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March, 2014

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Extracellular gluco-oligosaccharide degradation by *Caulobacter crescentus*

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Abstract

The oligotrophic bacterium *Caulobacter crescentus* has the ability to metabolize various organic molecules, including plant structural carbohydrates, as a carbon source. The nature of β -glucosidase (BGL)-mediated gluco-oligosaccharide degradation and nutrient transport across the outer membrane in *C. crescentus* was investigated. All gluco-oligosaccharides tested (up to celloheptose) supported growth in M2 minimal media but not cellulose or CM-cellulose. The periplasmic and outer membrane fractions showed highest BGL activity, but no significant BGL activity was observed in the cytosol or extracellular medium. Cells grown in cellobiose showed expression of specific BGLs and TonB-dependent receptors (TBDRs). Carbonyl cyanide 3-chlorophenylhydrazone lowered the rate of cell growth in cellobiose but not in glucose, indicating potential cellobiose transport into the cell by a proton motive force-dependent process, such as TBDR-dependent transport, and facilitated diffusion of glucose across the outer membrane via specific porins. These results suggest that *C. crescentus* acquires carbon from cellulose-derived gluco-oligosaccharides found in the environment by extracellular and periplasmic BGL activity and TBDR-mediated transport. This report on extracellular degradation of gluco-oligosaccharides and methods of nutrient acquisition by *C. crescentus* supports a broader suite of carbohydrate metabolic capabilities suggested by the *C. crescentus* genome sequence that until now have not been reported.

Introduction

Many bacteria and fungi produce enzymes to depolymerize plant polysaccharides into oligosaccharides and monosaccharides to be used as carbon sources for growth (Chang, 2007; Yip & Withers, 2004). *Caulobacter crescentus* is a Gram-negative, alphaproteobacterium found in soil, freshwater lakes, streams and marine environments. Genomic analyses of *C. crescentus* as well as other experimental evidence show the ability of this bacterium to metabolize plant carbohydrates, lignin and aromatic hydrocarbons allowing it to survive in low-nutrient environments (Blanvillain et al., 2007; Chatterjee & Bourquin, 1987; Hirsch, 1986; Hottes et al., 2004; Nierman et al., 2001; Poindexter, 1964). Furthermore, many other adaptations of *C. crescentus*, such as enhanced nutrient uptake by the membrane transporter-rich stalk of the sessile cells and slow growth or complete arrest of the cell cycle, contribute to its successful survival in low-nutrient conditions (Ireland et al., 2002; Laub et al., 2007).

Despite the genomic indication of broader carbohydrate metabolism by *C. crescentus* (Nierman et al., 2001), most of the studies conducted thus far have been limited to either monosaccharides or easily hydrolysable disaccharides, including lactose, sucrose and maltose (Blanvillain et al., 2007; Hottes et al., 2004; Neugebauer et al., 2005; Stephens et

al., 2007). Methods of nutrient acquisition from abundant cellulose- and xylan-derived oligosaccharides by *C. crescentus* have not been studied, except for a report of *C. crescentus* growth in cellobiose in minimal medium by Arellano et al. (2010). In this study, we explored cellobiose and gluco-oligosaccharide utilization by *C. crescentus* and the role of β -glucosidases (BGLs) and transporter proteins in this process.

Glycoside hydrolases (GHs) play a crucial role in the depolymerization of plant polysaccharides, such as cellulose and xylan, made up of monosaccharides linked via glycosidic bonds. Genomic analyses of *C. crescentus* (<http://www.cazy.org>) reveals the presence of 43 GHs, composed of 27 different GH families (Cantarel et al., 2009; Coutinho & Henrissat, 1999) and a conserved domain search (<http://www.ncbi.nlm.nih.gov/BLAST>) identifies nine putative BGLs among these 43 GHs. Expression of GHs and membrane transporters, especially TonB-dependent receptors (TBDRs), in *C. crescentus* in response to glucose and xylose as the sole carbon source in minimal medium compared to peptone-yeast extract (PYE) medium has been previously reported (Blanvillain et al., 2007; Hottes et al., 2004). These database analyses and experimental results indicate specific roles for these GHs and transporters in degrading the carbohydrate sources. This study identifies specific BGLs that may be involved in the breakdown of cellulose-derived oligosaccharides.

TBDRs have been known to transport larger molecules such as vitamin B complex, iron chelators and carbohydrates (Blanvillain et al., 2007). The *C. crescentus* genome contains 67 TBDRs and some of them have been identified as disaccharide or oligosaccharide transporters (Blanvillain et al., 2007; Eisenbeis et al., 2008; Lohmiller et al., 2008; Neugebauer et al., 2005). However, the substrate specificities of the majority of the TBDRs found in *C. crescentus* remain to be determined. This study intended to identify putative TBDRs that may be involved in cellobiose and/or gluco-oligosaccharide transport.

