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Original Article

Periplasmic β-glucosidase BglX from *E. coli* demonstrates greater activity towards galactose-containing substrates

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Abstract: Background: The diverse nature of carbohydrate structures and linkages requires a variety of enzymes responsible for sugar degradation. The *E. coli* periplasmic protein encoded by the *bglX* gene has been assigned to glycoside hydrolase family 3 and is predicted to function as a β-glucosidase. Objectives: We investigated the catalytic properties of the *E. coli* protein BglX and identified two functionally important amino acid residues. Methods: The *bglX* gene was cloned into a pET20b(+) vector, and three mutants, D111N, D287G, and E293Q, were generated using site-directed mutagenesis. Kinetic studies were performed on the wild-type and mutant enzymes. Results: Substrate specificity tests indicated that the BglX enzyme hydrolyzes β-glycosidic bonds in nitrophenyl-β-glycosides and demonstrates greater activity towards galactose-containing substrates compared to glucose derivatives. Monomeric glucose and galactose inhibit enzyme activity to a different degree in a substrate-dependent manner. In addition, BglX can hydrolyze lactose but not cellobiose, maltose, or laminarin. Subsequently, *E. coli* cells overexpressing active BglX have a growth advantage on minimal media supplemented with lactose as a carbon source. Mutation of D287 or D111 residues negatively affected the activity of BglX indicating their involvement in catalysis. Overexpression of BglX by *E. coli* cells did not increase biofilm formation. Conclusions: The low activity towards glucose-containing substrates and significantly elevated activity towards galactosides suggests that β-glucosidase activity may not be the primary function of the BglX enzyme.

Keywords: BglX, β-glucosidase, *E. coli,* periplasmic protein, enzyme activity

Introduction

Carbohydrates are important sources of nutrients for various organisms, including bacteria. Most saccharides found in nature are complex molecules. For example, they could be composed of several linked monomeric units or contain a carbohydrate bound to a non-carbohydrate moiety [1-3]. In order to release carbohydrates from these sources and utilize them as fuel, bacteria produce a variety of enzymes that differ in their specificities [4]. Enzymes capable of hydrolyzing the glycosidic bonds within sugar-containing compounds are known as glycoside hydrolases or glycosidases [5]. The large variety of enzymes belonging to the glycosidase superfamily is the result of the structural diversity of carbohydrates [6]. Due to their wide applications in medicine, biotechnology, food, and other industries, microbial glycoside hydrolases are thoroughly studied, with the search for novel enzymes triggering multiple metagenomic investigations [7, 8].

Many glycosidases produced by bacteria are assigned to glycoside hydrolase family 3 (GH3). GH3 enzymes are specialized in hydrolyzing β (1→4) glycosidic bonds, removing a saccharide residue from the non-reducing end of a glycoside [9]. Most enzymes in the GH3 family are involved in the metabolism of glucose-containing compounds and are thus classified as β-glucosidases (EC 3.2.1.21) [10-12]. Cytosolic β-glucosidases are common, but some bacteria secrete glycosidases into the extracellular space [13]. In Gram-negative bacteria, β-glucosidases are also found in the periplasm, the space between the inner and outer membranes [14]. The bacterial periplasmic space is rich in degradative enzymes involved in defense and

the supply of nutrients to the cell [15]. Periplasmic glycosidases in Gram-negative bacteria are implemented in the metabolism of osmoregulated periplasmic glucans (OPG) that aid in bacterial adaptation to changes in osmotic pressure [16, 17]. Additionally, some periplasmic proteins are involved in the synthesis or degradation of cellulose, an integral component of bacterial biofilms [18, 19].

Although monomeric glucose is the favored carbon source for *E. coli* [20], wild-type cells cannot consume β-glucosides. Under conditions of nutrient starvation, cell survival depends on mutations in the genome that activate cryptic systems which permit the organism to utilize available carbohydrates. The ability of *E. coli* to utilize β-glucosides as carbon sources has been attributed to proteins encoded within four operons - *asc*, *bgl*, *blc,* and *chb* [21]. Operon *chb* (formerly known as the *cel* operon [22]) allows bacteria to consume cellobiose, a glucose disaccharide produced from the degradation of cellulose [23]. Six proteins derived from this operon in wild-type *E. coli* are also involved in the processing of chitobiose, a glucosamine dimer formed during chitin hydrolysis [23]. Operon *bgl* contains a regulatory element and three structural genes responsible for the phosphorylation, uptake, and hydrolysis of phosphorylated β-glucosides [21]. The gene products of this operon are involved in the processing of aryl-β-glucosides, such as arbutin and salicin [24]. In addition, during the stationary phase, this operon is indirectly involved in regulating the transport of short peptides [25]. Septicemic *E. coli* strains contain the *blc* operon that encodes genes homologous to those of *bgl* [26]. Another cryptic operon, *asc,* contains structural genes for the transport and degradation of β-glucosides that are similar to those present in *bgl*. However, the two operons are regulated differently [27, 28].

Identified outside of the cryptic operons in *E. coli,* the *bglX* gene encodes the constitutively expressed BglX enzyme that has been functionally assigned as a β-glucosidase [29]. The purified enzyme demonstrated activity towards *o*-nitrophenyl-β-D-glucopyranoside but was not able to hydrolyze substrates containing fructose, xylulose, or *N*-acetylglucosamine in place of glucose [29]. The polypeptide sequence of BglX indicates the presence of an N-terminal signal peptide for transport into the periplasm. The periplasmic location of the enzyme implicates its role in providing glucose to the cell. However, neither elimination of the *bglX* gene nor overexpression of the BglX protein affected the growth of *E. coli* cells in the presence of the aromatic β-glucosides arbutin and salicin or the disaccharide cellobiose [29]. Thus, the exact physiological function of *E. coli* BglX remains unknown.

