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Cloning and Comparison of Third β-Glucoside Utilization (*bgl*EFIA) Operon with Two Operons of *Pectobacterium carotovorum* subsp. *carotovorum* LY34

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A third *bgl* operon containing *bgl*E, *bgl*F, *bgl*I, and *bgl*A was isolated from *Pectobacterium carotovorum* subsp. *carotovorum* LY34 (*Pcc* LY34). The sequences of BglE, BglF, and Bgll were similar to those of the phosphotransferase system (PTS) components IIB, IIC, and IIA respectively. BglF contains important residues for the phosphotransferase system. The amino acid sequence of BglA showed high similarity to various 6-phospho-β-glucosidases and to a member of glycosyl hydrolase family 1. Sequence and structural analysis also revealed that these four genes were organized in a putative operon that differed from two operons previously isolated from *Pcc* LY34, *bgl*TPB (accession no. AY542524) and *asc*GFB (accession no. AY622309). The transcription regulator for this operon was not found, and the EII complexes for PTS were encoded separately by three genes (*bgl*E, *bgl*F, and *bgl*I). The BglA enzyme had a molecular weight estimated to be 57,350 Da by SDS–PAGE. The purified β-glucosidase hydrolyzed salicin, arbutin, ρNPG, ρNPβG6P, and MUG, exhibited maximal activity at pH 7.0 and 40 °C, and displayed enhanced activity in the presence of Mg^{2+} and Ca^{2+}. Two glutamate residues (Glu_{178} and Glu_{378}) were found to be essential for enzyme activity.

Key words: *Pectobacterium carotovorum* subsp. *carotovorum* LY34; *bgl* operon; PTS system; 6-phospho-β-glucosidase

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*), classified previously as *Erwinia carotovora* subsp. *carotovora* (*Ecc*), is a pathogenic enterobacteria that causes soft-rot disease in plants. This phytopathogen degrades macromolecules that compose the plant cell wall, resulting in maceration of plant tissue. This macerating capacity comes from the secretion of a set of extracellular enzymes including pectinases, proteases, cellulases, and β-glucosidases, all of which occur in multiple enzymatic forms. Bacterial synthesis of these isozymes might ensure a more efficient degradation of polysaccharides present in the plant cell wall.[1)]

β-glucosidase is involved in the final step of cellulose degradation, in which cellobiose is degraded to glucose.[2)] β-glucosidases are widespread in bacteria, where they are involved in the metabolism of various carbohydrate substrates, including aromatic glycosides like arbutin and salicin.[2–6)]

The phosphoenolpyruvate:phosphotransferase system (PEP:PTS) is important for carbohydrate uptake, and is largely used for bacterial utilization of β-glucoside.[7–9)] PTS contains the general proteins enzyme I (EI) and HPr (histidine containing protein), as well as the substrate-specific enzyme II (EII).[10–12)] Reporting the sequence data on the carbohydrate transport proteins, Saier[13)] distinguished six families of sugar transporters of bacterial PEP:PTS. Representing the sugar-specific permease, EII complexes usually possess cytoplasmic IIA and IIB and a membrane-spanning IIC.[9,14)]

The *Pcc* strain LY34 used in the present study was originally isolated from Chinese cabbage with soft-rot symptoms. To analyze their importance in phytopathogenicity, three different kinds of cellulases were previously isolated: CelA, CelB, and CelC of *Pcc* LY34.[15)] Also, *bgl*TPB and *asc*GFB operons, which regulate the utilization of β-glucoside, were isolated and analyzed in our previous studies.[16,17)] These two operons from *Pcc* LY34 contain genes involved in sugar transport and metabolism. The *bgl*TPB operon possesses a positive regulator (operon-specific antiterminator: BglT) for the transcription of this operon, while the *asc*GFB operon possesses a negative regulator (operon-specific repressor: AscG). Furthermore, BglP comprises three domains (EIIB, EIIC, and EIIA) that are encoded by a single ORF, whereas AscF consists of two domains (EIIB and

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EIIC) in a single polypeptide. BglP and AscF proteins have high similarities to those of *E. coli* BglF and AscF respectively. The *bgl*B and *asc*B genes encode 6-phospho-β-glucosidase, which belongs to glycosyl hydrolase family 1.

In this report, we describe the putative *bgl*EFIA operon genes from *Pcc* LY34, the third operon of β-glucoside sugar utilization genes that are functionally expressed in *E. coli* from a *Pcc* LY34 genomic library. Sequence analysis of the active clone revealed a group of linked genes involved in sugar transport and metabolism, specifically, a putative *bgl*EFIA operon encoding a 6-phospho-β-glucosidase (*bgl*A). The structure of this operon differed from those of *bgl*TPB and *asc*GFB operons isolated from the same strain. The operon lacked a regulator gene for the transcription of this operon, and the EII complex was composed of three polypeptide chains encoded by three separate genes. The biochemical properties of the BglA protein were also examined.

Materials and Methods

Bacterial strains and growth conditions. Tryptone-yeast extract medium was used for the routine cultivation of *Pectobacterium carotovorum* subsp. *carotovorum* LY34 (*Pcc* LY34) strain. *Escherichia coli* DH5α, BL21 (DE3), and recombinant *E. coli* harboring the *bgl*A gene were cultured in LB medium containing 50 µg/ml ampicillin when appropriate.

Recombinant DNA techniques and DNA sequencing. Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were followed.[18] Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, Maryland, USA) and Boehringer Mannheim (Indianapolis, Indiana, USA). Other chemicals were purchased from Sigma Chemical (St. Louis, Missouri, USA). Nucleotide sequences were determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer., Norwalk, Connecticut, USA). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, California, USA). The BLAST program was used to find the protein coding regions. The nucleotide sequence data reported are available in the GenBank database under accession no. AY769096.