The Entner–Doudoroff (ED) pathway is one of the glucose catabolic pathways known and several environmental bacteria oxidize glucose to gluconate in the periplasm to feed the ED pathway (Lessie & Phibbs, 1984; Peekhaus & Conway, 1998). In *Escherichia coli*, the gluconate generated in the periplasm enters the cytoplasm via gluconate transporters found in the inner membrane and is then phosphorylated prior to entry into the ED pathway (Peekhaus et al., 1997). Glucose catabolism in *C. crescentus* occurs via the ED pathway and experimental evidence suggests that a phosphorylated glucose derivative enters this pathway in the cytoplasm (Hottes et al., 2004; Riley & Kolodziej, 1976). However, details of glucose catabolism via the ED pathway or the mechanism of glucose uptake across the inner and the outer membranes of *C. crescentus* remain to be established. Here we propose porin-facilitated diffusion of glucose across the outer membrane of *C. crescentus* into the periplasm and outline the transformation pathway that leads glucose derivatives into the ED pathway.

C. crescentus adapts to environmental changes, including nutritional scarcity, through a broad range of regulatory responses to modulate metabolic activities, including carbon acquisition (Britos et al., 2011). Our results indicate coordinated actions of outer

membrane and periplasmic BGLs and specific TBDRs for gluco-oligosaccharide degradation and glucose assimilation.

Methods

Culture conditions.

C. crescentus (CB15N) cells were grown in M2 minimal salt medium supplemented with 0.2% (w/v) glucose, cellobiose, gluco-oligosaccharides [degree of polymerization (DP)=3 to 7], carboxymethyl cellulose (CMC) or cellulose, differing from previously reported M2 medium (Ely, 1991; Hottes et al., 2004) only in the absence of EDTA. The culture medium was inoculated with a frozen stock of CB15N cells and shaken at 260 r.p.m., 30 °C. A positive control culture of PYE, a negative control culture of M2 minimal salt medium without any carbon source and an un-inoculated blank for each media type were incubated in parallel. Culture growth was monitored by measuring OD600.

Culture conditions described above were replicated, except 10 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to the minimal media containing cells and incubated for 10 min prior to the addition of glucose or cellobiose as described by Neugebauer et al. (2005). Control cultures without CCCP for each carbon source were run concomitantly. The OD600 was measured every 24 h for 72 h to monitor growth.

TLC and GC-MS analysis.

The extracellular fractions from each carbon source were analysed by TLC after 12 h of growth. Briefly, media concentrates were centrifuged (250 ml cultures, 9600 g for 10 min) to remove cells, filtered through 0.22 μ m syringe filters and lyophilized to yield a solid residue. Residues were redissolved in water, spotted onto silica gel GF plates and resolved using a mobile phase of 2:1:1 methanol/ethyl acetate/dichloromethane. The carbohydrates were visualized by spraying with 16 mM orcinol in 70% H₂SO₄ followed by heating at 110 °C for 10 min (Hrmova & Fincher, 1993).

GC-MS analysis was performed at the Metabolomics Center at the University of Illinois, Urbana, IL, on the extracellular media solid residue as prepared for the TLC analysis. Lyophilized residues were reacted with methoxyamine hydrochloride, then with N-methyl-N-(trimethylsilyl) trifluoroacetamide to convert sugars and sugar-derivatives into trimethylsilyl (TMS) derivatives (Rojas-Escudero et al., 2004). An Agilent HP-5MS capillary column (60 m×0.25 mm internal diameter and 0.25 mm film thickness) and an Agilent 7890A gas chromatograph coupled with a 5975C mass selective detector were used for separation and detection. The inlet and MS interface temperatures were 250 °C and the ion source temperature was 230 °C. Helium carrier gas was kept at a constant flow rate of 1.5 ml min⁻¹. The GC oven was held at 70 °C for 5 min, followed by a 50 °C min⁻¹ increase to 310 °C, and held at 310 °C for 10 min. Compounds were identified using the National Institute of Standards and Technology (NIST) and Wiley databases.

Preparation of extracellular media concentrate and the isolation of cellular fractions.

All handling of extracellular media and isolation of cellular fractions were performed either on ice or at 4 °C. Cell-free media (250 ml) collected from mid-exponential phase glucose and cellobiose cultures were treated with protease inhibitor cocktail (PIC) (Sigma-Aldrich) and concentrated to 10 ml using Amicon ultrafiltration cells fitted with 5000 kDa molecular mass cut-off filters at 4 °C under nitrogen gas. Concentrates were dialysed in 50 mM phosphate buffer (pH 7.5) to remove any glucose or cellobiose remaining from the original culture, and absence of these carbon sources was confirmed by the glucose oxidase assay described below. Dialysed concentrates were further concentrated with a 5000 kDa cut-off to a final volume of 5 ml for BGL assays.

Cell pellets obtained from the mid-exponential phase cellobiose and glucose cultures were washed twice with 4 °C, 10 mM Tris buffer (pH 8.0), resuspended in the same buffer, and used for all cellular fractionations. Outer membrane fractions were isolated as described by Neugebauer et al. (2005). Isolation of periplasmic and cytoplasmic contents was performed by a previously reported chloroform/Tris/HCl extraction procedure (Ames et al., 1984; Neu & Heppel, 1965) scaled to a 100 ml culture cell pellet. Periplasmic proteins were extracted into 10 ml 10 mM Tris buffer (pH 8.0) containing PIC. In order to prevent migration of spheroplasts into the periplasmic supernatant, the extract was centrifuged at 12000 g for 30 min and the supernatant containing periplasmic proteins was recovered and tested for cytoplasmic protein contamination by assaying for the cytoplasmic marker glucose-6-phosphate dehydrogenase prior to BGL activity assays as described by Lee & Ahn (2000).

