructofuranoside	0.02 (<1)
Fructose		0.02 (<1)
Mannose		0.01 (<1)

[a] Micromoles of pNPβGlc6P hydrolyzed per minute per milligram of protein. Values in parentheses are percentages of activity present in the extract prepared from cellobiose-grown cells.

also contained significant amounts of a smaller immunoreactive polypeptide with a M_r of ~40,000. Electrophoresis of cell extracts under nondenaturing conditions, followed by immersion of the gel in a buffered solution of 4MUβGlc6P, revealed formation of two zones of fluorescence by extracts from cells grown on β-glucosides (Fig. 4B). The 40-kDa polypeptide may represent a truncated but catalytically active form of the native enzyme.

Mechanism of inhibition of P-β-glucosidase. P-β-glucosidase activity in cell extracts was not detectable by visual (Fig. 1) or spectrophotometric procedures (Fig. 2) when the reaction mixture contained both $NADP^+$ and G6PDH. This was also the case for the purified enzyme (data not shown). However, the greater sensitivity afforded by fluorometry provided evidence for limited substrate hydrolysis prior to inactivation of P-β-glucosidase (Table 4). In these experiments, increasing amounts of P-β-glucosidase (5 to 20 μg) were added to reaction mixtures that also contained $NADP^+$ and G6PDH. Cellobiose-6P was added to each cuvette, and the amount of NADPH formed was measured by fluorometry. From the data (Table 4), it was estimated that ~48 nmol of NADPH (equivalent to 48 nmol of cellobiose-6P hydrolyzed) were generated per nmol of P-β-glucosidase prior to inactivation of the enzyme. This unequivocal demonstration of substrate cleavage pointed to a product of Glc6P oxidation (P-glucono-δ-lactone or 6-phospho-gluconate) as the probable inhibitor of P-β-glucosidase. Accordingly, these metabolites and all other components of the spectrophotometric assay were examined for their inhibitory effect on P-β-glucosidase (Table 5). P-glucono-δ-lactone was found to be an extremely potent inhibitor, and at a concentration of only ~0.3 μM this compound completely inactivated the enzyme. The importance of the phosphoryl moiety for inhibition is evident from the fact that the nonphosphorylated glucono-δ-lactone (at a 30,000-fold greater concentration) reduced P-β-glucosidase activity by only 30%.

Binding of P-glucono-δ-lactone to P-β-glucosidase. Radiolabeled P-glucono-δ-lactone was incubated with purified P-β-glucosidase, and binding was revealed by their coelution within the exclusion volume of a gel filtration column (Fig. 5). Denaturation of the P-β-glucosidase-inhibitor complex by heating in boiling water released the radiolabeled ligand. The ^{14}C-labeled material in the extract was identified by thin-layer chromatography and autoradiography as 6P-gluconate (Fig. 5, inset). Because P-glucono-δ-lactone is spontaneously hydrated to yield 6P-gluconate (see reaction 3), we believe that phosphoglucono-δ-lactone is bound to the enzyme prior to heat denaturation. From the amounts of protein and radioactivity recovered from the gel filtration column, it was calculated that the enzyme-inhibitor complex contained 0.26 mol of inhibitor per mol of P-β-glucosidase.

Cloning and molecular analysis of *pbgA*. The gene encoding P-β-glucosidase (*pbgA*) from *F. mortiferum* was cloned as described in Materials and Methods. Two thousand plaques from a phage library of *F. mortiferum* 25557 DNA were screened for reactivity with polyclonal antisera to P-β-glucosidase, and one positive clone was obtained. A plasmid designated pCelH-1, carrying a 3.2-kb *F. mortiferum* DNA fragment, was excised from the phage vector and transformed into *E. coli* XLOLR. Immunoblots of cell extracts demonstrated that *E. coli* harboring pCelH-1 expressed a 45-kDa polypeptide that cross-reacted with P-β-glucosidase antiserum (data not shown). The disparity between the M_r of the protein encoded by pCelH-1 and that of the native enzyme (~53,500), indicated that the