The main objectives of this study were to identify the catalytically important amino acid residues in the *E. coli* BglX protein and characterize its substrate specificity in order to elucidate the function of this enzyme.

Materials and methods

E. coli K-12 genomic DNA was from ATCC (Manassas, VA). Primers were custom ordered from Invitrogen (Carlsbad, CA). Restriction enzymes were from New England Biolabs (Ipswich, MA). The pET20b(+) vector was from EMB Chemicals, Inc. (Gibbstown, NJ). Competent cells BL21(DE3) were from Stratagene (La Jolla, CA). Luria Bertani (LB) broth, ammonium sulfate, ampicillin, Coomassie brilliant blue, isopropyl β-D-1-thiogalactopyranoside (IPTG), ethylenediaminetetraacetic acid (EDTA), potassium phosphate, sodium acetate, sodium chloride, sodium dodecyl sulfate (SDS), streptomycin sulfate, and tris(hydroxyl-methyl) aminomethane (Tris) were from Amresco (Solon, OH). Cellobiose, lactose, *o*-nitrophenyl-β-D-glucopyranoside (*o*NPGlc), *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal), 4-methylumbelliferyl-β-D-galactopyranoside (MUGal), and Tryptic Soy Broth (TBS) were from Sigma Aldrich (St. Louis, MO). D-Glucose, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc), and 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc) were from Acros Organics (Geel, Belgium). Maltose and D-galactose were from Fisher Scientific (Pittsburgh, PA). Laminarin (laminaritriose) was purchased from Spectrum Biochemical Mfg. Corp. (New Brunswick, NJ). Q-Sepharose Fast Flow was purchased from GE Healthcare (Piscataway, NJ). Hydroxyapatite was from Bio-Rad (Hercules, CA).

Cloning, expression, and purification of wild-type BglX

The *bglX* gene was PCR amplified from *E. coli* genomic DNA using primers containing recogni-

Table 1. Primers for cloning *bglX* and generation of the mutants

Gene	Primer Sequence (F - forward, R - reverse)
bglX	F: AAAAAACATATGAAATGGCTATGTTCAGTAGGAATC
	R: TTTGAATTCTTACAGCAACTCAAACTCGCCTTTC
D111N	F: CTCTTTTCTTTGCTTACAACGTGCTGCACGGTCAGC
	R: GCTGACCGTGCAGCACGTTGTAAGCAAAGAAAAGAG
D287G	F: GGCATCACCGTTTCCGGTCACGGTGCAATCAAAG
	R: CTTTGATTGCACCGTGACCGGAAACGGTGATGCC
E293Q	F: CACGGTGCAATCAAACAGCTGATTAAACATGGCACG
	R: CGTGCCATGTTTAATCAGCTGTTTGATTGCACCGTG

tion sites for the restriction enzymes *NdeI* and *EcoRI*. The sequences of the primers are presented in **Table 1**, and the mutation sites are underlined. The PCR product and expression vector pET20b(+) were digested with the same restriction enzymes, purified, and ligated, resulting in the pET20b(+)-*bglX* plasmid. This plasmid was transformed into BL21(DE3) *E. coli* cells for protein expression.

An overnight culture of BL21(DE3) cells containing pET20b(+)-*bglX* grown at 37°C in Luria-Bertani (LB) medium with ampicillin (100 μg/mL) was diluted 100 times into fresh LB media containing ampicillin and grown at 37°C with shaking at 200 rpm. Once the OD_{600} of the culture reached around 0.6-0.8, IPTG was added to the culture to a final concentration of 0.3 mM, and the culture continued to grow overnight. The cells were harvested by centrifugation at 6,000×g at 4°C for 10 min. The pellets were frozen at -20°C. The frozen pellets were resuspended completely in Tris buffer (10 mM, pH 7.0) containing 2 mM EDTA and sonicated for 30 seconds with one minute of cooling for a total of 6 cycles. The cell debris was removed by centrifugation at 11,000×g for 30 min, the supernatant was treated with streptomycin sulfate to a final concentration of 1% (w/v), and the precipitate was removed by centrifugation at 11,000×g for 20 min. Ammonium sulfate was added to the supernatant to 75% saturation, and the suspension was stirred for 30 min. The suspension was centrifuged at 11,000×g for 30 min. The pellets were dissolved in Tris buffer (20 mM, pH 7.4) and dialyzed overnight against the same buffer.

The dialyzed protein sample was loaded onto a Q Sepharose FF column which was equilibrated with Tris buffer (20 mM, pH 7.4). The proteins bound to the column were eluted with a linear gradient of 0-400 mM NaCl in 20 mM Tris, pH 7.4. Fractions with a high content of BglX based on SDS-PAGE analysis were pooled together and concentrated with an Amicon ultra centrifugal filter (MWCO 30 kDa) at 4,000×g. The concentrated protein sample was dialyzed against a 10 mM potassium phosphate buffer, pH 6.8 overnight at 4°C and then loaded onto a hydroxyapatite column that was equilibrated with the same buffer. The protein was eluted with a linear gradient of potassium phosphate buffer (10-400 mM, pH 6.8). The fractions containing BglX were pooled and stored at -20°C. The protein concentrations were calculated from the absorbance of the protein solutions at 280 nm using the extinction coefficient value of 82,740 $M^{-1}cm^{-1}$.

Generation of BglX mutants

The pET20b(+)-*bglX* plasmid was used as a template to generate BglX mutants D111N, D287G, and E293Q using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. The numbering of the mutated amino acid residues corresponds to their positions in the full-length BglX protein. The primers used for generation of the mutants are listed in **Table 1**; the sites of the mutations are underlined. All mutations were confirmed by DNA sequencing, and the resulting plasmids were transformed into BL21(DE3) *E. coli* cells for expression of the mutant proteins. All proteins were expressed and purified following the procedures described for the wild-type BglX.