The cytoplasmic fraction was isolated by forming spheroplasts as described by Larsen & Biedermann (1993). Briefly, the spheroplasts were resuspended in Tris buffer (pH 8.0) and sonicated (Sonic Dismembrator, Fisher Scientific) in the presence of PIC in ten repetitions of 1 min alternating intervals of sonication and ice-cooling to release cytoplasmic contents. The lysate was then centrifuged at 34000 g for 20 min at 4 °C to remove debris and the supernatant was saved for the BGL activity assay.

BGL activity assays.

The concentration of protein in each cellular fraction was determined by the Bradford method (Bradford, 1976) with standard BSA according to the manufacturer's instructions (Bio-Rad). BGL activity of each fraction towards p-nitrophenyl- β -D-glucopyranoside (pNPG) was measured as described by Lyman et al. (1995). One unit of activity was defined as the amount of enzyme necessary to produce 1 μ mol p-nitrophenol min⁻¹.

BGL activity towards cellobiose was measured by assaying the amount of glucose released using a glucose oxidase assay (sensitivity >0.01 mU) (Kunst et al., 1984).

Briefly, 100 μ l of extracellular or other cellular fraction was incubated with 400 μ l 0.1 M sodium acetate buffer (pH 4.5) containing 0.2% cellobiose at 37 °C for 1 h. Glucose oxidase assay reagent (1 ml) (Sigma-Aldrich) was added directly to the reaction mixture and incubated at 37 °C for 30 min. The reaction was stopped by adding 1 ml 6 M H₂SO₄ and the absorbance was measured at 540 nm. The amount of glucose released was calculated from a standard curve for glucose. One unit of activity was defined as the amount of enzyme necessary to produce 1 μ mol glucose min⁻¹.

Reverse transcription PCR (RT-PCR).

RNA was isolated from cells grown for 3 h in minimal media cultures containing cellobiose or glucose using an RNeasy mini kit (Qiagen). Three on-column DNase I digestions were performed to remove any contaminating DNA according to the manufacturer's instructions. Prior to cDNA synthesis (Iscrip, Bio-Rad), each RNA isolate was tested for contaminating genomic DNA by running PCRs using *E. coli* 16S rRNA universal forward primer B27F and the reverse primer U1492R (Kolbert & Persing, 1999; Orphan et al., 2001). Target genes were amplified by PCR using gene-specific primers for BGLs and TBDRs. The PCR products were separated by agarose (2.0%, w/v) gel electrophoresis and visualized by ethidium bromide staining. Genomic DNA was used to confirm equal amplification of all the genes tested in RT-PCR analyses under the specified conditions.

2D-gel electrophoresis.

Soluble protein contents of *C. crescentus* cells grown for 12 h in glucose and cellobiose were analysed by 2D-gel electrophoresis. Cells were pelleted and washed twice in 30 mM Tris/HCl (pH 8.5) and resuspended in 10 ml 30 mM Tris/HCl (pH 8.5) containing 2% Triton X-100 and PIC and lysed by 20 repetitions of alternating 1 min intervals of sonication and ice-cooling. The cell debris was removed by centrifugation at 34000 g for 20 min at 4 °C. Proteins in the supernatant were vortexed with 4 volumes of 10% trichloroacetic acid and 20 mM DTT in acetone at 20 °C. This mixture was left to precipitate at -20 °C overnight and centrifuged at 48000 g for 30 min to obtain a protein pellet, which was subsequently washed twice with 20 mM DTT in acetone at -20 °C. The pellet was air-dried and redissolved in 200 μ l IEF rehydration buffer containing 9 M urea, 50 mM DTT, 0.2% ampholytes, 3% CHAPS and 0.001% bromophenol blue (Sigdel et al., 2004). Protein samples were quantified using a protein assay dye (Bio-Rad) as per the manufacturer's instructions and approximately 125 μ g protein was loaded per 7 cm immobilized pH gradient (IPG) strip.

IEF was performed using 7 cm, pH 4–7 IPG strips after several rounds of optimization to select the proper pH range and the volt-hour (Vh) conditions for optimal focusing. The IPG strips were rehydrated passively at 20 °C with the protein sample suspended in rehydration buffer and overlaid with 1 ml mineral oil after 30 min and focused for 14000 Vh on a Protean IEF Cell (Bio-Rad). Prior to the second

dimension SDS-PAGE separation, the focused IPG strip was treated with equilibration buffer I and II as per the manufacturer's instruction and previously published procedures (Sigdel et al., 2004). Precast gels (10% Mini-Protean TGX, Bio-Rad) and molecular mass marker (250–10 kDa, Precision Plus Protein Standard Plug, Bio-Rad) were used for SDS-PAGE separations followed by staining with Coomassie blue R350.