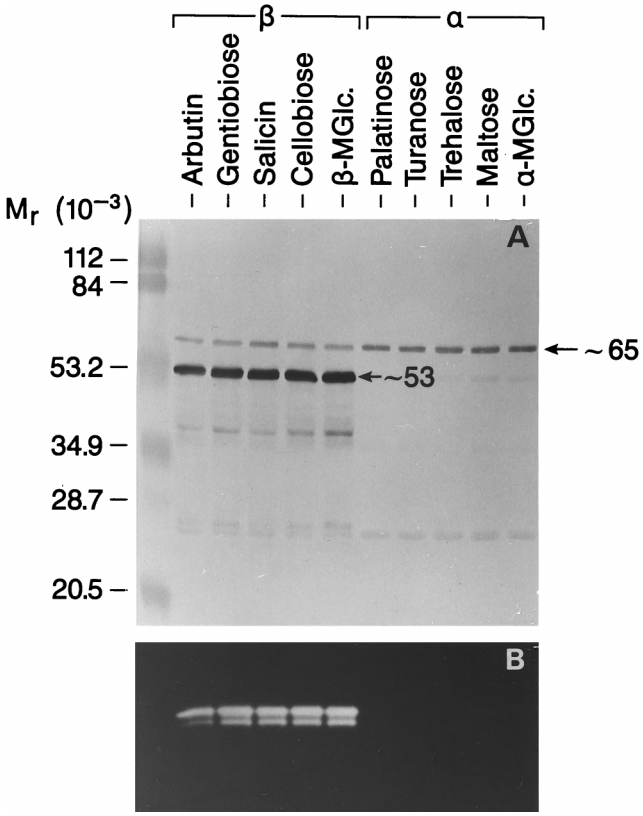

FIG. 4. Expression of P-β-glucosidase during growth of *F. mortiferum* on various glycosides, as revealed by Western immunoblot (A) and activity stain (B). For panel A, extracts (~15 μg) of cells grown previously on α- or β-glycosides were separated by SDS-PAGE. After electrotransfer to a nitrocellulose membrane, P-β-glucosidase (M_r, 53,000) was detected by immunoreaction with polyclonal antiserum. For panel B, proteins in cell extracts were separated by nondenaturing PAGE. The gel was immersed for 2 min in 50 mM HEPES buffer (pH 7.5) containing 0.1 mM 4MUβGlc6P, and P-β-glucosidase activity was revealed by formation of the intense blue-green fluorescence of 4-methylumbelliferone.

TABLE 4. Relationship between P-β-glucosidase concentration and amount of cellobiose-6P hydrolyzed prior to enzyme inactivation in the $NADP^+$ plus G6PDH coupled assay[a]

Nanomoles of P-β-glucosidase per 3-ml reaction mixture[b]	Nanomoles of cellobiose-6P hydrolyzed	Ratio $\left(\frac{\text{nmol cellobiose-6P hydrolyzed}}{\text{nmol of P-β-glucosidase}}\right)$
0.09 (4.9)	5.8	63
0.19 (9.9)	8.9	48
0.28 (14.8)	11.6	42
0.37 (19.8)	14.6	40
Avg		48.3 ± 10.4

[a] The fluorometric assay (3-ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.15 mM $NADP^+$, 2 U of G6PDH (yeast), and 0.09 to 0.37 nmol of purified P-β-glucosidase. The reactions were initiated by addition of 0.1 μmol of cellobiose-6P. The amount of NADPH formed (equivalent to cellobiose-6P hydrolyzed) prior to inhibition of P-β-glucosidase was determined by reference to a standard curve of NADPH. Fluorescence changes were measured in a McPherson model FL-750A fluorometer at excitation and emission wavelengths of 350 nm and 450 nm, respectively.

