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Characterization of the recombinant cellobiase from *cel*G gene in the beta-glucoside utilization gene operon of *Pectobacterium carotovorum* subsp. *carotovorum* LY34

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Abstract

A third *cel* operon containing *cel*E, *cel*F, and *cel*G genes was isolated from *Pectobacterium carotovorum* subsp. *carotovorum* LY34 (*Pcc* LY34) genomic DNA using a cosmid library. The amino acid sequences of CelE and CelF shared high sequence identity with the cellobiose-specific PTS enzymes IIB and IIC, respectively. CelF contained the disaccharide binding region essential for acquiring cellobiose molecules. The amino acid sequence of CelG shared high sequence identity with various β-glucosidases (cellobiases) belonging to the glycosyl hydrolase family 1. Sequence and structural analysis also demonstrated that this *cel* operon differs from three operons previously reported from *Pcc* LY34, including *bgl*TPB (accession number AY542524), *asc*GFB (accession number AY622309), and *bgl*EFIA (accession number AY769096). In this study, the *cel*F and *cel*G genes were expressed in the presence of cellobiose. Purified CelG was estimated to be approximately 54 kDa by SDS-PAGE and was able to hydrolyze salicin, arbutin, *p*NPG, cellobiose, and MUG, and exhibited maximal activity at pH 5.0 to 40 °C. Two glutamic acid residues (Glu_{172} and Glu_{370}) were shown to be essential for enzyme activity.

Keywords: *Pcc* LY34; Cosmid library; *cel*EFG; Cellobiase

1. Introduction

β-Glucosidases (EC. 3.2.1.21) are enzymes that hydrolyze β-glycosidic bonds between glucose and aryl or alkyl aglycone or oligosaccharides [1]. β-Glucosidases are ubiquitous in living organisms such as animals, plants, fungi, and bacteria [2]. Generally, these enzymes are present in related families, with glycosyl hydrolase families 1 and 3 being the most prominent [3–5]. The CAZy (carbohydrate active enzymes) classification system assembles glycosyl hydrolase (GH) enzymes into families according to sequence similarity, which also reflects shared structural features [6].

Complete degradation of cellulose requires a number of different hydrolases including cellulolytic endoglucanases and cellobiases (β-glucosidase). The β-glucosidases of cellulolytic and non-cellulolytic microorganisms are key enzymes for the assimilation of cellobiose, the biodegradation product of cellulose, and other plant-derived β-glucosides, such as arbutin and salicin [7]. In previous studies, the functional activity of cellobiases was observed in bacteria such as *Cellulomonas* sp. [8,9] or rumen bacteria [10], and in filamentous fungi, *Aspergillus* sp. [11], *Scytalidium thermophilum* [2], and *Phanerochaete chrysosporium* [12]. Among the seven families of PTS, systems related to cellobiose have been reported in several microorganisms including *Bacillus stearothermophilus cel*RABCD [13] and *Bacillus subtilis cel*RABCD [14]. Likewise, two cryptic PTS genes for cellobiose utilization are present in *Escherichia coli cel*ABCDF [15] and *Klebsiella aerogenes cas*RAB [16]. The most prominent cellulase system from many microorganisms is

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defective in cellobiase activity and restricts the conversion of cellobiose to glucose.

The uptake of microbial carbon sources requires transport proteins involved in group translocation of carbohydrates. Group translocation of carbohydrates is mediated by the bacterial phosphoenolpyruvate-phosphotransferase system (PEP:PTS). This system consists of two general energy-coupling proteins, enzyme I and HPr, as well as a sugar-specific permease commonly referred to as the enzyme II complex. This complex may consist of up to four distinct polypeptide chains. Each complex contains at least three functional domains: a hydrophobic transmembrane domain which binds and transports the sugar, a closely associated hydrophilic domain which contains the first phosphorylation site, and a second hydrophilic domain containing an additional phosphorylation site [17–20].