Tryptic digestion, MS and protein identification.

In-gel tryptic digestion, MS and proteomic identification were performed at Applied Biomics (Hayward, CA, USA) as per previously described methods (Tshala-Katumbay et al., 2009). Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer version 3.5 equipped with MASCOT search engine (Matrix Science) to search the National Center for Biotechnology Information (non-redundant) (NCBI nr) database. Searches were performed without constraining protein molecular mass or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage allowed in the search parameters. Candidates with either protein score confidence intervals (C.I.) % or Ion C.I. % greater than 95 were considered significant.

Results

C. crescentus growth and degradation of cellobiose and gluco-oligosaccharides

Cell growth monitored by OD600 measurements for 108 h in cellobiose, cellotriose, cellotetraose and CMC are shown in comparison to glucose in Fig. 1(a). An interesting trend of higher OD600 values in oligosaccharides with an even number of DP (e.g. cellotetraose) than those with an odd number of DP (e.g. cellotriose) was observed in all the oligosaccharides tested. The OD600 measurements of CMC cultures did not show any increase, and the OD600 of cellulose could not be used as an approximation of cell numbers due to suspended particulate matter in the cultures caused by the insoluble cellulose crystals. However, a standard plating procedure did not produce any colonies. All cultures were visually examined under a microscope and all cultures except cellulose and CMC cultures contained viable cells.

The TLC analysis of the extracellular media of glucose, cellobiose, cellotriose, cellotetraose, CMC and cellulose cultures collected after 12 h of growth is shown in Fig. 1(b) along with molecular standards. The chromatogram shows presence of glucose as well as all possible smaller oligosaccharide derivatives in cellotriose- and cellotetraose-containing culture media. The cellulose and CMC culture media did not show any degradation products. GC-MS analyses of the cellobiose extracellular media (Fig. 1c) confirmed the disappearance of cellobiose and appearance of glucose. In addition to glucose, two glucose-derivatives, gluconate and 2-ketogluconate, were also detected in the extracellular medium. TMS-derivatization

usually results in multiple peaks for a single compound in a GC trace due to variations in derivatization (Neeser & Schweizer, 1988). Degradation of larger gluco-oligosaccharides into smaller gluco-oligosaccharides and glucose in the extracellular medium was confirmed by HPLC and ¹H-NMR spectroscopic analyses (data not shown).

Fig. 1. (a) Comparison of *C. crescentus* growth in M2 minimal medium supplemented with 0.2% (w/v) glucose, gluco-oligosaccharides or CMC. All data points are the mean of two independent growth experiments. (b) TLC analysis of the cell-free growth media collected after 12 h of growth, shown with corresponding standards. Mobile phase was 2:1:1 methanol/ethyl acetate/dichloromethane and the carbohydrates were detected by spraying with orcinol/H₂SO₄ and heating at 110 °C for 10 min. (c) Time-dependent GC-MS analysis of the cellobiose extracellular medium over a 36 h growth period. TMS-derivatized cellobiose medium residues were separated by GC and compounds were identified by MS and NIST/Wiley database search.

BGL activity

In order to identify the subcellular location of BGL activity, assays were conducted on subcellular fractions and extracellular media from both glucose- and cellobiose-grown cultures using pNPG (Fig. 2a) and cellobiose (Fig. 2b) as substrates. The

highest specific activity [~ 250 U (mg protein) $^{-1}$] towards the pNPG substrate was observed in the periplasmic fraction isolated from cellobiose cultures (Fig. 2a). Outer membrane fractions from both glucose and cellobiose cultures showed nearly 100 U (mg protein) $^{-1}$ specific activities. No measurable activity was detected towards pNPG in the cytoplasmic or in extracellular media fractions of both glucose and cellobiose cultures.

Fig. 2. β -Glucosidase activity of cellular fractions and extracellular medium of mid-exponential phase glucose and cellobiose cultures. Activities were calculated by measuring (a) p-nitrophenol release from pNPG and (b) glucose release from cellobiose. All data points are the mean of duplicate assays of three independently isolated fractions.

Periplasmic contents isolated from glucose and cellobiose cultures showed higher specific BGL activity towards the cellobiose substrate compared to that of all other fractions (Fig. 2b). The highest activity [~ 100 U (mg protein) $^{-1}$] was observed in the periplasmic fraction of the cellobiose culture. Unlike the pNPG assay, the cellobiose assay showed measurable activity in the cytoplasmic fractions of both glucose and cellobiose cultures. Although the outer membrane fraction isolated from the mid-exponential phase cellobiose culture did not show any measurable activity towards cellobiose, the 48 h culture showed BGL activity of 12 U (mg protein) $^{-1}$. BGL activity of most of the fractions collected at early hours of growth did not show sufficient activity measurable by these assay methods.

Expression of BGL, TBDR and OprB genes

The nine BGL genes for expression analysis were chosen based on the conserved domain search that predicted potential BGL activity. The TBDR genes were selected based on the genetic organization of TBDRs and BGLs as shown in Fig. 3 with anticipated coordinated expression of TBDRs and BGLs (or GH, in the case of CC_2804). Among the BGL gene expression tested, expression of CC_0968, CC_1756 and CC_1757 was detected in both glucose and cellobiose cultures (Fig. 4a).