Enzyme activity assay with model substrates (pNPGlc, oNPGlc, oNPGal)

The chromogenic substrates *p*NPGlc, *o*NPGlc, and oNPGal were used to test the ability of BglX and its mutants to hydrolyze β-glycosidic bonds formed by glucose or galactose. All assays were conducted at room temperature and absorbance values were recorded using an Agilent 8453 Diode Array Spectrophotometer (Agilent Technologies, Santa Clara, CA). The reaction mixture containing 850 μL of Tris buffer (50 mM, pH 7.0) and 100 μL of substrate solution was used as a blank at 405 nm. The reactions were initiated by the addition of 50 μL

of enzyme solution, and the change in absorbance was measured over 5 min. The protein concentrations were held constant (1-2 μM). Substrate concentrations were varied between 1 and 40 mM for *o*NPGlc, 0.05-15 mM for *p*NPGlc, and 0.01-3 mM for *o*NPGal. Reaction rates were calculated using extinction coefficients determined under the same experimental conditions: 10,440 $M^{-1}cm^{-1}$ for *p*-nitrophenol (*p*NP) and 3,055 $M^{-1}cm^{-1}$ for *o*-nitrophenol (*o*NP). The kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation.

In-gel activity testing

Samples containing BglX or mutant proteins obtained after initial purification steps (ammonium sulfate precipitation and dialysis) were loaded onto a 10% native polyacrylamide gel in duplicate and electrophoresed under standard conditions. The resulting gel was cut in half, and one part was developed with Coomassie blue to detect protein positions. The other half was subjected to fluorometric analysis using either 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc) or 4-methylumbelliferyl-β-D-galactopyranoside (MUGal). This portion of the gel was soaked for 15 min in sodium acetate buffer (100 mM, pH 5.0) with occasional shaking, followed by incubation at 37°C for 30-60 min with 10 mM MUGlc or MUGal dissolved in the same buffer. The zymograms were analyzed under UV light to detect enzyme activity based on the release of methylumbelliferone.

Enzyme activity assay with disaccharides (cellobiose, lactose, maltose)

The activity of BglX and its mutants towards the hydrolysis of the disaccharides lactose, maltose, and cellobiose was studied using the Glucose (GO) Assay Kit (Sigma-Aldrich, St. Louis, MO). First, 160 μL of Tris buffer (50 mM, pH 7.0), 20 μL of a 20 μM solution of BglX (or mutant), and 20 μL of a disaccharide solution at various concentrations (0.5-40 mM) were incubated at room temperature for 30 min in an Eppendorf tube. The reaction was stopped by heating the tube at 95°C for 3 min, and the precipitate was removed by centrifugation at 13,000×g for 3 min. Fifty microliters of the supernatant were added to a mixture of 900 μL of GO reagent (composed of glucose oxidase, horseradish peroxidase, and *o*-dianisidine) and 50 μL of water. After 30 min of incubation at room temperature, the absorbance of the solution was measured at 540 nm. The amount of free glucose was calculated from the calibration curve obtained under similar experimental conditions using standard glucose solutions. Control reactions in which the BglX protein solution was replaced with 20 μL of water were performed in a similar manner to evaluate the rate of uncatalyzed hydrolysis of the disaccharides. The kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation.

BglX activity with laminarin

The ability of the BglX enzyme to hydrolyze laminarin was tested by mixing 50 μL of Tris buffer (50 mM, pH 7.0), 50 μL of laminarin solution to a final concentration of 40 mM, and a BglX solution in the same buffer to a final concentration of 10 μM in an Eppendorf tube. The reaction mixture was incubated at room temperature, and aliquots were spotted on a TLC (Thin Layer Chromatography) plate after 1, 14, 24, and 36 hours. The plate was developed using a mobile phase containing ethyl acetate:*n*-butanol:water (2:7:1). The plate was dried, sprayed with 5% (v/v) solution of concentrated sulfuric acid in ethanol, and heated on a hot plate. The sugar-containing compounds appeared as brown spots.

Inhibition of BglX activity by glucose and galactose

To test whether high concentrations of glucose or galactose could affect the activity of BglX and its mutants, inhibition studies were conducted. First, the reactions were performed without an inhibitor. In a 1-mL quartz cuvette, 850 μL of Tris buffer (50 mM, pH 7.0) and 100 μL of substrate solution (final concentration of 1.25 mM *o*NPGal or 5.0 mM *p*NPGlc) were mixed and used to blank the spectrophotometer at 405 nm. The reaction was initiated by the addition of 50 μL of a 20 μM solution of BglX (or mutants), and the change in absorbance was observed over 10 min. The reactions were repeated in a similar manner, except that the 100 μL of Tris buffer was replaced with the same volume of either a glucose or galactose solution (final concentration of 125 mM).

Effect of carbon source on the growth of E. coli cells overexpressing BglX or its mutants

Overnight cultures of BL21(DE3) *E coli* cells harboring pET20b(+)-*bglX* or pET20b(+) vectors carrying the mutated *bglX* gene were serially diluted (10^{-1}-10^{-8}) in either LB medium or minimal M9 medium (MM9) and plated on the corresponding agar plates supplemented with either glucose (0.4% w/v) or lactose (1.5% w/v). All plates also contained ampicillin (100 μg/mL). The plates were incubated at 37°C overnight, and bacterial growth was observed.