We have previously reported three β-glucoside utilization systems, including *bgl*TPB (AY542524) [21], *asc*GFB (AY622309) [22], and *bgl*EFIA (AY769096) [23] from *Pectobacterium carotovorum* subsp. *carotovorum* LY34. Also, early reports described the presence of three different cellulases including CelA (AF025768) [24], CelB (AF025769) [25], and CelC (AY188753) [26] in the wild-type LY34 strain, suggesting that it encodes a complex system of cell wall-hydrolyzing enzymes. They were previously analyzed for their importance in phytopathogenicity and their capability for utilization of different carbon sources. In this study we isolated a fourth β-glucoside utilization (*cel*) operon from a LY34 genomic library, and expressed it in and purified it from *Escherichia coli*. Comparison of its sequence with the previously reported three β-glucosidases from same strain, and with other β-glucosidases, revealed that it belongs to glycosyl hydrolase family 1.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Tryptone-yeast extract medium was used for the routine cultivation of *P. carotovorum* subsp. *carotovorum* LY34 (*Pcc* LY34) strain. *E. coli* DH5α, BL21 (DE3), EPI300, and recombinant *E. coli* harboring the *cel*G gene were cultured in LB medium containing 50 μg/ml ampicillin, 50 μg/ml kanamycin, and 12.5 μg/ml chloramphenicol, respectively, when appropriate.

2.2. 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 as described by Sambrook and Russell [27]. Restriction enzymes and DNA modifying enzymes were purchased from Promega (Madison, Wisconsin, USA) and gel extraction kit and plasmid DNA isolation kit were purchased from iNtRON Biotechnology (Seongnam, Korea). 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 number DQ987482.

2.3. Construction of cosmid library and cloning of the *celEFG* operon

A genomic library was constructed in the cosmid vector pCC1FOS as previously described [28]. Total genomic DNA from *Pcc* LY34 was sheared into approximately 40 kb fragments using a syringe needle, size-fractionated on a 5–40% 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, containing 1 mM 4-methylumbelliferyl β-D-glucoside (MUG) for β-glucosidase activity (a positive colony has a fluorescent halo). A cosmid clone (pHY1) bearing β-glucosidase activity was isolated. For subcloning, pHY100 was partially digested with *Sau*3AI. Three to 6 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 (4.6 kb) was obtained (pHY100) and sequenced. For high expression of *cel*G, the PCR product generated with primers 5′-GGATCCATGCACGATCATCTTCAG-3′ (sense, containing a *Bam*HI site as underlined) and 5’-AAGCTTTCAAAAGCCATTCTCAGCC-3’ (antisense, containing a HindIII site as underlined) was cloned into expression vector pET-28a(+) (Novagen, Darmstadt, Germany) using BamHI and HindIII sites, resulting in the addition of a C-terminal $(His)_6$ tag. The resulting plasmid was designated pET-28a(+)/CelG (pHY200). The absence of mutations within the coding region of CelG was verified by DNA sequencing.

2.4. Site-directed mutagenesis

Site-directed mutagenesis of the *cel*G gene to create the E172A and E370A mutations was performed using the following oligonucleotide primers: E172A, 5′-TTTACGTTCAATGAGCCAGTGGTGCCG-3′ (sense) and 5′-CGGCACCACTGGGCGATTGAACGTAAAC-3′ (antisense); E370A, 5′-TGCTTTATTTCGGAAAATGGCATGGGG-3′ (sense) and 5′-CATGCCATTTTCCGAAATAAAGCAGCG-3′ (antisense). The 50 μl of reaction mixtures contained 1 μl of pET-28a(+)/*cel*G DNA (95 ng/μl), 100 pmol 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 as pHY300 and pHY400, respectively.

2.5. Enzyme assay

β-Glucosidase activity was determined by using *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), 4-hydroxyphenyl β-D-glucopyranoside (arbutin), 2-(hydroxymethyl) phenyl β-D-glucopyranoside (salicin), 4-methylumbelliferyl β-D-glucoside (MUG), cellobiose as substrates. The standard assay consisted of incubating enzyme with 5 mM *p*NPG in 50 mM sodium phosphate buffer (pH 7.0) for 30 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 *p*-nitrophenol released from the *p*NPG was determined by measuring absorbance at 405 nm. One unit of β-glucosidase was defined as the amount of enzyme required to release 1 mol of *p*-nitrophenol per minute 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 *cel*G 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. 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 was stopped by 0.5 ml of 1 M Na_2CO_3, as described previously. Cellobiase activity was assayed by dinitrosalicylic acid (DNS) method and was measured absorbance at 510 nm. 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 2.0 to 10.0; all assays were performed at 40 °C. To determine the effect of temperature on the enzymatic activity, samples were incubated at temperatures from 10 to 80 °C for 1 h. The effects of various metal ions classified by a concentration of 10 mM on β-glucosidase activity were determined.