[b] Values in parentheses are micrograms of P-β-glucosidase (1 nmol of enzyme = 53.2 μg of protein).

clone might not contain the complete P-β-glucosidase gene (*pbgA*). Indeed, this supposition was confirmed by DNA sequence analysis of the 3.2-kb fragment of *F. mortiferum* DNA in pCelH-1 (Fig. 6). An open reading frame was identified (nucleotides 286 to 1398) whose deduced amino acid sequence shared up to 57% identity with P-β-glucosidase(s) from *E. coli*, *B. subtilis*, and *E. chrysanthemi*. Comparative alignment of the aforementioned sequences, and the discrepancy in M_r of the cloned protein, indicated that a fragment of DNA encoding 80 to 90 amino acid residues was missing from the 5′ end of *pbgA*. We amplified this region of *pbgA* by PCR with one primer whose sequence was derived from pCelH-1 and one degenerate primer based on the N-terminal amino acid sequence of P-β-glucosidase from *F. mortiferum*. When the PCR product (nucleotides 1 to 254, Fig. 6) was translated, the first 42 amino acids matched those determined by Edman degradation for the N terminus of the purified P-β-glucosidase. The *pbgA* gene

TABLE 5. Effects of various compounds upon the activity of P-β-glucosidase from *F. mortiferum* ATCC 25557

Compound added to assay	Concentration (mM)	P-β-glucosidase activity[a]
Complete assay (control)		34.6 (100)
Glucose-6P	0.1	30.8 (89)
	1.0	34.0 (98)
6P-gluconate	0.1	34.6 (100)
	1.0	21.6 (62)
$NADP^+$	0.1	34.5 (100)
	1.0	37.4 (108)
NADPH	0.1	35.1 (102)
	1.0	35.0 (101)
G6PDH[b]		30.2 (87)
G6PDH and $NADP^+$	0.1	No activity
P-glucono-δ-lactone	0.015[c]	16.7 (48)
	0.030[c]	11.7 (34)
	0.300[c]	No activity
Glucono-δ-lactone	10	24.5 (71)

[a] Micromoles of pNPβGlc6P hydrolyzed per minute per milligram of protein. Values in parentheses are percentages of the control.

[b] Two units of yeast G6PDH.

[c] Micromolar concentration.

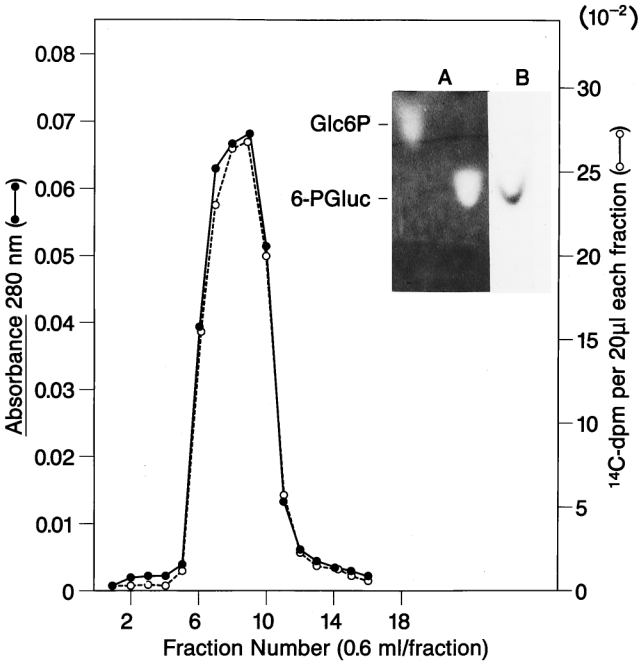

FIG. 5. Binding of the inhibitory [U-^{14}C]P-glucono-δ-lactone to P-β-glucosidase from *F. mortiferum*. Radiolabeled P-glucono-δ-lactone was mixed with P-β-glucosidase, and after dialysis, the inactive enzyme preparation was passed through a PD-10 gel filtration column. Assays for protein (●) and radioactivity (○) revealed an overlapping peak, indicative of the coelution of enzyme and inhibitor. Inset: (A) Migration of Glc6P and 6P-gluconate standards by microcrystalline thin-layer chromatography. (B) Migration and identification of the radioactive material (6P-gluconate) obtained after hot water extraction of the (P-β-glucosidase-inhibitor) complex.