Biofilm formation assay

Overnight cultures of BL21(DE3) *E. coli* cells harboring pET20b(+), pET20b(+)-*bglX* or a vector carrying the mutated *bglX* gene grown in LB with ampicillin (100 μg/mL) were diluted 1:50 into fresh TSB media. After one hour of incubation, IPTG was added to a concentration of 0.3 mM. For each bacterial strain, 150 μL aliquots were distributed into eight wells of a 96 well-plate. Eight wells contained 150 μL aliquots of TSB medium that were later used to prepare a blank for absorbance measurements. Three plates were prepared in the same fashion. The plates were incubated at 37°C for three days. After incubation, the liquid was removed from the wells by inverting the plates. The plates were gently submerged in distilled water to eliminate loose cells, and then the water was removed by inverting and shaking the plates. For biofilm staining, 150 μL of 0.1% (m/v) aqueous solution of crystal violet was added into each well. The plates were incubated at room temperature for 15 min, the solution was removed by inverting the plates, and the wells were washed three times by submerging the plates in distilled water. After the water was removed, the plates were air-dried. To dissolve the stained biofilms, 150 μL of 30% (v/v) acetic acid was added into each well, and the plates were incubated at room temperature for 15 min with gentle shaking. For each plate, the eight wells containing the same *E. coli* strain were combined, and the absorbance of the pooled solutions was measured at 585 nm.

Statistical analysis

The data was analyzed in Microsoft Excel and presented as mean ± standard deviation (n = 3). For biofilm formation, an independent t-test was employed to compare the absorbances of the control and the bacterial cells expressing various BglX proteins. The statistical significance was set at $P < 0.05$.

Results

Expression of BglX protein and construction of mutants

The *bglX* gene encoding the full-length protein was cloned into the pET20b(+) expression vector. The BglX protein was subsequently overexpressed in *E. coli* BL21(DE3) cells and purified using ammonium sulfate precipitation and anion-exchange chromatography. The functional importance of the predicted catalytic residues in BglX was studied using site-directed mutagenesis. Three single mutants were constructed: D287G, D111N, and E293Q. The mutant proteins were overexpressed in *E. coli* BL21(DE3) cells and purified following the wild-type BglX purification protocol. The purity of the proteins after chromatographic purification was confirmed by SDS-PAGE (Figure S3).

Enzyme activities with model substrates

The enzymatic activities of the wild-type and mutant proteins with chromogenic model aryl-β-glycosides were evaluated. The turnover numbers (k_{cat}), Michaelis constants (K_M), and catalytic efficiencies (k_{cat}/K_M) are summarized in **Table 2**. The wild-type BglX demonstrated similar turnover numbers for both glucose-containing substrates; however, the K_M for *o*NPGlc was significantly higher than the K_M for *p*NPGlc. The E293Q mutant displayed a matching trend in kinetic parameters. Its catalytic constants for the two substrates were similar, although the values were lower compared to those for the wild-type. This mutant's K_M for the *ortho*-substituted phenol derivative was also higher than its K_M for the *para*-substituted analog. Neither the D111N nor the D287G mutant had detectable activity towards these substrates. All four proteins were active in the hydrolysis of the β-glycosidic bond formed by D-galactose in *o*NPGal. The k_{cat} of the wild-type enzyme for the reaction with *o*NPGal was two orders of magnitude greater compared to that for *o*NPGlc and at least five-fold higher than those of the mutants. Interestingly, the Michaelis constants for this substrate were lower than those for its glucose analog.

Table 2. Enzyme activity with model substrates and lactose

Substrate	Enzyme	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}min^{-1}$)
*p*NPGlc	wt-BglX	1.02 ± 0.12	0.95 ± 0.08	1.07 ± 0.04
*p*NPGlc	D287G	NA	NA	NA
*p*NPGlc	E293Q	0.46 ± 0.05	3.2 ± 0.3	0.14 ± 0.03
*p*NPGlc	D111N	NA	NA	NA
*o*NPGlc	wt-BglX	1.08 ± 0.12	12.0 ± 1.0	0.090 ± 0.003
*o*NPGlc	D287G	NA	NA	NA
*o*NPGlc	E293Q	0.47 ± 0.05	6.7 ± 1.8	0.070 ± 0.012
*o*NPGlc	D111N	NA	NA	NA
*o*NPGal	wt-BglX	282 ± 30	0.21 ± 0.02	1343 ± 15
*o*NPGal	D287G	22.8 ± 2.4	0.14 ± 0.02	163 ± 6
*o*NPGal	E293Q	54.0 ± 0.6	0.21 ± 0.02	257± 21
*o*NPGal	D111N	46.2 ± 4.8	0.51 ± 0.02	91 ± 6
Lactose	wt-BglX	2.64 ± 0.24	6.8 ± 1.0	0.39 ± 0.02
Lactose	D287G	NA	NA	NA
Lactose	E293Q	0.33 ± 0.05	6.7 ± 1.0	0.049 ± 0.001
Lactose	D111N	NA	NA	NA

NA: no activity.

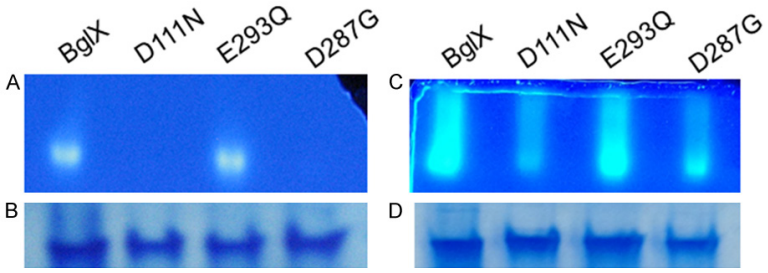

Figure 1. In-gel activity of BglX and mutants. Two fluorogenic substrates were used to test in-gel enzymatic activity of BglX and its mutants: (A) 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc) and (C) 4-methylumbelliferyl-β-D-galactopyranoside (MUGal), (B and D) show the protein load.