2.6. Expression and purification of the enzyme

E. coli strain BL21 (DE3) carrying pET-28a(+)/CelG was grown at 37 °C to mid-log phase in LB medium containing 50 μg/ml kanamycin. 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 (6000 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 (6000 rpm, 30 min) to remove cell debris. The solubilized recombinant CelG with His-tag (CelG-His) was applied on a HisTrap kit (Amersham Pharmacia Biotech, Pennsylvania, USA). CelG 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). 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 [29].

2.7. Total RNA extraction and RT-PCR

Total RNA was isolated from 100 ml cultures of *Pcc* LY34 and recombinant *E. coli* harboring *cel*EFG, in M63 and M9 minimal media containing 1% cellobiose, using easy-spin™ total RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's protocol. To detect a gene expression, reverse transcription polymerase chain reaction (RT-PCR) was performed with mRNA using combinations of designed primers: *cel*F, 5′-CGTTTAACCTGTTCATCCAGTCCGC-3′ (sense) and 5′-AAACGGCAGGAACAGCAGCG-3′ (antisense); *cel*G, 5′-GTGAAAGCCTATCGTGAACAGTCTC-3′ (sense) and 5′-CCACATATGGTAGCCGAGGC-3′ (antisense). Reverse transcription was performed for 30 min at 45 °C then PCR was performed under the following condition: denaturation for 5 min at 94 °C followed by 25 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension for 1 min at 72 °C, and final extension for 5 min at 72 °C.

3. Results and discussion

3.1. Cloning and nucleotide sequence analysis of celE, celF, and celG genes

A cosmid library of *Pcc* LY34 genomic DNA was screened for clones expressing β-glucosidase activity on M9 media containing 1 mM 4-methylumbelliferyl β-D-glucopyranoside (MUG). One cosmid clone exhibiting β-glucosidase activity was isolated and digested by *Sau*3AI restriction enzyme. Subclones were screened using the same assay. One positive clone (pHY100) was isolated and contained an insert of approximately 4.6 kb. This insert was sequenced and single restriction sites for *Bgl*I, *Cla*I, *Hin*dIII, *Nae*I, and *Sca*I were identified (Fig. 1). Sequence analysis also showed the presence of three open reading frames (ORF) designated *cel*E, *cel*F, and *cel*G, putatively encoding proteins of 107, 440, and 465 amino acids in length, respectively (Fig. 2). All of these proteins were in the same reading frame.

The first ORF (*cel*E) is 324 nucleotides in length and potentially encodes a protein of 107 amino acids (Fig. 2). Sequence comparison suggests this ORF encodes a cellobiose-specific enzyme IIB component protein with a predicted molecular mass of 11,639 Da and a p*I* value of 5.30. We also identified that a cell attachment signature site, RGD, predicted to play an important role in cell adhesion.