Additionally, CC_0797 and CC_1105 expression was detected only in the cellobiose sample, while expression of CC_2136 was observed only in the glucose culture. Expression of BGL genes CC_2006, CC_2052 and CC_2227 was not detected in any of the samples analysed. Expression of the TBDR genes CC_0970, CC_1754 and CC_2804 was tested; however, only the expression of CC_0970 was detected and it was expressed in both glucose and cellobiose cultures. All primer pairs for the genes showed comparable amplification with genomic DNA template under the same PCR conditions.

Fig. 3. Genetic organization of putative BGLs, TBDRs and LacI family transcription regulators (TR). P1, P2 and P3 are promoter regions for genes shown downstream. A stop-start codon overlap observed between CC_1756 and CC_1757 is shown with an asterisk.

Fig. 4. (a) RT-PCR analyses for the expression of BGLs and TBDRs at 3 h of growth in glucose and cellobiose minimal media cultures of *C. crescentus*. Numbers above the amplicons indicate the gene locus tag (CC number). Expected amplicon size ranges from 80 to 180 bp except the rRNA amplicon, which is 1500 bp. (b) RT-PCR analyses of CC_2888 (OprB porin) expression in glucose (G), cellobiose (C) and PYE (P) cultures of *C. crescentus*. Expected amplicon size for CC_2888 was 260 bp. Absence of DNA contamination was verified by control PCR without the reverse-

transcriptase step for 16S rRNA templates (No RT_16S rRNA) with *E. coli* 16S rRNA universal primers B27F and U1492R.

Common biological properties observed between *C. crescentus* and several species of *Pseudomonas* (Nierman et al., 2001) and the absence of OmpF, an outer membrane porin that allows passive diffusion of hydrophilic solutes (Nierman et al., 2001; Nikaido, 1994) in *C. crescentus*, prompted us to search the *C. crescentus* genome for OprB genes, resulting in only one: CC_2888. In order to seek further information about potential role of OprB porin in glucose transport, we tested the expression of CC_2888 using RT-PCR. Surprisingly, we found expression of OprB gene CC_2888 in glucose- and cellobiose-containing minimal media cultures (Fig. 4b), but not in the PYE culture.

Proteomic analyses of the whole-cell lysates from glucose and cellobiose cultures were performed to identify differentially produced proteins, particularly TBDRs. Visual comparison of the gels revealed several distinguishable spots in the cellobiose gel, including high molecular mass proteins of comparable size to TBDRs (Fig. 5). Three of the five protein spots analysed were identified as TBDRs: CC_0970 (spot 2), CC_0999 (spot 1) and CC_1666 (spot 3). Spot 4 was identified as serine protease HtrA (CC_2758), which is associated with the stress condition of cells (Wu et al., 2011), and spot 5 as a translation elongation factor (CC_1922).

Fig. 5. 2D-gel electrophoresis images of the soluble fraction of the whole-cell lysates of *C. crescentus* grown in (a) glucose- and (b) cellobiose-containing minimal medium for 12 h. First dimension separation was performed using a pH 4–7 IPG strip and the second dimension separation was performed on a pre-cast gel. Numbered protein spots (1–5) on the cellobiose gel were picked for protein identification by MALDI-TOF MS.

Effect of CCCP on cell growth

TBDR-mediated transport of nutrients across the outer membrane in Gram-negative bacteria is dependent on a proton motive force (pmf) generated across the inner membrane (Postle & Larsen, 2007). In order to test whether glucose and cellobiose transport across the outer membrane is dependent upon the pmf-driven process, the cell growth, which would be an indirect indicator of carbon source availability, in glucose and cellobiose cultures was monitored in the presence and absence of the pmf disruptor CCCP. Cellobiose cultures incubated with 10 μ M CCCP showed only a small OD600 value after 48 h of growth, but the OD600 value increased to $\sim$ 0.61 at 72 h, whereas the cellobiose culture without CCCP showed normal growth (compare Fig. 1(a) with Fig. 6). The glucose cultures incubated with 10 μ M CCCP showed growth similar to that of the glucose culture without CCCP (Fig. 6).

Fig. 6. *C. crescentus* growth in 0.2% (w/v) glucose- or cellobiose-containing M2 minimal media in the presence of 10 μ M CCCP. OD600 measurements were made at 48 h and 72 h into growth and all data points are the mean of three independent growth experiments.

Discussion

This study demonstrates the ability of *C. crescentus* to utilize cellobiose and gluco-oligosaccharides as its sole carbon source and provides insight into the degradation and the uptake mechanisms of cellobiose and gluco-oligosaccharides. Although many disaccharides, such as sucrose, maltose and lactose, have been shown to be transported across the outer membrane of *C. crescentus* (Arellano et al., 2010; Blanvillain et al., 2007; Lohmiller et al., 2008; Neugebauer et al., 2005), the results presented here, in contrast, indicate extracellular degradation of cellobiose and gluco-oligosaccharides.