In-gel activity testing

Two fluorogenic substrates were used in these studies: one containing glucose (MUGlc) and the other containing galactose (MUGal) (**Figure 1**). The results presented in Figure S1 demonstrate that the positions of the BglX protein bands on the gel correspond to the areas where the fluorescent compound, methylumbelliferone, was formed. In the experiment with MUGlc, only two fluorescent bands were observed - one from the wild-type and the other from the E293Q mutant. The wild-type BglX yielded a band with higher intensity. On the gel treated with MUGal, all four proteins showed formation of the fluorescent product, although the intensities of the bands varied between the proteins.

Inhibition studies

The effects of free D-glucose or D-galactose on BglX enzymatic activity towards either *p*NPGlc or *o*NPGal as a substrate were evaluated (**Figure 2**). In the presence of 125 mM galactose, the enzyme activity decreased by 30 to 50% regardless of the nature of the substrate. The effect of glucose, however, depended on the substrate used. Glucose at a concentration of 125 mM suppressed the enzymatic activity towards *p*NPGlc by almost 95%, while the reaction with *o*NPGal was inhibited by no more than 40%.

Activities with disaccharides and laminarin

The glycosidic bond specificity of BglX was evaluated using three disaccharides - cellobiose, maltose, and lactose. None of the BglX proteins were able to cleave the glycosidic bonds in cellobiose or maltose; however, the wild-type and the E293Q mutant hydrolyzed lactose. Their kinetic parameters are presented in **Table 2**. The Michaelis constants were quite similar, but the turnover number for the wild-type was an order of magnitude greater than that for the E293Q mutant. In addition to the disaccharides, the activity of the wild-type BglX towards the trisaccharide laminarin was assessed by incubating the enzyme and laminarin for up to 36 hours at room temperature. The release of free glucose was not observed.

Effect of carbon source on bacterial growth

The effect of overexpression of the wild-type BglX or the mutant enzymes on the growth of *E. coli* cells was tested by growing the cells on minimal media containing either D-glucose or

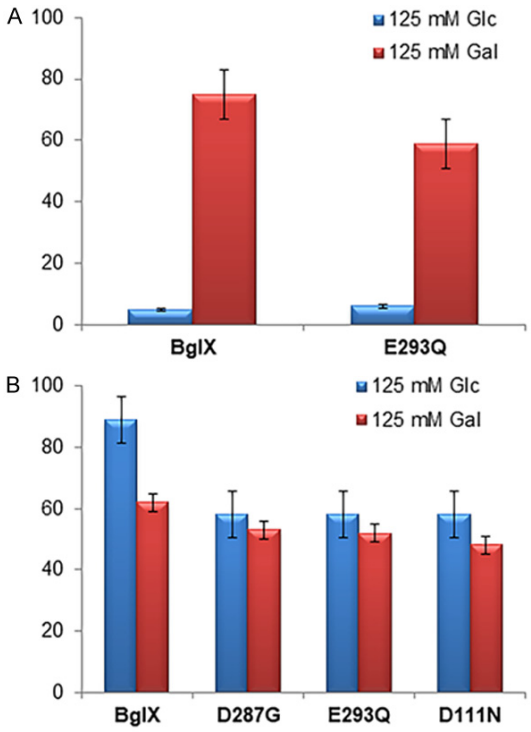

Figure 2. Percent of remaining enzyme activity in the presence of glucose and galactose. A. Enzyme activity was evaluated using 5.0 mM *p*NPGlc as a substrate, D287G and D111N had no detectable activity with this substrate; B. Enzyme activity was evaluated using 1.25 mM *o*NPGal as a substrate. Enzyme activity without an inhibitor was considered as 100%. The error bars illustrate the standard deviation from the mean (n = 3).

lactose as carbon sources. All bacterial strains thrived on the agar plates with nutrient-rich LB media used as a control (**Figure 3A**). In addition, all cells demonstrated similar but slower growth rates on minimal media supplemented with glucose. In contrast, the bacterial cells showed differences in growth rates when lactose was added as the carbon source (**Figure 3B**). The cells overexpressing BglX formed more colonies at higher dilution than any other cells.

Biofilm formation assay

To assess the possible involvement of BglX in the formation of biofilm, cells overexpressing the wild-type or mutant BglX were grown in a 96-well plate for several days, and the biofilm was stained with crystal violet. The absorbances of the dissolved biofilms at 585 nm shown in **Figure 4** were a measurement of the biofilm quantities. The cells expressing BglX formed about the same amount of biofilm as the *E. coli* cells containing the empty pET20b(+) vector used as a control. There were no statistically significant differences in absorbances between the control and the cells expressing either wild-type BglX or the mutant proteins.

Discussion

The *bglX* gene in *E. coli* encodes BglX, a periplasmic protein consisting of 765 amino acid residues [29]. The first twenty amino acid residues at the N-terminus comprise a signal peptide for periplasmic delivery, and the cleavage of this fragment yields a functional protein of 81.3 kDa. The overproduction of a protein that requires transport into the periplasm could lead to so-called "jamming" of the secretion machinery [30]. In these cases, the transport system becomes overwhelmed with a large amount of protein, leaving some of it in the cytosol with the intact signal peptide. To circumvent this issue, we cloned a truncated version of the *bglX* gene encoding only the active portion of the protein into a pET20b(+) vector (primers' sequence is shown in Table S1), and expressed the protein in BL21(DE3) cells. The cells demonstrated substantial production of truncated BglX based on SDS-PAGE analysis (Figure S2); however, this protein had no enzymatic activity. The lack of activity is most likely due to the inability of the protein to fold properly. In bacteria, the secretion of periplasmic proteins is accomplished by two main mechanisms - the *Sec*-pathway and the *Tat*-pathway [31]. In the first mechanism, a protein synthesized in the cytoplasm is taken into the periplasm where it is folded into the correct structure with the help of periplasmic *Sec* chaperones. In the second mechanism, the folded protein is translocated through the inner membrane. Apparently, BglX requires the assistance of chaperones to fold correctly in the periplasm. Since the truncated version of BglX yielded inactive protein, the full-length BglX was cloned into the expression vector allowing for the production of the functional enzyme.