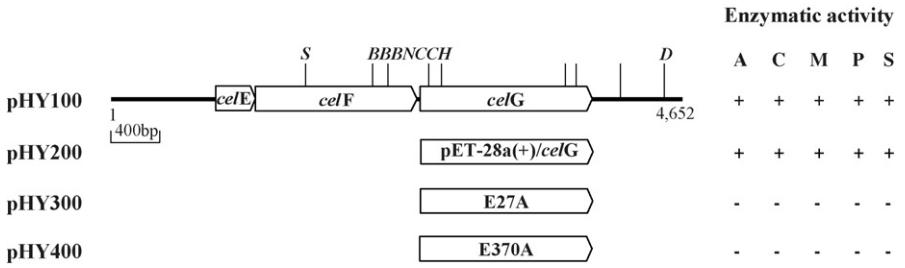

Fig. 1. Physical map of recombinant DNA pHY100 carrying *cel*F, *cel*F, and *cel*G genes of the *P. carotovorum* subsp. *carotovorum* LY34. The ORFs are shown by arrows. The cleavage sites of restriction enzymes *Bgl*I (*B*), *Cla*I (*C*), *Dra*I (*D*), *Hin*dIII (*H*), *Nae*I (*N*), and *Sca*I (*S*) are shown. pHY100 was constructed by cloning a 4.6 kb fragment of cosmid DNA (pHY1) into the pBluescript II SK + vector. pHY200 was derived by cloning into pET-28a(+) expression vector. pHY300 and pHY400 were derived by site-directed mutagenesis (E172A and E370A) from pHY200. β-Glucosidase activity (CelG/pHY200) was determined using *p*NPG (P), MUG (M), salicin (S), arbutin (A), and cellobiose (C) as substrates.

The second ORF (*cel*F) is 1323 nucleotides in length, and its start codon overlaps with the last two bases of the *cel*E stop codon. Like CelE, this gene encodes a cellobiose-specific enzyme IIC component protein belonging to the lactose–cellobiose superfamily. The deduced protein is 440 amino acids in length and has a calculated p*I* of 8.86. The enzyme IIB component protein is phosphorylated by IIA at a cysteine or histidine residue in the active site. The CelF protein has nine transmembrane domains as predicted by the SOSUI computer program. Comparison with other PTS IIC component proteins describes the consensus sequence [FLM]-[GN]-[IV]-[TSN]-E-P-[AIV]-[ILMV]-[FY]-G-[VILMA]-[NPT]-[LI], which is a known disaccharide binding site. The conserved domain FNINEPILFGAPI of *Pcc* LY34 CelF also encodes this conserved disaccharide binding site. It is proposed this region may bind cellobiose molecules outside of the cell, or may be degraded by extracellular enzymes such as cellulases. One portion of this sequence (NINE) is predicted to be involved in phosphoryl transfer to the bound sugar.

The third ORF (*cel*G) begins 25 nucleotides downstream of *cel*F and is 1398 nucleotides long. It is predicted to encode a protein of 465 amino acids. A putative ribosome-binding site, AGAGA, is positioned eight bases from the start of the coding region. The predicted CelG protein shares sequence identity with β-glucosidases from a variety of organisms, and belongs to family 1 of the glycosyl hydrolases. Glu_{27} and Glu_{370} are conserved in two glycosyl hydrolase family 1 signature sequences and are thought to be involved in catalysis and substrate binding of β-glucosidase. The amino acid sequence of the β-glucosidase from *Pcc* LY34 was compared with the sequences of cellobiases belonging to the glycosyl hydrolase family 1 from several organisms (Fig. 2).

3.2. Expression of celF and celG genes

We confirmed the expression of *cel*F and *cel*G genes by performing reverse transcription-polymerase chain reaction (RT-PCR) experiments. *Pcc* LY34 and recombinant *E. coli* harboring *cel*EFG were cultured in M63 and M9 minimal media, respectively, containing 0.1% cellobiose at 37 °C for 6 h. Total RNA was isolated and *cel*F and *cel*G sequences were amplified using gene specific primers. Electrophoresed of PCR product showed two bands of approximately 450 and 600 bp were amplified. Likewise the RT-PCR experiments confirmed that *bgl*TPB, *bgl*EFIA, and *asc*GFB operons could not use cellobiose on minimal medium supplemented with 2 mM cellobiose (data not shown).

3.3. Characterization of CelG

CelG was overexpressed in *E. coli* and purified using column filtration techniques as described in Materials and Methods. A sample of each fraction was analyzed by SDS-PAGE, and only one protein band (approximately 54 kDa) was present in each sample after the final purification step (Fig. 3). The activity of CelG on *p*NPG was determined at 40 °C in various buffers ranging in pH from 4.0 to 9.0 (Fig. 4). Maximal activity was observed at pH 5.0. The activity of CelG on *p*NPG was also assessed at various temperatures at pH 5.0. Maximal activity was observed at 40 °C (Fig. 4). To check effect of divalent cations on relative activity of CelG the β-blucosiase was incubated for 30 min at 40 °C and pH 5.0 to 5 mM concentration of metal ions and then measured under standard conditions and expressed relative to the activity measured on 1 mM cellobiose without added ion. Metal ions such as Hg^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} exhibited 11, 87, 90, 34, 52, 36 and 28 relative activity, respectively. However, all metal ions inhibited enzyme activity and given no significant data.