*Construction of cosmid library and cloning of the bgl*EFIA *operon.* A genomic library was constructed in the cosmid vector pCC1FOS as previously described.[19] Total genomic DNA from *Pcc* LY34 was sheared into approximately 40-kb fragments using a syringe needle, size-fractionated on a 5 to 40% linear sucrose gradient, and then end-repaired to yield blunt, 5′-phosphorylated ends. The resulting DNA fragments were ligated with cloning-ready pCC1FOS vector and then packaged using a lambda DNA packing kit (Epicentre, Wisconsin, USA). The library was screened on M9 media,[18] containing 1 mM 4-methylumbelliferyl β-D-glucoside (MUG) for β-glucosidase activity (a positive colony has a fluorescent halo). A cosmid clone (pAY3) bearing β-glucosidase activity was isolated. For subcloning, pAY3 was partially digested with *Sau*3AI. Three-to six-kb fragments of the cosmid DNA from this partial digestion were ligated into the *Bam*HI site of pBluescript II SK+ vector and transformants in *E. coli* DH5α were screened as above. The positive subclone was obtained (pAY300).

Cloning of the bglE, bglF, bglI, and bglA genes. To amplify *bgl*E, *bgl*F, *bgl*I, and *bgl*A from the *Pcc* LY34 chromosome, specific oligonucleotide primers were designed based on the DNA sequence data. The primers for *bgl*E, *bgl*F, *bgl*I, and *bgl*A were 5′-TTCCCCGATTGCGCTATTTATG-3′ (sense), 5′-GATGGAGCGCAGATGAGCAGAAC-3′ (antisense); 5′-CGCTGTTGAGATAAAAGCCGTAG-3′ (sense), 5′-CACAGAAGCCGCGTTCAGCAGTTC-3′ (antisense); 5′-CTGATTGTCGTCTCATCCTTAATC-3′ (sense), 5′-CTCCAGCTCATCGCCATTGGGGAAAATACG-3′ (antisense); and 5′-ATCCAGACTGCTTTGATTGGTGCC-3′ (sense), 5′-AACCCCGCTTACACACTACATTCT-3′ (antisense) respectively. The purified PCR products of the 1.4-, 1.7-, 0.8-, and 1.8-kb fragments respectively were cloned into pGEM-T Easy vector (Promega, Wisconsin, USA) and sequenced. The resulting plasmids were designated pAY310, pAY320, pAY330, and pAY340. For high expression of *bgl*A, the PCR product generated with primers 5′-GGATCCATGTCTGTTCAACAATTACCG-3′ (sense, containing a *Bam*HI site as underlined) and 5′-AAGCTTGTAGCTCGGCACCGTTGCT-3′ (antisense, containing a *Hind*III site as underlined) was cloned into expression vector pET-21a (+) (Novagen, Darmstadt, Germany) using *Bam*HI and *Hind*III sites, resulting in the addition of a C-terminal $(His)_6$ tag. The resulting plasmid was designated pET-21a(+)/BglA (pAY510). The absence of mutations within the coding region of BglA was verified by DNA sequencing.

Site-directed mutagenesis. Site-directed mutagenesis of the *bgl*A gene to create the E178A and E378A mutations was performed using the following oligonucleotide primers: E178A, 5′-TGGATGACTTTCAATGCGATCAACAACCAGC-3′ (sense) and 5′-GCTGGTTGTTGATCGCATTGAAAGTCATCCA-3′ (antisense); E378A, 5′-CAATGTTCATCGTCGCAAACGGTTTTGGCGC-3′ (sense) and 5′-GCGCCAAAACCGTTTGCGACGATGAACATTG-3′ (antisense). The 50 µl of reaction mixtures contained 1 µl of pET-21a(+)/BglA DNA (80 ng/µl), 100 *p*mol of each primer, 5 µl of 2 mM dNTP mixture, 5 µl of 10 × *pfu* DNA polymerase buffer containing 20 mM $MgSO_4$, and 2.5 U

of cloned *Pfu* DNA polymerase purchased from Stratagene (La Jolla, California, USA). PCR products were incubated on ice for 5 min, and then 1 µl of *Dpn*I restriction enzyme (10 U/µl) was added for 1 h incubation at 37 °C. *Dpn*I-treated plasmids were then transformed into *E. coli* DH5α according to the manufacturer's specifications (Site-directed mutagenesis kit, Stratagene, La Jolla, California, USA). The resulting plasmids were designated pAY520 and pAY530 respectively.

Enzyme assay. β-glucosidase activity was determined using ρ-nitrophenyl β-D-glucopyranoside (ρNPG),[20,21] 4-hydroxyphenyl β-D-glucopyranoside (arbutin), 2-(hydroxymethyl) phenyl β-D-glucopyranoside (salicin), and 4-methylumbelliferyl β-D-glucoside (MUG)[22] as substrates. The standard assay consisted of incubating enzyme with 5 mM ρNPG in 50 mM sodium phosphate buffer (pH 7.0) for 10 min in a total volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml of 0.2 M glycine–NaOH (pH 10.5), and the amount of ρ-nitrophenol released from the ρNPG was determined by measuring absorbance at 405 nm.[23,24] One unit of β-glucosidase was defined as the amount of enzyme required to release 1 mole of ρ-nitrophenol per min under the assay conditions. All assays were carried out in 50 mM sodium phosphate buffer (pH 7.0) at 40 °C, unless otherwise noted. In order to detect the β-glucosidase activity for salicin as substrate, an appropriate aliquot of cell suspension of the *bgl*A clone was added to 800 µl of 30 mM salicin in 50 mM phosphate buffer (pH 7.0). After 30 min of incubation, the enzymatic reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The production of saligenin from salicin was detected as described previously.[5,25] Enzyme activity for the arbutin substrate was measured by washing the culture and resuspending it in 0.8 ml of 50 mM phosphate buffer (pH 7.0). The reaction was started by adding 0.1 ml of 10 mM $MgCl_2$ and 0.1 ml of 30 mM arbutin, and was stopped by 0.5 ml of 1 M Na_2CO_3, as described previously.[26]