It is reported that some species of the order Caulobacterales are able to depolymerize cellulose and CMC in enriched soil samples (Mašková et al., 1988; Song et al., 2013); however, our attempts to grow *C. crescentus* in these carbon sources were not successful. Furthermore, analysis of the genomic information of *C.*

crescentus did not yield any enzymes with putative cellulase activity leading us to conclude that *C. crescentus* is not capable of degrading crystalline cellulose or CMC under the experimental conditions described in this study.

Although BGL activity is not observed in extracellular media, the membrane fraction showed BGL activity in both glucose and cellobiose cultures. In addition, the detection of gluco-oligosaccharide degradation products in the extracellular media indicated the presence of membrane-bound BGL. Prediction of the subcellular location for the nine BGLs, using PSORTb version 3.0.2 and Gneg-mPLOC for Gram-negative bacterial proteins (Shen & Chou, 2010; Yu et al., 2010), showed four out of nine BGLs are predicted to localize to the periplasm (Table 1). This prediction is supported by the observation of the highest level of BGL activity in the periplasmic fraction (Fig. 2). Additional database analyses (LipoP1.0/SignalP 4.1 servers) for signal sequences revealed all three putative membrane-associated proteins: CC_1756, CC_1757, CC_2227 (Table 1) possess a signal peptidase I (SPase I) cleavage site, indicating their subcellular localization is beyond the inner membrane, i.e. periplasm and/or outer membrane (Auclair et al., 2012; Juncker et al., 2003). Dodd et al. (2011) proposed a model for xylan degradation by rumen and human colonic bacterioides involving outer membrane-bound extracellular xylanase that possesses an SPase II cleavage site; however, the mechanism of protein localization on the outer leaflet of the outer membrane remains unknown (Tokuda, 2009). Therefore, the data presented here allow us only to state that gluco-oligosaccharides undergo extracellular as well as periplasmic degradation. Confirmation of the subcellular locations of the BGLs identified in this study needs further investigation. An excellent review by Tokuda & Matsuyama (2004) provides insights for any future studies of BGL sorting to other outer surfaces of the outer membrane.

Table 1. Putative BGLs, GH family, and their predicted subcellular location		
BGL	Predicted enzyme activity/GH family*	Predicted subcellular location†
CC_0797	(1,4)- β -D-Glucan glucohydrolase/GH3	Periplasm
CC_0968	BGL/GH3	Periplasm
CC_1105	BGL/GH3	Periplasm
CC_1756	BGL/GH3	Outer membrane
CC_1757	Glycosyl hydrolase/GH30	Inner membrane/periplasm
CC_2006	Glycosyl hydrolase/GH3	Cytoplasm
CC_2052	(1,4)- β -D-Glucan glucohydrolase/GH3	Periplasm
CC_2136	BGL/GH1	Cytoplasm
CC_2227	BGL/GH9	Membrane/extracellular

* GH family information was obtained from the CAZy database.

† Subcellular location was predicted using PSORTb (version 3.0.2) and Gneg-mPLOC.

Observation of higher growth rate in oligosaccharides with an even number of DP may indicate substrate selectivity of one or more of the BGLs expressed. An extensive hydrogen-bonding network observed between the oligosaccharide substrate and the substrate binding and catalytic residues of the glycosidic enzymes indicates the substrate length might be a determinant in the overall glycosidic enzyme rate (Li et al., 2007; Zhou et al., 2004). Hrmova et al. (1998) reported an increased catalytic efficiency with increasing degree of polymerization in a GH1 family BGL from barley. Therefore, individual enzymological characterization of the BGLs identified in our study is necessary to explain the observed difference in oligosaccharide utilization.

All the nine BGLs of this study fall into four GH families (Table 1): GH1, GH3, GH9 and GH30 (www.CAZy.org). Six BGL genes, whose expression is tested in this study, belong to the GH3 family (Table 1). GH3 family BGLs catalyse the removal of monosaccharide residue, including glucose, from the non-reducing ends of a variety of substrates (Harvey et al., 2000). CC_2227 belongs to the GH9 family that primarily contains endoglucanases and some GH9 family members contain a C-terminal carbohydrate-binding module (CBM) that makes them processive (Sakon et al., 1997). However, a conserved domain search for the presence of a CBM in CC_2227 did not show any CBM. The remaining two BGLs, CC_2136 and CC_1757, belong to the GH1 and GH30 families, respectively. The GH1 family exhibits BGL and β -galactosidase activities, whereas the GH30 family exhibits β -glucosylceramidase, β -(1,6)-glucanase and β -xylosidase activities (Keen et al., 1996).

The chromogenic substrate pNPG can be cleaved by any BGL to release p-nitrophenol that is measured in the pNPG assay. However, the cellobiose substrate used in the glucose oxidase assay is cleaved only by BGLs that demonstrate substrate specificity towards cellobiose, i.e. cellobiase activity. This substrate specificity difference between these two assays accounts for the lower BGL activity measured in the glucose oxidase assay than the pNPG assay for any subcellular fraction. This difference in activity indicates that only a subset of the BGLs expressed has substrate specificity towards cellobiose and these cellobiases are concentrated in the periplasm as indicated by the higher glucose oxidase activity compared to other subcellular fractions.