Site-directed mutagenesis is a valuable tool for studying the relationship between protein structure and function. Therefore we used this method to replace two Glu residues with Ala at sites in CelG that might be important for catalytic function. Mutant plasmids were sequenced on both strands to confirm that only the intended mutation was introduced. Enzyme activity assays were performed with CelG clones carrying the E172A and E370A mutations, and then confirmed that its activities were not detectable in all mutants (E172A and E370A). These results indicate that Glu_{172} and Glu_{370}, which are both conserved in the β-glucosidase sequences of family 1, are essential for CelG activity.

3.4. Comparison of four operons for β-glucoside utilization

In order to study the biological role of β-glucosidases in Pcc LY34, we recently studied a number of cellulolytic

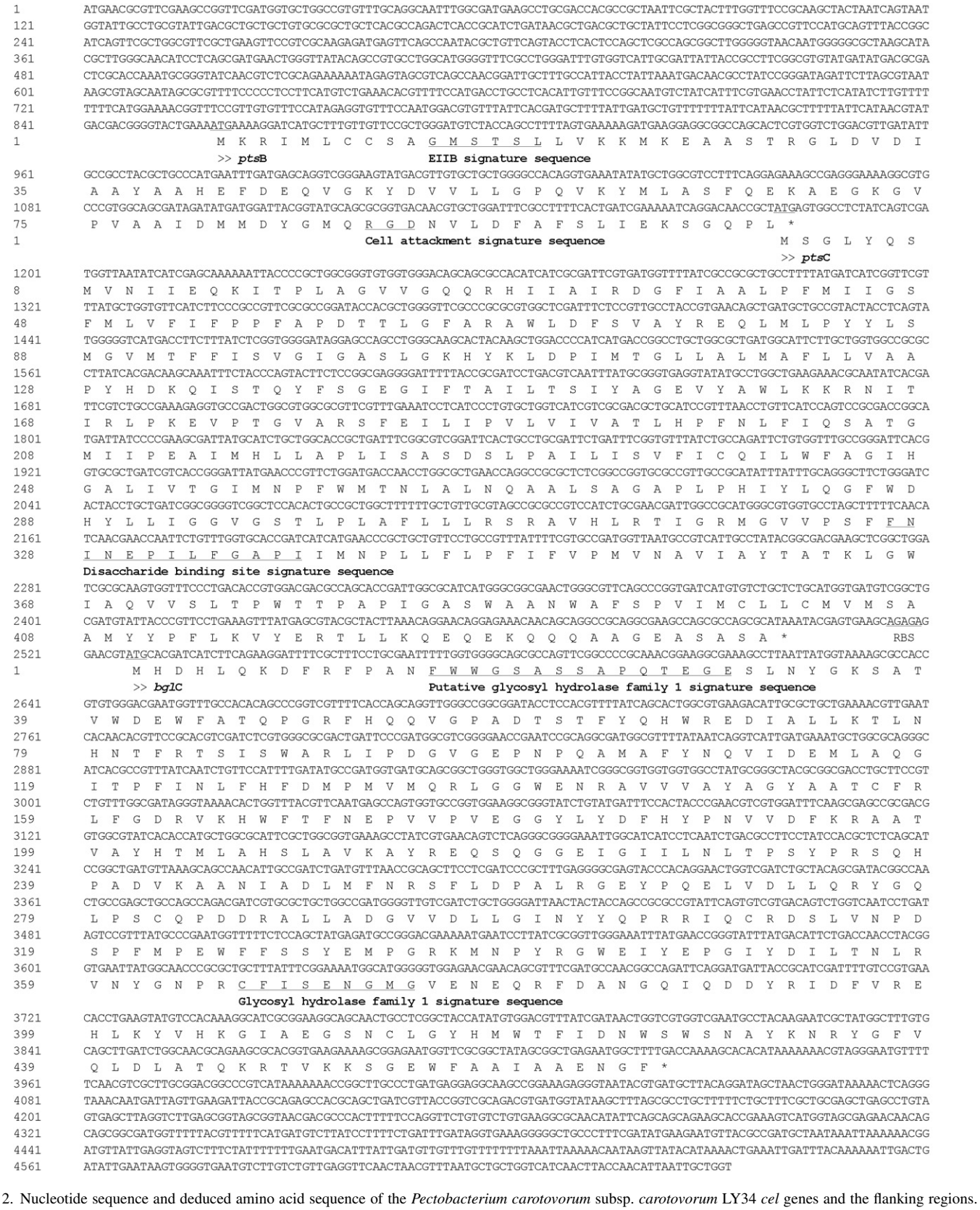

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *Pectobacterium carotovorum* subsp. *carotovorum* LY34 *cel* genes and the flanking regions. The deduced amino acid sequences for each ORF are placed below the nucleotide of corresponding codon. Putative Shine-Dalgarno sequences for ribosomal binding (RBS) are underlined. Proteins are labeled at their respective start codons. The signature sequence and disaccharide binding site for PTS of CelF are underlined. The glycosyl hydrolase family 1 signature sequences are also underlined and labeled.

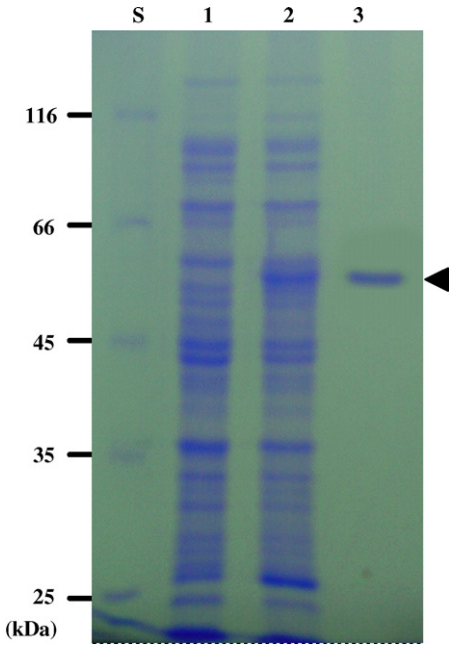

Fig. 3. Electrophoretic analysis of the purified β-glucosidase. Separation was performed on a 12.5% (w/v) SDS-polyacrylamide gel. Lane 1; protein standard marker, lane 2; crude extract from BL21 (DE3) containing pET-28a(+)/CelG, lane 3; crude extract from IPTG-induced BL21 (DE3) containing pET-28a(+)/CelG, lane 4; purified β-glucosidase (CelG) from HiTrap kit (Amersham). The gel was stained with 0.025% Coomassie blue R-250. Molecular weight markers used were β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), restriction endonuclease Bsp 981 (25,000), β-lactoglobulin (18,400), and lysozyme (14,400).