The effects of pH and temperature on β-glucosidase activity were examined with the purified recombinant enzyme. The effect of pH on β-glucosidase activity was determined in the standard assay, but with pH values ranging from pH 4.0 to 9.0; all assays were performed at 40 °C. To determine the effect of temperature on the enzymatic activity, samples were incubated at temperatures from 20 to 60 °C for 30 min. Thermostability data were obtained by pre-incubating BglA at various temperatures and then measuring residual activity under the standard assay condition. The effects of various metal ions classified by a concentration of 10 mM on β-glucosidase activity were also examined. The kinetic constants, K_m and V_{max}, corresponding to the β-D-glucosidase activity of purified mature BglA for the ρ-nitrophenyl β-D-glucopyranoside were examined by using the Michaelis-Menten equation.[27] Phospho-β-glucosidase activity was assayed using ρ-nitrophenyl β-D-glucopyranoside-6-PO_4 (ρNPβG6P). Preparation of ρNPβG6P was by the procedure described earlier for the synthesis of ρNPαG6P.[17,28,29]

Expression and purification of the enzyme. E. coli strain BL21 (DE3) carrying pET-21a(+)/BglA was grown at 37 °C to mid-log phase in LB medium containing 50 µg/ml ampicillin. Expression was then induced by adding IPTG to a final concentration of 0.5 mM, and growth was continued for 6 h. The cells were harvested by centrifugation (6,000 rpm, 10 min) and washed twice with 10 mM Tris–HCl buffer (pH 7.0). The cells were resuspended in the same buffer disrupted by sonication at 4 °C, and centrifuged (6,000 rpm, 30 min) to remove cell debris. The solubilized recombinant BglA with His-tag (BglA-His) was applied on a HisTrap kit (Amersham Pharmacia Biotech, Pennsylvania, USA). BglA was eluted with 100 mM imidazole. The enzyme samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The active fractions were combined and purified by ion exchange chromatography on a Q-Sepharose column (Amersham Pharmacia Biotech, Pennsylvania, USA).[30] For the final purification step, the dialyzed sample was loaded onto an anion exchange Mono Q HR (Amersham Pharmacia Biotech, Pennsylvania, USA) column pre-equilibrated with 20 mM MOPS buffer, pH 6.5. The fractions with β-glucosidase activity eluted as a single protein peak and the purity of the enzyme was assessed by SDS–PAGE. The protein concentration was determined by the method of Bradford.[31]

Results

Cloning and nucleotide sequence analysis of the putative bglEFIA operon

A cosmid library of *Pcc* LY34 genomic DNA was screened for clones expressing β-glucosidase activity on M9 media containing 1 mM MUG. A cosmid clone expressing β-glucosidase activity was isolated and designated pAY3. *Sau*3AI subclones of pAY3 were then screened using the same assay, yielding the positive clone pAY300 containing a 3.7-kb insert (Fig. 1). A 3,738-bp DNA fragment was sequenced and analyzed (accession no. AY769096). Sequence analysis identified the presence of four open reading frames, *bgl*E, *bgl*F, *bgl*I, and *bgl*A, putatively encoding proteins of 101-, 437-, 108-, and 480-aa respectively (Fig. 2). The *bgl* genes were cloned separately, as described in "Materials and Methods."

Structure of the putative bglEFIA operon of Pcc LY34 and comparison to other bgl operons

The National Center for Biotechnology Information's BLAST e-mail server (USA) was used to search the peptide sequence databases for proteins homologous to the Bgl proteins. The *Pcc* LY34 BglE, BglF, BglI, and

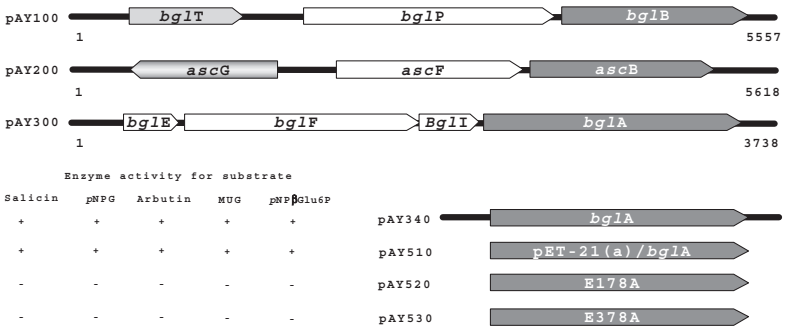

Fig. 1. Comparison of the Putative β-Glucoside Utilization Operons from *Pectobacterium carotovorum* subsp. *carotovorum* LY34.

Physical map of recombinant DNA fragments. pAY100 (accession no. AY542524), pAY200 (accession no. AY622309), and pAY300 (accession no. AY769096) were constructed by cloning 5.5-, 5.6-, and 3.7-kb fragments of cosmid DNA (pAY1, pAY2, and pAY3) into pBluescript II SK+ vector. pAY340 was derived by cloning the PCR products from pAY300 into pGEM-T Easy vector. pAY510 was derived by cloning into pET-21a(+) expression vector. pAY520 and pAY530 were derived by site-direct mutation (E178A and E378A) from pAY510. β-glucosidase activity (BglA/pAY500) was determined using ρ-nitrophenyl β-D-glucopyranoside (ρNPG), 4-hydroxyphenyl β-D-glucopyranoside (arbutin), 2-(hydroxymethyl) phenyl β-D-glucopyranoside (salicin), 4-methylumbelliferyl β-D-glucoside (MUG), and ρ-nitrophenyl β-D-glucopyranoside-6-PO_4 (ρNPβGlu6P).