extends from nucleotide 1 to 1398 (Fig. 6) and encodes a polypeptide of 466 amino acids whose calculated molecular weight of 53,538, is in excellent agreement with the M_r of 53,564 ± 42 obtained for the purified protein by MALDI-MS. A region of dyad symmetry that may function as a factor-independent terminator (5) is located between nucleotides 1430 and 1456 of the sequence. A second, partial open reading frame (*pbgB*) is located between nucleotides 1754 and 3400. The deduced amino acid sequence of this incomplete polypeptide (549 residues) exhibits 30% identity to endo-1,4-β-glucanase(s).

DISCUSSION

In this communication we report the first purification, cloning, and sequence analysis of P-β-glucosidase from an anaerobic bacterium. In some of its physicochemical properties, the enzyme from *F. mortiferum* is similar to the P-β-glucosidase(s) described from *E. coli* (49) and *K. pneumoniae* (25). P-β-glucosidase was induced by growth of *F. mortiferum* on β-glucosides, and the purified enzyme catalyzed the hydrolysis of a wide variety of C6-phosphorylated β-glucosides. P-β-glucosidase from *F. mortiferum* requires the Glc6P moiety to be present in its substrates, and the nonphosphorylated analog (pNPβGlc) was not cleaved by the enzyme. The phosphorylated C-4 epimer (oNPβGal6P) was hydrolyzed at only 5% of the rate of pNPβGlc6P. The enzyme is nonspecific with respect to the β-linked aglycon moiety which may be represented by D-glucose attached via C-2 as in sophorose-6P, C-3 (laminaribiose-6P), C-4 (cellobiose-6P), C-6 (gentiobiose-6P), an aliphatic group (methyl-α-glucoside-6P), an aromatic ring com-

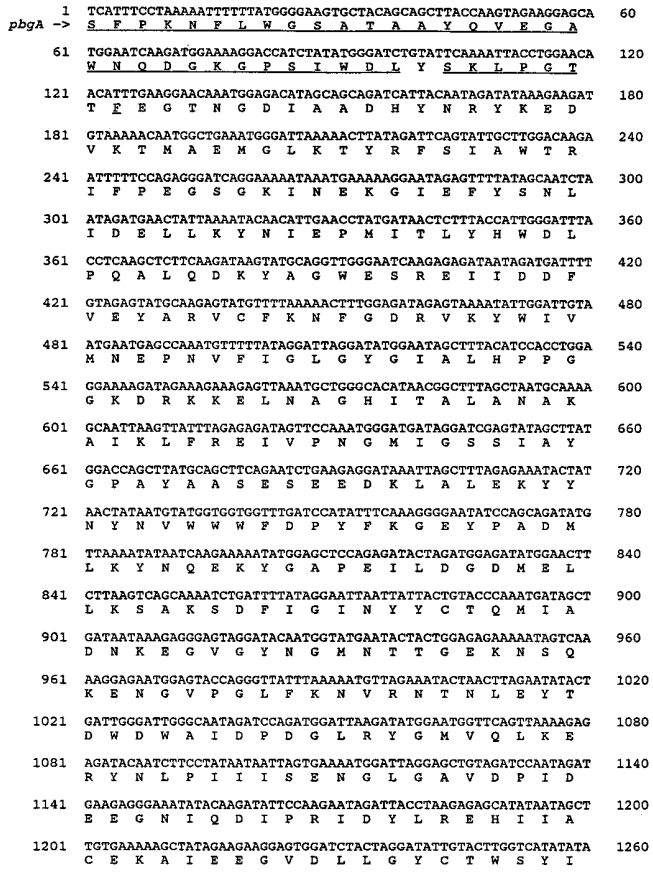

FIG. 6. Nucleotide and deduced amino acid sequences of *pbgAB*. The nucleotide sequence of the coding strand of *pbgAB* is presented. Nucleotides 1 to 254 were determined from the cloned PCR product of *pbgA* encoding the first 85 amino acids from the N terminus of P-β-glucosidase. Nucleotides 255 to 3402 were determined from a phage clone. The N-terminal amino acid sequence obtained by Edman degradation of the purified enzyme is underlined. A region of dyad symmetry following *pbgA* is doubly underlined. The gene *pbgB* encodes a putative endo-1,4-β-glucanase.