enzymes, such as endoglucanses (CelA, CelB, and CelC) and β-glucosidases (AscB, BglA, and BglB. In this study, we isolated a fourth β-glucoside utilization celEFG operon from the same strain. This operon differed structurally from the previously reported operons (Fig. 5), yet like the others it is involved in the uptake of carbon using the PT system. The translated amino acid sequences of Pcc LY34 cel operon genes share 95.1%, 99.1%, and 96.6% sequence similarity with the corresponding genes of *E. carotovora* subsp. *atroseptica* SCRI1043. Likewise, they share 66.7%, 71.6%, and 68.2% similarity with the corresponding genes of *Photobacterium profundum* SSP, and 63.4%, 84.1%, and 80.8% similarity with those of *Yersinia intermedia* ATCC 29909 (data not shown).

Among the PT systems, the EII complex of the putative celEFG operon contained factors (CelE and CelF) corresponding to the EIIB component (permease specific direct phosphoryl donor) and to the EIIC component (sugar-specific permease or receptor). The Pcc LY34 phosphotransferase (PTS) genes, CelE and CelF, are highly homologous to that of *E. carotovora* subsp. *atroseptica* SCRI1043, *P. profundum* SSP, and *Y. intermedia* ATCC 29909, which are all associated with transport and utilization of cellobioses. However, the *cel*EFG operon genes formed an incomplete PTS enzyme lacking the IIA protein (family specific indirect phosphoryl donor). This suggests that the cellobiose utilization system of the LY34 strain could function with IIA enzyme which encoded in another locus on the chromosome of LY34. Kotrba et al. [19] has suggested that several other *E. coli* PTS permeases, including maltose-specific $IICB^{Mal}$, sucrose-specific $IIBC^{Scr}$, trehalose-specific $IICB^{Tre}$, and arbutin-salicin-cellobiose-specific $IICB^{Asc}$, also lack their cognate IIAs. However, it is possible that these systems use the IIA^{Glc} protein of another system.