BglA proteins are associated with transport and utilization of β-glucosides (Table 1). Based on these similarities, we hypothesized that the *Pcc* LY34 proteins could functionally replace CelA and CelB in *Photorhabdus luminescens* subsp. *laumondii* TTO1 and BglA in *E. coli* (Table 2). The putative operon *bgl*EFIA differs from two putative operons, *bgl*TPB and *asc*GFB, previously isolated from *Pcc* LY34 (Fig. 1).

The first ORF (*bgl*E) spans 306 nucleotides and encodes a deduced protein of 101 amino acids. There is a potential ribosome-binding site, GAGG, located 6 bases upstream from the start codon of this ORF (Fig. 2). The *Pcc* LY34 BglE protein exhibits 55.0, 54.0, and 51.5% similarity with *P. luminescens* subsp. *laumondii* TTO1 CelA, *V. cholerae* phosphotransferase system IIB component, and *Clostridium acetobutylicum* phosphotransferase system IIB component respectively (Table 1).

The second ORF (*bgl*F) is 1,314-bp long, and the deduced protein is 437-aa long. The start site of this ORF is 16-bp downstream from the end of *bgl*E. There is a putative ribosome-binding site, GGAGGA, located 3 bases upstream from the start of this ORF (Fig. 2). The deduced protein from this ORF displayed similarity to several GenBank entries, with very high similarity with the phosphotransferase system components IIC from *V. vulnificus* YJ016, CelB from *P. luminescens* subsp. *laumondii* TTO1, and diacetylchitobiose-specific IIC from *Bacillus cereus* ATCC 14579 (Table 1, 2). Lai[32] compared PTS-dependent EII permease homologies and observed patterns of hydrophobicity, suggesting a central membrane-spanning IIC domain flanked by hydrophilic IIB and IIA domains located in the N-terminus and C-terminus respectively of CelA. But as a single protein, BglF did not have a central membrane-spanning IIC domain flanked by hydrophilic IIB and IIA domains as in BglP and AscF of *Pcc* LY34 (Fig. 3A, B).

The third ORF (*bgl*I) started 11-bp upstream of the terminator of *bgl*F. *bgl*I was 327-bp long and was deduced to be a protein of 108 amino acids (Fig. 2). Preceding this ORF, no ribosome-binding site was found. The *Pcc* LY34 BglI protein has 52.9, 51.5, and 49.5% similarity with the phosphotransferase system components IIA from *E. coli* CFT073, CelC from *Yersinia pestis* KIM, and CelC from *P. luminescens* subsp. *laumondii* TTO1 (Table 1, 2).

The fourth ORF (*bgl*A) begins 348-bp downstream from *bgl*F and is 1,443 nucleotides long. It is predicted to encode a protein of 480 amino acids. A putative ribosome-binding site, TAAGAAG, is positioned 8 bases from the start of the coding region (Fig. 2). The predicted BglA protein, which has homology to β-glucosidase from a variety of organisms, is classified in family 1 of the glycosyl hydrolases.[33] BglA also contained two family 1 glycosyl hydrolase signature regions. We found a high level of identity (approximately 76%) among the *Y. pestis* KIM BglA, *E. coli* BglA, and *S. typhimurium* LT2 BglA proteins (Table 1).

Purification and characterization of BglA

Using an *E. coli* strain that overexpressed BglA, the protein was purified using column filtration techniques, as described in "Materials and Methods." Protein fractions from the purification steps were analyzed by SDS–PAGE, and only one protein band (approximately 57 kDa) was present after the final purification step (Fig. 4). The effect of pH on the activity of BglA against ρNPG was determined at 40 °C in various buffers ranging from pH 4.0 to 9.0 (Fig. 5A). Maximal activity was observed at pH 7.0. The temperature dependence of BglA activity toward ρNPG was determined by measuring activity at various temperatures at pH 7.0. Maximal activity was observed at 40 °C (Fig. 5B). Thermo-