Expression of BGL genes CC_0968, CC_1756 and CC_1757 in both glucose and cellobiose cultures indicates that these BGL genes are either constitutively expressed or glucose dependent, whereas CC_0797 gene expression is dependent only on cellobiose (Fig. 4a). Interestingly, global promoter identification analyses conducted for *C. crescentus* (<http://pepper.molgenrug.nl/>) (de Jong et al., 2012) show that CC_0968 or the overlapping and likely translationally coupled genes CC_1756 and CC_1757 (Fig. 3, marked with asterisk) are encoded in operons with widely spaced genes. The promoters for these operons (marked P1 and P3 in Fig. 3) are shown upstream of the CC_0969–CC_0968 and CC_1754–CC_1757 gene clusters with LacI-family transcription regulators (CC_0969 and CC_1753, respectively). Widely spaced genes in operons are known to be highly expressed and are usually

expressed under complex regulatory conditions (Ohashi-Kunihiro et al., 2007; Pradhan et al., 2009; Price et al., 2006). Identification of the regulatory sequences and characterization of corresponding inducers/repressors of these genes would further reveal the genetic regulation associated with these BGLs and TBDRs in terms of broader gluco-oligosaccharide metabolism in *C. crescentus*.

Among the 67 TBDRs present in the *C. crescentus* genome, CC_0970 and CC_1754 are located in the vicinity of BGL genes (Fig. 3) and both have been reported as glucose-induced genes through conserved motif identification by Hottes et al. (2004). The TBDR CC_2804, which is coded along with two putative xylosidases (CC_2802 and CC_2803), has been previously shown to be expressed in glucose- or maltose-supplemented minimal media cultures of *C. crescentus* (Neugebauer et al., 2005). Therefore, we hypothesized that the TBDR CC_2804 would be expressed in glucose or cellobiose cultures based on the operonic nature of gene organization and included it in our gene expression analysis. However, the RT-PCR analyses showed only the expression of CC_0970 (Fig. 4a), concurring with the identification of CC_0970 as a glucose-induced gene. Expression of CC_1756 and CC_1757, but not the TBDR CC_1754, may be due to the complex regulatory nature of these translationally coupled, widely spaced genes and provides opportunity for further investigations of gene regulation.

Identification of three TBDRs (CC_0999, CC_0970 and CC_1666) in our proteomic analysis indicates high levels of TBDR-mediated transport across the outer membrane as previously reported (Blanvillain et al., 2007; Hottes et al., 2004; Neugebauer et al., 2005; Schauer et al., 2008). This observation is further supported by the slow cell growth, but not complete arrest, observed in the cellobiose medium containing CCCP compared to the glucose medium with CCCP (Fig. 6) further supporting a pmf-dependent active cellobiose uptake, as previously observed for TBDR-mediated maltodextrin transport (Neugebauer et al., 2005). Based on the data presented here, we propose that the TBDR CC_0970 is involved in cellobiose transport across the outer membrane of *C. crescentus* and its expression may have been induced by the glucose generated by extracellular degradation of cellobiose. However, proteomics data suggest additional TBDRs may be involved in the cellulose-derived oligosaccharide transport and that hypothesis should be tested in the future.

Absence of any significant decrease of cell growth in glucose medium in the presence of CCCP suggests that glucose transport is not dependent on TBDRs or other pmf-dependent processes in *C. crescentus*, and thus may be accomplished by passive diffusion via outer membrane porins (Nikaido, 1994; Shrivastava et al., 2011; Tamber & Hancock, 2003; van den Berg, 2012). OprB is a glucose-induced porin, first identified in *Pseudomonas aeruginosa*, and known to transport a wide range of hydrophilic molecules, including carbohydrates, by facilitated diffusion. (Adewoye & Worobec, 1999; Hancock & Carey, 1980; Wylie & Worobec, 1995). Expression of OprB gene CC_2888 in glucose- and cellobiose-containing minimal

media cultures (Fig. 4b) indicates a carbon-substrate-dependent expression of CC_2888 as well as an apparent requirement for glucose for OprB expression.

Detection of gluconate and 2-ketogluconate in the cellobiose-containing extracellular medium (Fig. 1c) provides clues about the status of glucose prior to entry into the ED pathway. In *C. crescentus*, glucose in the periplasm may be subjected to two successive oxidations to produce gluconate and 2-ketogluconate by glucose and gluconate dehydrogenases, respectively, as observed in many *Pseudomonas* species, such as *P. fluorescens*, which also use the ED pathway (Iwamoto & Imanaga, 1991; Matsushita et al., 1982). Gluconate and 2-ketogluconate are then transported across the inner membrane into the cytosol by yet to be identified transporters of *C. crescentus* and converted into glucose 6-phosphate, 6-phosphogluconate, or 2-keto-6-phosphogluconate for the entry into the ED pathway (Fig. 7) (Lessie & Phibbs, 1984). Adding support to our model, genomic database analyses of *C. crescentus* revealed a carbohydrate kinase (CC_1496) with high sequence similarity and conserved domains to 2-keto-3-deoxygluconate kinase, in an operon with 2-keto-3-deoxy-6-phosphogluconate aldolase (CC_1495) that breaks 2-keto-3-deoxy-6-phosphogluconate into pyruvate and D-glyceraldehyde-3-phosphate in the ED pathway. Furthermore, absence of any phosphorylated intermediates in the GC-MS analysis supports the physical separation of non-phosphorylated and phosphorylated intermediates by the inner membrane (Fig. 7). It is likely that hydrophilic gluconate and 2-ketogluconate may have diffused from the periplasm into the extracellular medium via porins as observed in *Pseudomonas* species, where gluconic acid diffuses from the periplasm into the extracellular environment via porins to solubilize insoluble phosphates to meet phosphorous needs (de Werra et al., 2009).