pound (salicin-6P), *p*-hydroquinone (arbutin-6P), *p*-nitrophenol (pNPβGlc6P), or a double-ring substituent as is the case for 4MUβGlc6P.

That P-β-glucosidase from *F. mortiferum* should be inhibited in the presence of $NADP^+$-dependent G6PDH was unexpected. To our knowledge this inhibitory phenomenon has not previously been reported during the in vitro assay of other P-β-glucosylhydrolases. Paradoxically, this coupled assay is the method of choice for assay of P-β-glucosidase activity in cells of *E. coli* and *K. pneumoniae*. Initially, it was felt that failure to demonstrate P-β-glucosidase activity in extracts of *F. mortiferum* by spectrophotometric assay might be attributable either to an inhibitory association between G6PDH and P-β-glucosidase or to the inhibitory effects of pH, buffer species, or metal or sulfate ions in the reaction mixture. Although considered, these explanations were eliminated by two important findings. The first was that $NADP^+$, G6PDH, and P-β-glucoside substrate were prerequisite for enzyme inactivation. The second was that the results from fluorimetric analysis showed that cleavage of nanomolar amounts of substrate (and Glc6P formation) occurred prior to inhibition of P-β-glucosidase. Our attention centered on P-glucono-δ-lactone as the probable inhibitor because this compound is the immediate product of G6PDH-catalyzed oxidation of Glc6P. Subsequent experiments (see Table 5) established that the phosphorylated glucono-δ-lactone is a potent inhibitor of P-β-glucosidase from *F. mortiferum*. The inhibitor remained bound to the enzyme after dialysis and during gel filtration chromatography. Whether the association between the two is via a covalent linkage or by ion pairing cannot be ascertained from the available data.

```
PßG    1   SFPKNFLWGSATAAYQVEGAWNQDGKGPSIWDL-----YSKLPGTTFE 43
BglB   1 MKAFPETFLWGGATAANQVEGAWQEDGKGISTSDLQPHGVMGKMEPRILG 50

PßG   44 GTN-GDIAADHYNRYKEDVKTMAEMGLKTYRFSIAWTRIFPEGSG-KINE 91
BglB  51 KENIKDVAIDFYHRYPEDIALFAEMGFTCLRISIAWARIFPQGDEVEPNE 100

PßG   92 KGIEFYSNLIDELLKYNIEPMITLYHWDLPQALQDKYAGWESREIIDDFV 141
BglB 101 AGLAFYDRLFDEMAQAGIKPLVTLSHYEMPYGLVKNYGGWANRAVIDHFE 150
                          *
PßG  142 EYARVCFKNFGDRVKYWIVMNEPNVFIGLGY-GIALHPPGGKDRKKELNA 190
BglB 151 HYARTVFTRYQHKVALWLTFNEINMSLHAPFTGVGLAEESGEA--EVYQA 198

PßG  191 GHITALANAKAIKLFREIVPNGMIGSSIAYGPAYAASESEEDKL-ALEKY 239
BglB 199 IHHQLVASARAVKACHSLLPEAKIGNMLLGGLVYPLTCQPQDMLQAMEEN 248

PßG  240 YNYNVWWWFDPYFKGEYPADMLKYNQEKYGAPEILDGDMELLKSAKSDFI 289
BglB 249 RRW--MFFGDVQARGQYPGYMQRFFRDHNITIEMTESDAEDLKHT-VDFI 295
                                          †
PßG  290 GINYYCTQMIADNKEGVGYNGMNTTGEKNSQKENGVPGLFKNVRNTNLEY 339
BglB 296 SFSYYMTGCVSHD--------------ESINKNAQGNILNMIPNPHLKS 330
                                    *
PßG  340 TDWDWAIDPDGLRYGMVQLKERYNLPIIISENGLGAVDPIDEEGNIQDIP 389
BglB 331 SEWGWQIDPVGLRVLLNTLWDRYQKPLFIVENGLGAKDSVEADGSIQDDY 380

PßG  390 RIDYLREHIIACEKAIEEGVDLLGYCTWSYIDLLS-WLNGYKKQYGFIYV 438
BglB 381 RIAYLNDHLVQVNEAIADGVDIMGYTSWGPIDLVSASHSQMSKRYGFIYV 430

PßG  439 DRKNN----LERKKKASYFWYKDVIASNGEKL         466
BglB 431 DRDDNGEGSLTRTRKKSFGWYAEVIKTRGLSLKKITIKAP 470
```