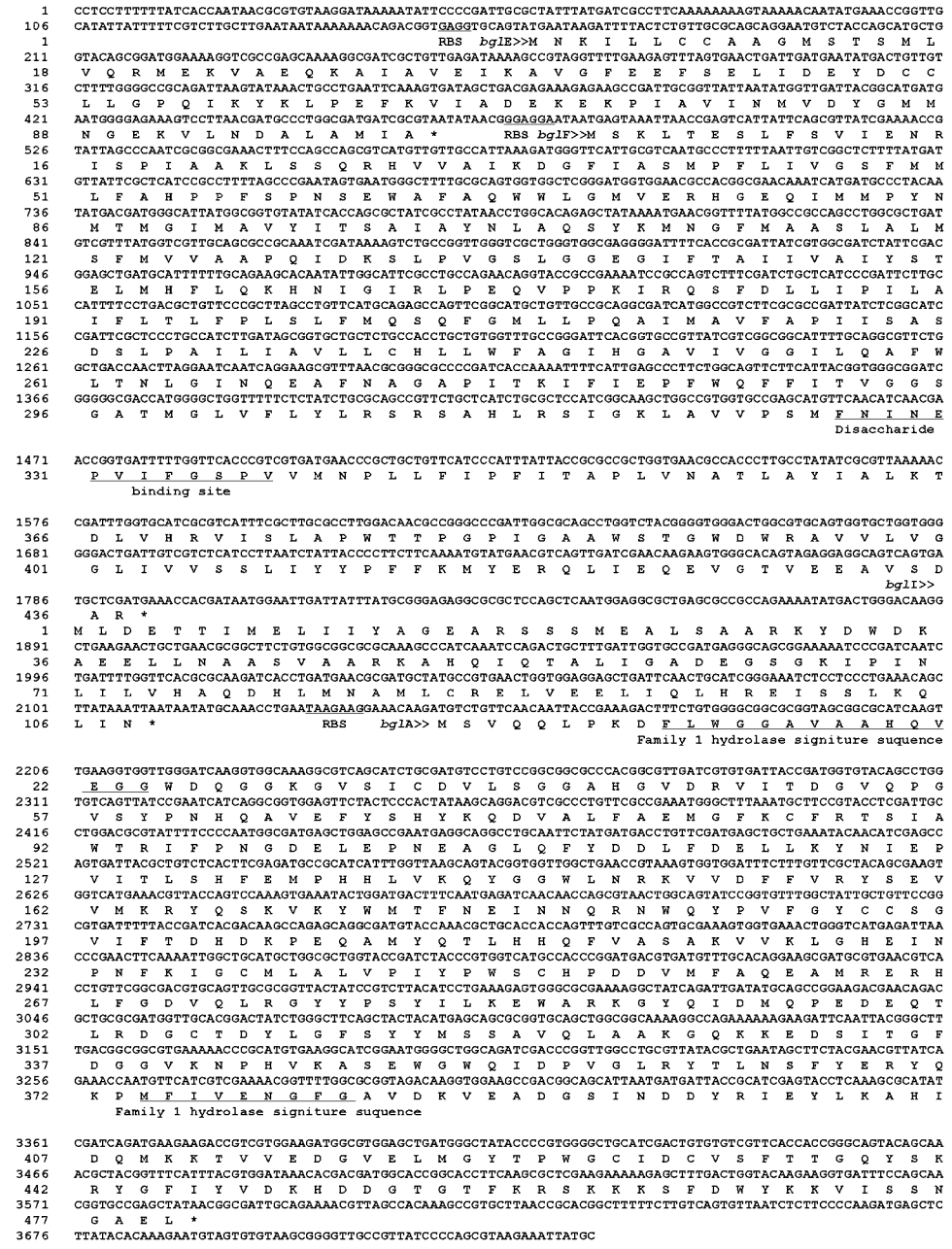

Fig. 2. Nucleotide Sequence of *Pectobacterium carotovorum* subsp. *carotovorum* LY34 *bgl* Genes and the Flanking Regions.
The deduced amino acid sequences for each ORF are placed below the nucleotide of the corresponding codon. Putative Shine-Dalgarno sequences for ribosomal binding site (RBS) are underlined and labeled. Proteins are labeled at their respective start codons. The signature sequence for disaccharide binding site is underlined and labeled. The family 1 hydrolase signature sequences are also underlined and labeled.

stability data were obtained by pre-incubating BglA at various temperatures and then measuring residual ρNPG hydrolyzing activity under the standard assay condition. After 10 min of incubation at 60 °C, the activity dropped by 38% (Fig. 5C). β-glucosidase activity was also measured at pH 7.0 and 40 °C in the presence of various metal ions. Divalent cations such as Zn^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, and Co^{2+} inhibited enzyme activity while Mg^{2+} and Ca^{2+} had positive effects (Table 3). The addition of 15 mM Mg^{2+} yielded the maximum activity on ρNPG (Fig. 5D). The kinetic parameters of β-glucosidase (K_m and V_{max}) were determined using ρNPG as the substrate. The K_m value of BglA was 0.23 mM and the V_{max} value was 36 μmol/min/mg of protein. The values of K_m and V_{max} were not detected in single-point mutants E178A and E378A (Table 4).

Identification of essential residues for β-glucosidase activity

In vitro site-directed mutagenesis can be invaluable in studying protein structure-function relationships. In the present study, we used site-directed mutagenesis to replace two Glu residues with Ala at sites in BglA that might serve catalytic functions. Mutant plasmids were

sequenced on both strands to confirm that only the intended mutation was introduced. Enzyme activity assays were then performed with BglA mutants carrying Glu178Ala and Glu378Ala. These mutations abolished enzyme activity for the β-glucoside (Table 4). These results indicate that Glu_{178} and Glu_{378}, which are both conserved in the β-glucosidase sequences of family 1, are essential for BglA activity (Table 2).

Table 1. Pairwise Similarity (%) of BglE, BglF, BglI, and BglA of *Pcc* LY34

	Similarity (%) with related amino acid sequences[a]			
Sequence	1	2	3	4
BglE	100.0	55.0	54.0	51.5
CAE15128		100.0	47.5	42.6
D82219			100.0	43.6
A96947				100.0
BglF	100.0	53.6	51.1	41.2
BAC95664		100.0	55.3	38.0
CAE15129			100.0	36.9
AAP09357				100.0
BglI	100.0	52.9	51.5	49.5
AAN80414		100.0	49.5	50.0
AAM84827			100.0	42.5
CAE15130				100.0
BglA	100.0	80.7	76.2	76.1
AAM86483		100.0	79.0	79.5
Q46829			100.0	95.4
AAL21926				100.0

[a] Calculated with CLUSTAL W and the PAM250 residue weight table. The sequences are from the following sources: BglE, BglF, BglI, and BglA from *Pcc* LY34 (1); CAE15128 (2), CAE15129 (3), and CAE15130 (4) from *Photorhabdus luminescens* subsp. *laumondii* TTO1; D82219 (3) from *Vibrio cholerae*; A96947 (4) from *Clostridium acetobutylicum*; BAC95664 (2) from *Vibrio vulnificus* YJ016; AAP09357 (4) from *Bacillus cereus* ATCC 14579; AAN80414 (2) from *E. coli* CFT073; AAM84827 (3) and AAM86483 (2) from *Yersinia pestis* KIM; Q46829 (3) from *E. coli*; AAL21926 (4) from *Salmonella typhimurium* LT2.