Most known glucosidases contain two amino acid residues in the active site that are imperative for the cleavage of a glycosidic bond - one serves as a nucleophile and the other participates in acid-base catalysis [32-34]. The D287 residue has been predicted to function as a nucleophile in the active site of the BglX enzyme [29]. This residue is located within a conserved

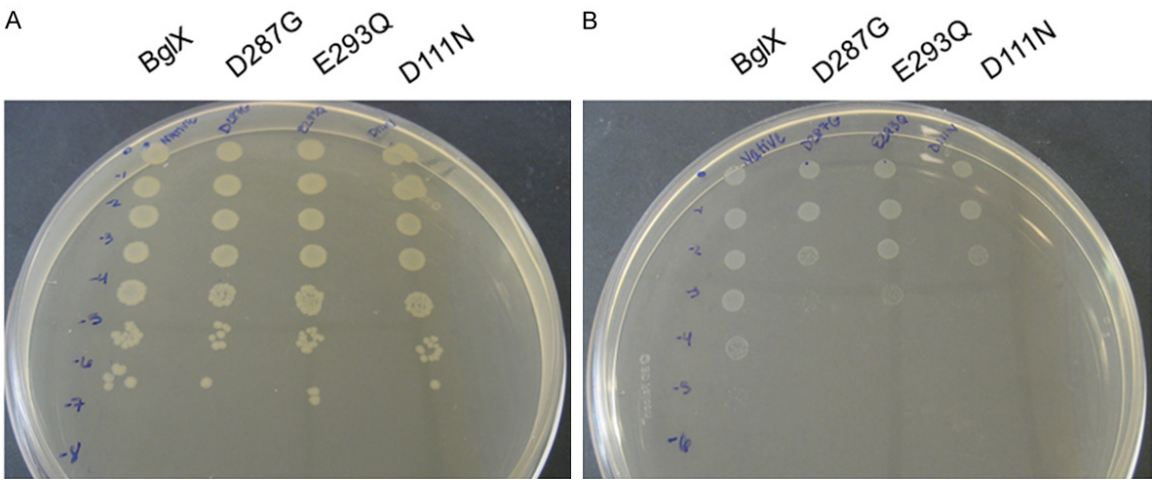

Figure 3. Effect of carbon sources on bacterial growth. Ten-fold serially diluted bacterial strains overexpressing BglX or the mutant enzymes grown on agar plates: (A) Nutrient-rich LB media; (B) Minimal media supplemented with lactose.

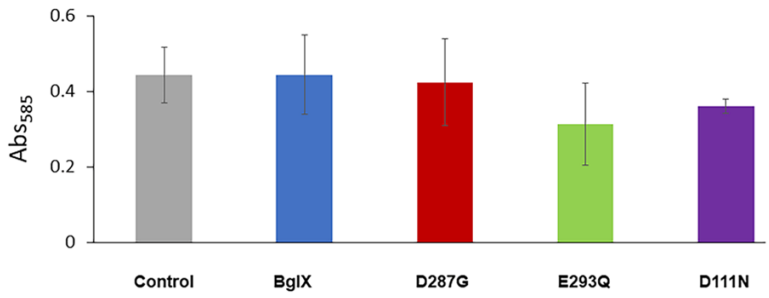

Figure 4. Evaluation of the amount of biofilm. Cells overexpressing BglX or mutants were grown for three days at 37 °C in a 96-well plate containing TSB media. The wells were washed with water, and the biofilms were stained with crystal violet. After dissolving the stained films in acetic acid, the absorbance of the solutions was measured at 585 nm. The error bars illustrate the standard deviation from the mean (n = 3).

GXXXSDH/W motif [35]. The residue that might act as an acid-base catalyst, D111, was chosen after analysis of the BglX 3D-structure predicted by Phyre2 [36] using β-glucosidase from barley seed as a template [37]. It should be noted, though, that these two β-glucosidases have a low percentage of identity in their primary structures. D287 was substituted with a glycine residue, as its side-chain hydrogen atom is incapable of assisting in nucleophilic catalysis. D111 was replaced with asparagine; an amino acid with a comparably sized side chain that cannot donate and accept a proton like the carboxyl group of aspartate. One other residue of potential importance is E293. We proposed that this residue may be involved in forming interactions between the enzyme and a substrate. Here, E293 was mutated to glutamine.

Since BglX is predicted to function as a β-glucosidase, we first tested the substrates *p*NPGlc and *o*NPGlc which both contain a β-glycosidic bond formed by D-glucose. The activity of BglX towards these two substrates was quite low - the enzyme was only able to hydrolyze about one molecule of substrate per minute. For comparison, a periplasmic β-glucosidase from *Rhodothermus marinus,* DSM 4252, has a k_{cat} with *p*NPGlc about 3,500-fold higher than that of *E. coli* BglX [38]. Obviously, the kinetic parameters of β-glucosidases vary significantly between various organisms. However, in most cases, the enzymes can convert up to three hundred molecules of their native substrates per second and usually exhibit K_M values below 1 mM [10]. The mutations of D287 and D111 rendered the enzymes inactive towards glucose-containing substrates. The loss of activity by the D287G and D111N mutants confirmed the participation of these amino acid residues in the hydrolysis of aryl-glucosides. To our surprise, the mutants were able to hydrolyze the galactose-containing substrate *o*NPGal. All proteins demonstrated lower K_M values and significantly

higher turnover numbers with *o*NPGal than with its glucose analog. We confirmed these results by testing the in-gel enzymatic activity of BglX and its mutants. The intensities of the fluorescent bands with MUGal correlated with the catalytic efficiency data in the *o*NPGal kinetic assay. Overall, the activity decreased in the following order: wild-type > E293Q > D287G > D111N.