Although many PT systems from other organisms are functionally uncharacterized, phylogenetic data predicts the classification of seven PTS permeases (IIC component protein) families, including the glucose (Glc), fructose (Fru), lactose (including aromatic β-glucoside and cellobiose), galactitol (Gat), glucitol (Gut), mannose (Man), and L-ascorbate (Asc) families [20]. These permeases were identified and classified by doing a search of the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) using functionally characterized PTS proteins obtained from the transporter classification database (TCDB; http://www.tcdb.org), as well as by studying the potential secondary structural membrane proteins (SOSUI; http://bp.nuap.nagoya-u.ac.jp/sosui/). Based on these data we propose that CelF is the cellobiose-specific enzyme IIC component protein, that it contains nine transmembrane helices, and that it may function as a cellobiose transporter.

CelG (β-glucosidase) shares 28.7%, 30.9%, and 30.2% sequence identity with BglB, BglA, and AscB, respectively, from the same strain. It also shares sequence identities of 33.9%

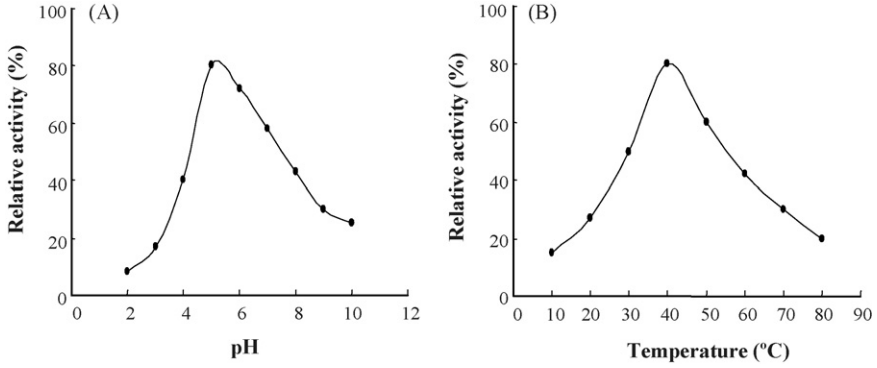

Fig. 4. Effect of pH and temperature on the activity of CelG. (A) Effect of pH on the relative activity of CelG. Enzyme activity was assayed at 40 °C for 30 min in sodium phosphate buffers of indicated pH. (B) Effect of temperature on the relative activity of CelG. Enzyme activity was assayed at pH 5.0 for 30 min at the indicated temperature.

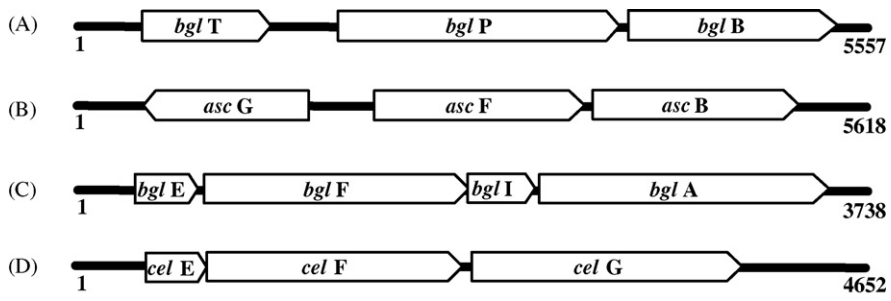

Fig. 5. Comparison of the putative β-glucoside utilization operons from Pcc LY34. (A) *bgl*TPB operon (accession number AY542524). (B) *asc*GFB operon (accession number AY622309). (C) *bgl*EFIA operon (accession number AY769096). (D) *cel*EFG operon (accession number DQ987482).

and 32.3% with BglA and BglB, respectively, from *Paenibacillus polymyxa* [30,31], 34.4% with BglA from *Thermotoga maritima* [32], 34.3% with BglA from *Thermotoga neapolitana* [33], and 33.8% with *agb* from *Agrobacterium* sp. ATCC 21400 [34]. These analyses suggest that CelG is phylogenetically related to gentibiases (cellobiases) belonging to glycosyl hydrolase family 1 (Fig. 6).