Discussion

There have been several physiological and genetic studies of the β-sugar metabolic system. Many bacteria contain more than one β-sugar catabolic pathway, with *E. coli* containing four β-glucoside sugar systems.[34] Previously, *bgl*TPB and *asc*GFB operons for uptake and utilization of β-sugars were isolated from *Pcc* LY34. In the present study, we isolated four genes (*bgl*E, *bgl*F, *bgl*I, and *bgl*A) that form the genetic organization of a putative operon. The respective genes encode (I) the putative IIB component of PTS system, BglE; (II) PTS permease BglF, a phosphotransferase system component IIC protein; (III) the putative IIA component of PTS system, BglI; and (IV) the β-glucosidase, BglA, that contains two conserved regions of glycosyl hydrolase family 1 (Fig. 2). This operon differed from the two operons above (*bgl*TPB and *asc*GFB) in that it lacked an operon-specific antiterminator (BglT) and an operon-specific repressor (AscG). The *bgl*EFIA system might at least specify a transport system and a hydrolase that acts on salicin, arbutin, ρNPG, and MUG.

The molecular weight of β-glucosidase (BglA) was estimated by SDS–PAGE to be 57,350 Da. β-glucosidase exhibited maximal activity at pH 7.0 and 40 °C, with enhanced activity in the presence of Mg^{2+} and Ca^{2+} (Table 3, Fig. 5D). The optimal activity of BglA showed similarly optimal activity at the same pH and temperature to that previously reported for BglB and AscB, with Mg^{2+} also enhancing these enzymes.[16,17] Furthermore, BglA showed 52.7 and 52.2% identity with BglB and AscB respectively. Three β-glucosidases, BglA, BglB, and AscB, clearly belong to glycosyl hydrolase family 1, containing a conserved enzyme that employs a double-displacement mechanism of catalysis.[35] In view of two glutamic acid residues, acting as an acid/base and a nucleophile respectively in the hydrolysis of β-glucosidic bonds in the β-glucosidase from

Table 2. Alignment of the Regions Surrounding the Homologous Region of BglE and BglI, Conserved Regions of BglF, and Putative Acid/Base and Nucleophile of BglA and Some Typical Representatives of Glycosyl Hydrolase Family 1

	BglE		BglF		BglI		BglA (β-glucosidase)	
	EIIB		EIIC		EIIA		Acid/base	Nucleophile
BglE	CCAAGMSTS	**BglF**	FNINEPVIFGSPV	**BglI**	VHAQDHLMN	**BglA**	TFN**E**INNQ	MFIV**E**NGFG
CAE15128	CCAAGMSTS	**BAC95664**	FQINEPVIFGSPV	**AAN80414**	IHAQDHLMN	**AAM86483**	TFN**E**INNQ	LFIV**E**NGFG
D82219	CCSAGMSTS	**CAE15129**	FNINEPLLFGSPI	**AAM84827**	VHAQDHLMN	**Q46829**	TFN**E**INNQ	LFIV**E**NGFG
A96947	FCSAGMSTS	**AAP09357**	FNINEPVIFGLPI	**CAE15130**	VHAQDHLMN	**AAL21926**	TFN**E**INNQ	LFIV**E**NGFG
AAL00638	VCNAGMSTS	**AAO81318**	FNINEPVIFGVPI	**CAG21150**	THIQDHIMT	**AAN44371**	TFN**E**INNQ	LFIV**E**NGFG
AAP29103	CCAAGMSSS	**H70216**	FNINEPIMFGAPI	**AAL20239**	VHAQDHLMT	**H82185**	TFN**E**INNQ	IFVV**E**NGLG
CAD00451	VCAAGMSTS	**BAC12654**	FNISEPTIFGAPV	**AAF94442**	VHAQDHLMT	**CAE14566**	TFN**E**INNQ	IFIV**E**NGLG
AAC66322	VCGAGMSTS	**AAM23871**	FNINEPIIFGAPI	**AAN43082**	VHAQDHLMT	**P42973**	TFN**E**INNQ	LFIV**E**NGFG
	* ****.*		*.* ** .** *.		* ***.*.		********	.*.**** *

The sequences are from the following sources: BglE, BglF, BglI, and BglA from *Pcc* LY34; CAE15128, CAE15129, CAE15130, and CAE14566 from *Photorhabdus luminescens* subsp. *laumondii* TTO1; D82219 from *Vibrio cholerae*; A96947 from *Clostridium acetobutylicum*; AAL00638 from *Streptococcus pneumoniae* R6; AAP29103 from *Bacillus anthracis* str. *Ames*; CAD00451 from *Listeria monocytogenes*; AAC66322 from *Borrelia burgdorferi* B31; BAC95664 from *Vibrio vulnificus* YJ016; AAP09357 from *Bacillus cereus* ATCC 14579; AAO81318 from *Enterococcus faecalis* V583; H70216 from *Borrelia burgdorferi*; BAC95664 from *Vibrio vulnificus* YJ016; AAM23871 from *Thermoanaerobacter tengcongensis*; AAN80414 from *E. coli* CFT073; Q46829 from *E. coli*; AAM84827 and AAM86483 from *Yersinia pestis* KIM; CAG21150 from *Photobacterium profundum*; AAL20239 and AAL21926 from *Salmonella typhimurium* LT2; AAF94442 from *Vibrio cholerae* 01 biovar eltor str. N16961; AAN43082 and AAN44371 from *Shigella flexneri* 2a str. 301; H82185 from *Vibrio cholerae*; P42973 from *Bacillus subtilis*.