FIG. 7. Alignment of the deduced amino acid sequences of P-β-glucosidase(s) from *F. mortiferum* (PβG, this study) and *E. coli* (BglB, reference 36). Asterisks identify conserved and catalytically active glutamyl residues. The dagger indicates the glutamyl residue of the duplicated ENG motif in the enzyme from *F. mortiferum*. The single underline designates the glycine triplet G(X)G(X)(X)G that may represent the βαβ-fold of a nucleotide-binding domain. Colons indicate similar residues and vertical lines indicate identical residues between the sequences.

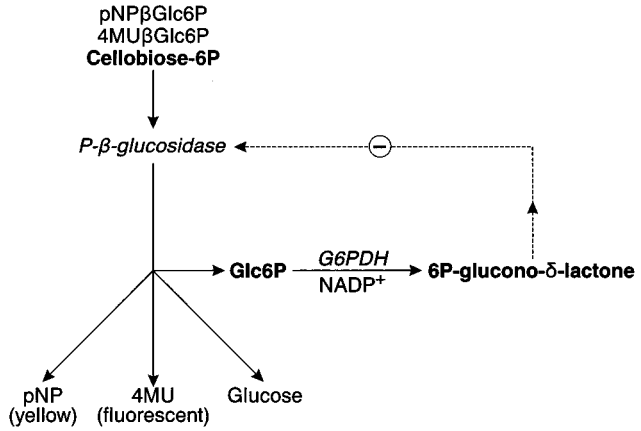

FIG. 8. Schematic representation of the in vitro formation and inhibitory effect of P-glucono-δ-lactone on P-β-glucosidase from *F. mortiferum.*