These results may explain how the incomplete *cel*EFG system acts on cellobiose by means of transport and hydrolysis. This suggests that *Pcc* LY34 has unique and complex catabolic mechanisms to cause soft-rot disease in plants, and to use β-sugar as a nutrition source. Further studies are required to examine the relationship between the cellobiose–lactose specific PTS system and cellobiase.

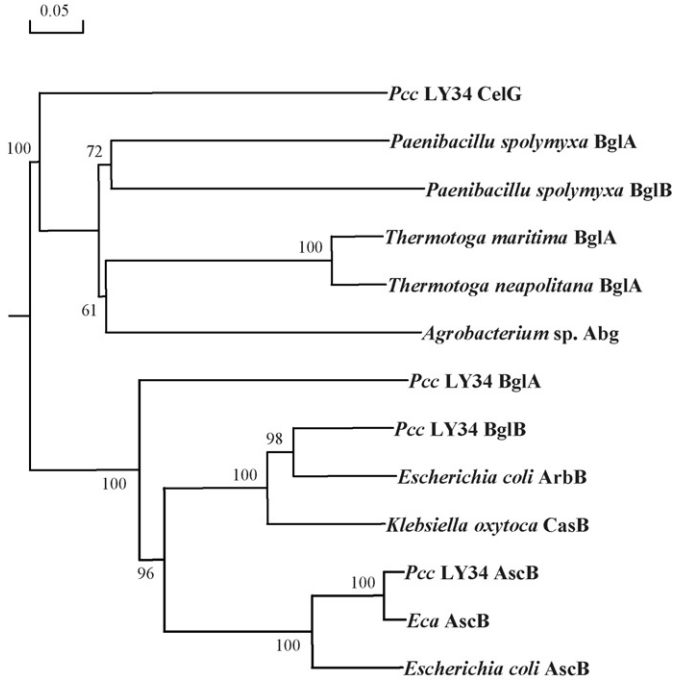

Fig. 6. Phylogenetic placement of CelG amino acid sequences from *Pcc* LY34. Numbers above each node are confidence levels (100%) generated from 1000 bootstrap trees. Phylogenetic tree between cellobiase (CelG) and gentibiases (cellobiases) belong to glycosyl hydrolase family 1. The aligned proteins are from the following sources: AscB, BglB, BglA, and CelG from *Pcc* LY34.

4. Conclusion

Cellulose and cellulolytic enzymes may be advantageous to the soft-rot bacteria such as *Pcc* LY34 by increasing the availability of energy and nutrients obtained from carbon sources. Cellulose degradation by the LY34 strain is predicted to proceed through a combination of hydrolytic reactions performed by cellulose specific enzymes secreted. Cellobiose, the main product of the reaction, is further hydrolyzed into two molecules of glucose by β-glucosidases, called cellobiases. We isolated and characterized a cellobiose utilization *cel* operon from *P. carotovorum* subsp. *carotovorum* LY34 (*Pcc* LY34) genomic DNA using a cosmid library. This operon consists of a three gene, *cel*E (cellobiose-specific enzyme IIB component), *cel*F (cellobiose-specific enzyme IIC component), and *cel*G (cellobiase). *cel*EFG operon structurally differed from three operons, *bgl*TPB, *asc*GFB, and *bgl*EFIA from *Pcc* LY34. The PTS proteins, CelE and CelF, formed an incomplete PTS enzyme lacking the IIA protein. The CelG belonged to the glycosyl hydrolase family 1, was expressed in the presence of cellobiose, and was able to hydrolyze salicin, arbutin, *p*NPG, cellobiose, and MUG. Maximal enzymatic activity was exhibited at pH 5.0 and 40 °C. Two glutamic acid residues (Glu_{172} and Glu_{370}) were shown to be essential for enzyme activity.

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