Next, we examined the enzymatic activity of BglX and its mutants in the presence of high concentrations of glucose or galactose. In general, the activity of glycosidases are affected by free monosaccharides in three ways: they may be inhibited, stimulated, or unaffected by high concentrations of those sugars [39, 40]. We observed that glucose produced a significant inhibitory effect on enzymatic activity when *p*NPGlc was used as a substrate but only slightly reduced the activity towards *o*NPGal. High concentrations of galactose only moderately decreased the enzymatic activity of BglX and the mutants when either *p*NPGlc or *o*NPGal were used as substrates. These results combined with the retention of activity towards *o*NPGal by the D287G and D111N mutants suggest that glucose- and galactose-containing substrates may bind differently within the enzyme's active site.

We evaluated three disaccharides as potential natural substrates for BglX. Cellobiose and maltose are composed of two glucose residues linked by a 1→4-glycosidic bond. In cellobiose, the glycosidic bond is in β-configuration, while maltose contains an α-glycosidic bond. Lactose consists of a galactose residue bonded to a glucose residue via a β (1→4)-glycosidic bond. Only lactose was hydrolyzed by the wild-type BglX and E293Q mutant. Both enzymes exhibited the same trends in kinetic parameters with lactose as those observed previously with *o*NPGal - very similar K_M values and significantly higher k_{cat} for the wild-type BglX. In agreement with the activity studies towards lactose, the cells that overexpressed the wild-type BglX had a noticeable growth advantage when plated on minimal media supplemented with lactose. The cells expressing E293Q mutant followed. The slow growth rates of the cells overexpressing the D111N and D287G mutants correlated with the inability of those mutants to hydrolyze lactose.

The ability of BglX to cleave a different type of glycosidic bond was assessed using laminarin, a trisaccharide of glucose residues connected via β (1→3) bonds [41]. A study of periplasmic BglX from *P. aeruginosa* demonstrated its activity with short gluco-oligosaccharides like sophorose and laminaritriose, containing β (1→2) and β (1→3) glycosidic bonds, respectively [42]. In contrast, incubation of the wild-type *E. coli* BglX with laminarin established that the enzyme was not able to cleave the β (1→3) bond. Another striking difference in the behavior of these two enzymes is that *P. aeruginosa* BglX is unable to hydrolyze lactose, while its *E. coli* analog has this ability. A more recent study on the adaptation of *Caulobacter crescentus* to osmotic stress showed the ability of its BglX to hydrolyze the β (1→2) bond in sophorose [43]. This bond specificity led to the hypothesis that the enzyme may participate in osmoregulation through the hydrolysis of OPG and/or biofilm synthesis and suggested that BglX works in collaboration with the esterase EstG to hydrolyze cyclic glucose structures [43]. The inactivation of the *bglX* genes by transposon insertion in *P. aeruginosa* [42] and avian pathogenic *Escherichia coli* [44] leads to decreased biofilm formation. We compared the sequences of the three BglX enzymes. The BglX enzyme from *E. coli* has about 62% sequence identity to the enzyme from *P. aeruginosa* and only 42% to BglX from *Caulobacter.* All three enzymes are active with the model substrate *p*NPGlc. Our analysis of biofilm formation by *E. coli* cells overexpressing the wild-type BglX or its mutants indicated no statistically significant difference between the cells. Consequently, the overexpression of BglX did not result in an increase in biofilm production. The lack of the overexpression effect may be explained if BglX relies on another enzyme(s) to modify the OPG before acting on them. Further investigation into the possibility of this specific enzymatic collaboration in *E. coli* cells is required.

Conclusions

In this study, we demonstrated the functional importance of two amino acid residues, D111 and D287, for *E. coli* BglX activity. Mutation of the D287 residue to glycine and D111 to asparagine completely abolished enzymatic activity towards β-glucosides; however, both mutants retained some activity towards β-galactosides.

Glucose strongly inhibited enzymatic activity with glucose-containing substrates while it only moderately decreased activity towards compounds containing galactose. Galactose only slightly reduced activity, with no obvious differences between glucose- and galactose-containing substrates. Furthermore, BglX can hydrolyze lactose but not cellobiose or laminarin. Taken together, these observations suggest differences in the binding of gluco- and galactosaccharides within the active site of the BglX enzyme. Low activity with glucose-containing substrates suggests that β-glucosidase activity may not be the primary function of the BglX enzyme. Our results indicate that despite its functional prediction, *E. coli* BglX demonstrates higher activity towards β-galactosides compared to structurally related glucose derivatives, a finding that could further direct our search of this enzyme's physiological role.

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Disclosure of conflict of interest

None.