The inhibitory effect that we describe for P-glucono-δ-lactone toward P-β-glucosidase from *F. mortiferum* is reminiscent of the inhibition of β-glucosidase(s) by glucono-δ-lactone (17, 18, 21, 42). Indeed, it is from these latter studies, and from current concepts of β-glucosidase catalysis (20, 40, 41, 50, 51), that we can propose reasonable explanations for the P-glucono-δ-lactone-mediated inhibition of P-β-glucosidase from *F. mortiferum*. β-Glucosidases (EC 3.2.1.21) and P-β-glucosidases (EC 3.2.1.86) comprise the subgroup A (BGA) family 1 of glycosylhydrolases, and alignment of the deduced amino acid sequences reveals extensive similarity among these enzymes (6–8, 12, 13, 33). Both β- and P-β-glucosidases catalyze the hydrolytic cleavage of the linkage between the anomeric C-1 and the glycosidic oxygen atom of their substrates via a reaction that is formally a nucleophilic substitution at C-1 (40, 41). For β-glucosidase, this double-displacement reaction proceeds with the formation of a transition-state oxocarbonium ion. Because of the coplanarity of C-5, O-5, C-1, and C-2 atoms in the pyranose ring, the glucosyl cation assumes a half-chair stereochemical configuration. In a series of elegant experiments with β-glucosidase from *Agrobacterium tumefaciens* Withers et al. (50, 51) identified Glu-358 as the active-site nucleophile that participates in the formation, stabilization, and covalent binding of the oxocarbonium ion to the enzyme. These investigators also showed that a second glutamyl residue (Glu-170) functions as an acid/base catalyst during the formation, and breakdown, of the glucosyl-enzyme complex (46). Inactivation of β-glucosidase by glucono-δ-lactone is rationalized by the fact that the trigonal planimetry of the inhibitor molecule (9) is similar to that of the transition-state oxocarbonium ion. The two catalytically active glutamyl residues identified in β-glucosidase from *A. tumefaciens* are positionally conserved in the ENG and NE(P/I) motifs of all members of the BGA family (6–8, 33, 48, 52), including P-β-glucosidase from *F. mortiferum* (Fig. 7). It is reasonable to assume that substrate hydrolysis by P-β-glucosidase proceeds via a mechanism similar, if not identical, to that proposed for β-glucosidase from *A. tumefaciens*. However, for P-β-glucosidase-catalyzed hydrolysis, the presumptive transition-state intermediate would be the 6-phospho-oxocarbonium ion and, by analogy, inhibition of P-β-glucosidase from *F. mortiferum* as a consequence of binding of P-glucono-δ-lactone to the active site is an attractive concept. However, on the basis of a common catalytic mechanism, one would expect inactivation, not only of the enzyme from *F. mortiferum*, but also of P-β-glucosidase(s) from both *E. coli* and *K. pneumoniae*. Clearly, this is not the case and we must consider the possibility that P-glucono-δ-lactone may bind to a sequence or domain that is perhaps unique to the fusobacterial enzyme. Comparative alignment of amino acid sequences of P-β-glucosidase from *F. mortiferum* and *E. coli* (Fig. 7) revealed some intriguing differences between the two proteins. Significantly for P-β-glucosidase from *F. mortiferum*, the ENG motif that contains the active-site glutamyl residue (Glu-370) is repeated in residues 322 to 324 of the amino acid sequence. Furthermore, this duplicated motif is preceded by 15 residues that have no counterpart in P-β-glucosidase from either *E. coli* or *E. chrysanthemi*. This extra sequence contains two positively charged lysyl residues (positions 303 and 317) that potentially may ion pair with the negatively charged phosphoryl moiety of the inhibitor. It is also of interest that three (of the four) glycine residues that are present exhibit the characteristic G(x)G(x)(x)G motif that defines the βαβ-fold of the nucleotide-binding domain of many NAD(P)-dependent enzymes (3, 38, 47). Whether Glu-322, the glycyl triplet, or the lysyl residues participate in binding of P-glucono-δ-lactone by P-β-glucosidase from *F. mortiferum* has yet to be determined. Although conjectural, it is possible that the interaction of P-glucono-δ-lactone with one, or more, of these residues may elicit a conformational change within the protein that is accompanied by loss of catalytic function. Alternatively, by steric hinderance, the bound inhibitor may sim-

ply prevent access of substrate to the active site. Answers to these questions are presently being sought by sequence deletion and site-directed mutagenesis experiments.

The summary of our findings (Fig. 8) emphasizes that Glc6P is both a product of one enzymatic activity (P-β-glucosidase) and a substrate for another (G6PDH). As depicted, these in vitro reactions constitute a feedback mechanism whereby the catalytic activity of G6PDH (i.e., formation of P-glucono-δ-lactone) causes inactivation of P-β-glucosidase. It is axiomatic that the operation of such a feedback in cells of *F. mortiferum* would either inhibit or prevent growth of the organism on β-glucosides. Remarkably, G6PDH activity was not detectable in cell extracts of *F. mortiferum* and feedback inhibition is therefore precluded. By contrast, cells of *K. pneumoniae* and *E. coli* possess a P-β-glucosidase that is not inactivated by P-glucono-δ-lactone, and these organisms contain high levels of $NADP^+$-dependent G6PDH.

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