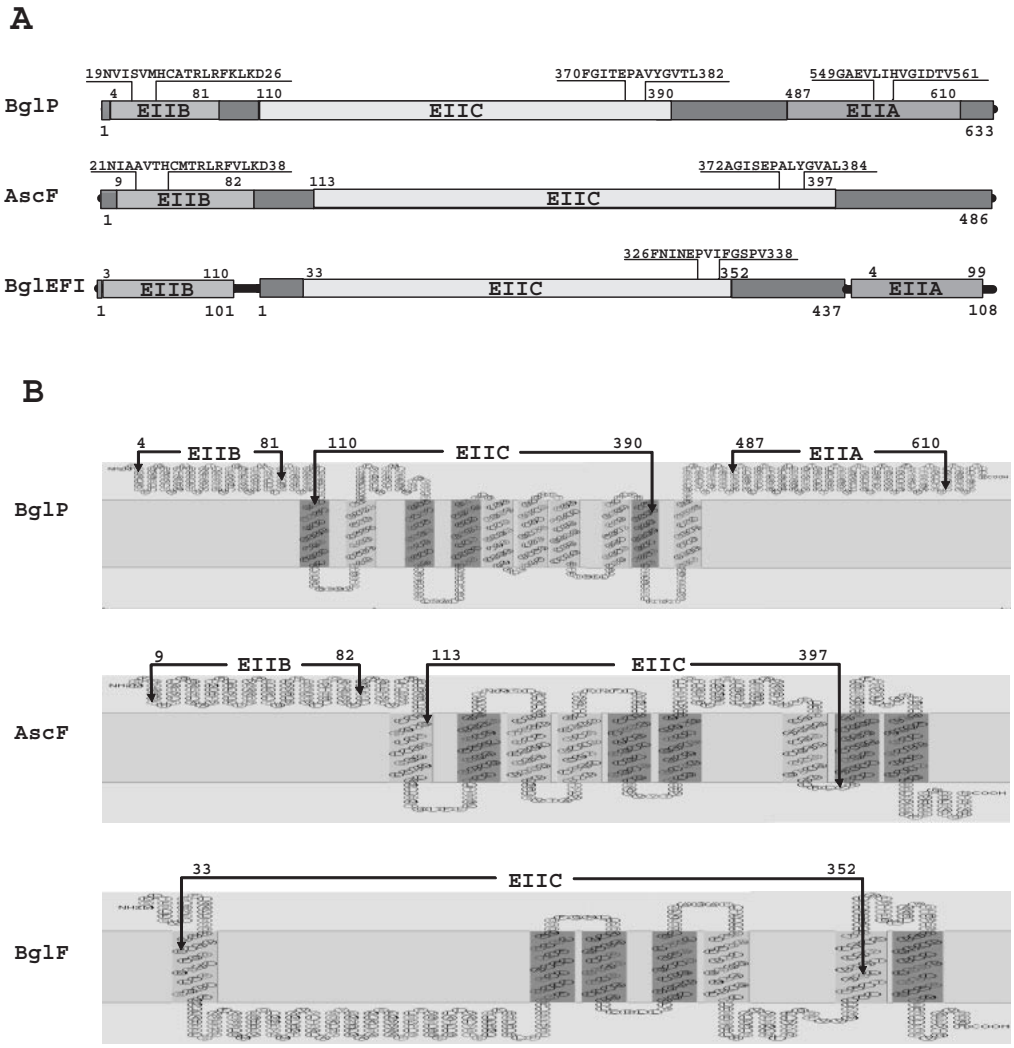

Fig. 3. Comparison of Phosphotransferase EII Domains from *Pectobacterium carotovorum* subsp. *carotovorum* LY34.

A, PTS EII Domains of BglP, AscF, and BglEFI. Three EII complexes of BglP, AscF, and BglEFI respectively are exhibited. The numbers at the extremes of the putative EII domain represent the first and last amino acid residues of the putative EIIA, EIIB, and EIIC regions respectively. Disaccharide-binding sites in EIIC domains are designated above the regions of the polypeptide chains. Residues corresponding by homology to those important for phosphoryl transfer (EIIA and EIIB) are indicated. B, Maps of the putative transmembrane proteins across membranes by SOSUI of proteomics tool (http://sosui.proteome.bio.tuat.ac.jp/sosui_subunit.html). The respective domains of EII complexes are shown. Designated numbers indicate the positions of the corresponding amino acids.

Agrobacterium faecalis,[35,36] site-directed mutagenesis was performed on BglA (Table 2). Based on the results, we propose that Glu_{178} and Glu_{378} are catalytic sites in BglA.

The PTS proteins encoded by *bgl*E, *bgl*F, and *bgl*I were similar to those from the IIB, IIC, and IIA components respectively of PTS (Table 1, 2). These four genes (*bgl*E, *bgl*F, *bgl*I, and *bgl*A) are also contiguously transcribed in the same direction of the reading frame (Fig. 1). This further suggests that this gene cluster might form an operon involved in the utilization of carbon sources.