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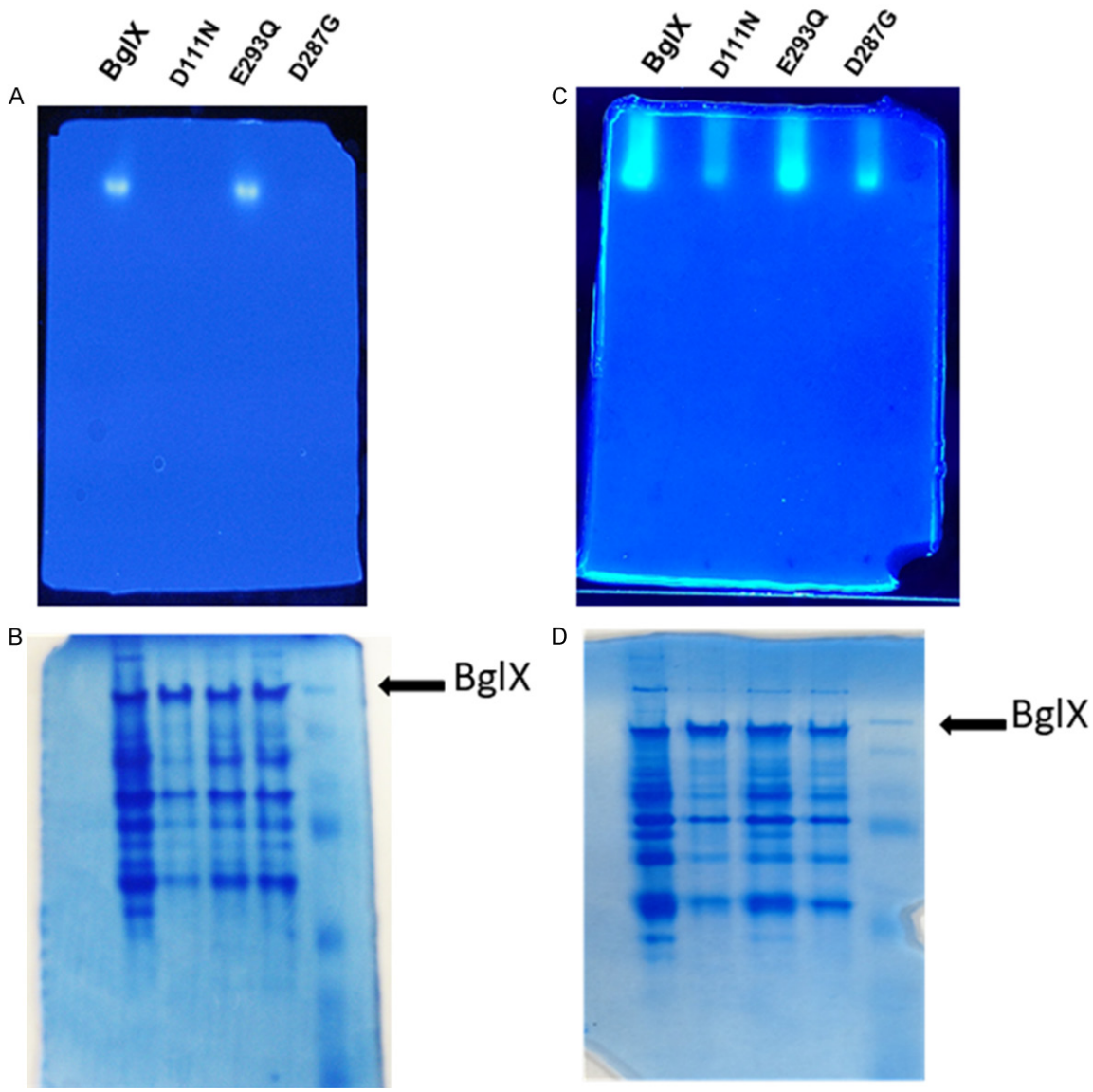

Figure S1. In-gel activity of BglX and mutants. Two fluorogenic substrates were used to test in-gel enzymatic activity of BglX and its mutants: (A) 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc) and (C) 4-methylumbelliferyl-β-D-galactopyranoside (MUGal), (B and D) show the protein load, gels were developed with Coomassie Blue dye.

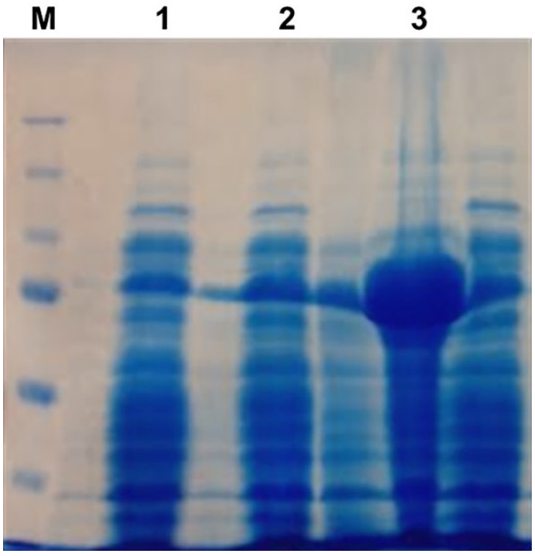

Figure S2. SDS-PAGE analysis of *E. coli* cells for truncated BglX expression. Lane 3 represents the colony with the highest expression of t-BglX.

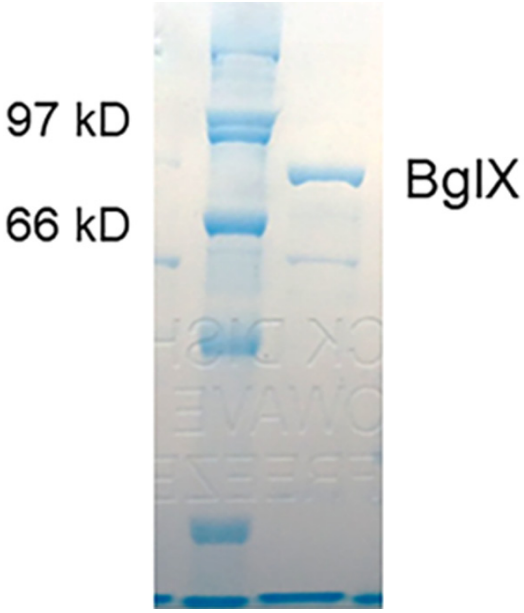

Figure S3. SDS-PAGE analysis of the purified BglX.

Table S1. Primers for cloning truncated *bglX*

Gene	Primer Sequence (F - forward, R - reverse)
t-bglX	F: AAAAAAACATATGGATGATTTATTCGGCAACCATCCATTA
	R: TTTGAATTCTTACAGCAACTCAAACTCGCCTTTC

The restriction sites for *NdeI* and *EcoRI* are underlined.