PTS EII typically consists of two hydrophilic domains (EIIA and EIIB) that bind to the sugar substrate and a membrane spanning domain for transport (EIIC)[14,32,37] whereas the IIA, IIB, and IIC components can occur as individual proteins.[13,38] Currently, six families of sugar-specific PTS transporters have been identified.[39] The EII complex (sugar-specific permease) of the putative *bgl* operon from *Pcc* LY34 contained BglE, BglF, and BglI, which correspond to EIIB, EIIC, and EIIA respectively. BglF, BglP, and AscF belong to the glucose-glucoside (Glc) PTS system which transports glucose, *N*-acetyl-glucosamine, and various α- and β-glucosides of the six PTS family. Hydropathy analysis using GCG-ALOM has indicated that BglF contains 7 transmembrane helices,[40] and thus differs from BglP and AscF transmembrane patterns. BglF separates with BglE (EIIB) and BglI (EIIA), whereas BglP and AscF contain the EIIA or EIIB domain (Fig. 3B). Hence, we propose that this putative EII complex might belong to one of the currently known six PTS families and might function as a β-sugar transporter in the *Pcc bgl* operon. Additionally, it is assumed that different compositions of these

three proteins might exist to serve different roles in various bacterial environments. This implies that there are various complex catabolic mechanisms of β-oligosugar in *Pcc* LY34. Further experimentation is needed to further clarify the PTS system.

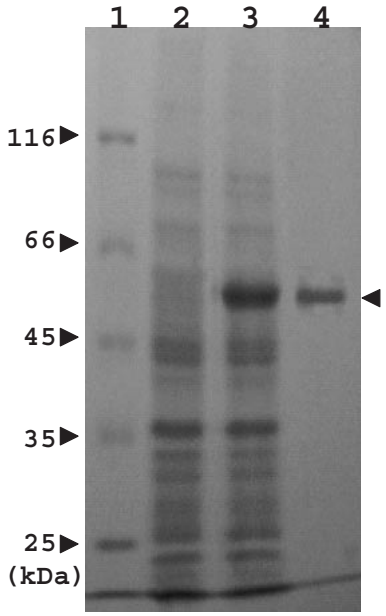

Fig. 4. Electrophoretic Analysis of Purified β-Glucosidase.

Separation was performed on a 12.5% (W/V) SDS–polyacrylamide gel. Lane 1, marker; lane 2, crude extract from un-induced BL21 (DE3) containing pET-21a(+)/BglA; lane 3, crude extract from IPTG-induced BL21 (DE3) containing pET-21a(+)/BglA; lane 4, purified β-glucosidase from HiTrap kit (Amersham). The gel was stained with 0.025% Coomassie blue R-250. The molecular weight markers used were β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactate dehydrogenase (35,000 Da), restriction endonuclease Bsp 981 (25,000 Da), β-lactoglobulin (18,400 Da), and lysozyme (14,400 Da).

In summary, we found that *Pcc* LY34 possesses at least three sets of genes with potential for β-glucoside utilization. It is believed that cellulolytic enzymes serve as cell wall-modifying enzymes, since their action renders other polysaccharide components in plant cell walls more susceptible to hydrolysis. It might be advantageous for *Pcc* LY34 to have a variety of mechanisms for β-glucoside utilization. The cloning of additional β-glucosidases of *Pcc* LY34 should help towards a better understanding of this complex system.

Table 3. Effect of Metal Ions on BglA

Ion (10 mM)	Relative activity[a]
Mg^{2+}	110
Ca^{2+}	109
Cu^{2+}	90
Mn^{2+}	75
Co^{2+}	69
Hg^{2+}	0
Zn^{2+}	56

[a] β-glucosidase (0.05 unit in 200 ml of 50 mM phosphate buffer, pH 7.0) was exposed for 30 min at room temperature to various concentrations of metal ions. The activity was then measured under standard conditions and expressed relative to the activity measured on ρ-nitrophenyl β-D-glucopyranoside (100) without added ions.

Table 4. Specific Activity and Kinetic Parameters for Hydrolysis of ρNPG by the BglA, E178A, and E378A Mutants

	Kinetic parameter[a]	
	K_m (mM)	V_{max} (μmol/min/mg protein)
BglA	0.23	36
E178A	ND	ND
E378A	ND	ND

[a] The parameters were determined at 40 °C (pH 7.0).
ND, not detectable.

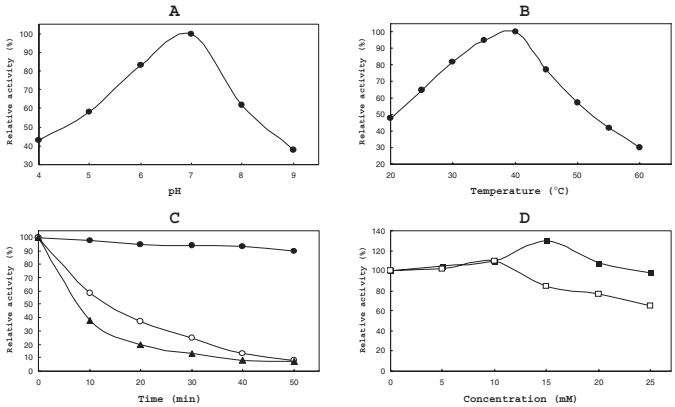

Fig. 5. Effect of pH, Reaction Temperature, Pre-Incubation Temperature, and Magnesium/Calcium Ions on the Activity of BglA.

A, Effect of pH on the relative activity of BglA. Enzyme activity was assayed at 40 °C for 30 min in sodium phosphate buffers of indicated pH. B, Effect of temperature on the relative activity of BglA. Enzyme activity was assayed at pH 7.0 for 30 min at the indicated temperatures. C, Effect of time and temperature on the relative activity of BglA. Enzyme activity was assayed at 40 °C (●), 50 °C (○), and 60 °C (▲), pH 7.0 for 60 min at the indicated reaction times. D, Effect of concentration of Mg^{2+} (■) and Ca^{2+} (□) on the relative activity of BglA. Enzyme activity was assayed at 40 °C.

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