Fig. 7. Proposed model for cellobiose and gluco-oligosaccharide degradation, uptake and utilization by *C. crescentus*. Locus tag numbers of the BGL, TBDR and porin (OprB) genes that may be involved in the gluco-oligosaccharide metabolism and transport are given in parentheses. IM, inner membrane; PS, periplasmic space; OM, outer membrane.

Based on the results presented here, we propose a model for cellobiose and gluco-oligosaccharide degradation, uptake and further metabolism by *C. crescentus* (Fig. 7). Determination of the roles played by specific BGLs, TBDRs and the porin identified in this study would further describe the nature of cellulose-derived oligosaccharide use as a carbon source by *C. crescentus* and other similar environmental bacteria.

Acknowledgements

We thank Pramodha S. Liyanage, Dilani G. Gamage, Suzanne M. Walker and Brant Riegel for their assistance in growth, GC-MS and 2D-gel electrophoresis experiments. We gratefully acknowledge the help of Drs Zhiqing Yan for recording NMR spectra, Thomas Canam for HPLC analysis, and Wasala Herath and Frank Golich for careful reading of the manuscript. This research was supported by an award from Research Corporation for Science Advancement to G.R.P, and PIF, CFR and URSCA grants from Eastern Illinois University to G. R. P. and others. M.J.P. received the Richard and Ellen Keiter Fellowship for summer research from Eastern Illinois University.

References

- Adewoye, L. O. & Worobec, E. A. (1999). Multiple environmental factors regulate the expression of the carbohydrate-selective OprB porin of *Pseudomonas aeruginosa*. *Can J Microbiol* 45, 1033–1042.
- Ames, G. F., Prody, C. & Kustu, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol* 160, 1181–1183.
- Arellano, B. H., Ortiz, J. D., Manzano, J. & Chen, J. C. (2010). Identification of a dehydrogenase required for lactose metabolism in *Caulobacter crescentus*. *Appl Environ Microbiol* 76, 3004–3014.
- Auclair, S. M., Bhanu, M. K. & Kendall, D. A. (2012). Signal peptidase I: cleaving the way to mature proteins. *Protein Sci* 21, 13–25.
- Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., Denancé, N., Vasse, J., Lauber, E. & Arlat, M. (2007). Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. *PLoS ONE* 2, e224.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.

Britos, L., Abeliuk, E., Taverner, T., Lipton, M., McAdams, H. & Shapiro, L. (2011). Regulatory response to carbon starvation in *Caulobacter crescentus*. *PLoS ONE* 6, e18179.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V. & Henrissat, B. (2009). The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* 37 (Database), D233–D238.

Chang, M. C. (2007). Harnessing energy from plant biomass. *Curr Opin Chem Biol* 11, 677–684.

Chatterjee, D. K. & Bourquin, A. W. (1987). Metabolism of aromatic compounds by *Caulobacter crescentus*. *J Bacteriol* 169, 1993–1996.

Coutinho, P. M. & Henrissat, B. (1999). Carbohydrate-active enzymes: an integrated database approach. In *Recent Advances in Carbohydrate Bioengineering*, pp. 3–12. Edited by Gilbert, H. J., Davies, G., Henrissat, B. & Svensson, B. . Cambridge: Royal Society of Chemistry.

de Jong, A., Pietersma, H., Cordes, M., Kuipers, O. P. & Kok, J. (2012). PePPER: a webserver for prediction of prokaryote promoter elements and regulons. *BMC Genomics* 13, 299.

de Werra, P., Péchy-Tarr, M., Keel, C. & Maurhofer, M. (2009). Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Appl Environ Microbiol* 75, 4162–4174.

Dodd, D., Mackie, R. I. & Cann, I. K. (2011). Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. *Mol Microbiol* 79, 292–304.

Eisenbeis, S., Lohmiller, S., Valdebenito, M., Leicht, S. & Braun, V. (2008). NagA-dependent uptake of N-acetyl-glucosamine and N-acetyl-chitin oligosaccharides across the outer membrane of *Caulobacter crescentus*. *J Bacteriol* 190, 5230–5238.
Ely, B. (1991). Genetics of *Caulobacter crescentus*. *Methods Enzymol* 204, 372–384.

Hancock, R. E. W. & Carey, A. M. (1980). Protein D1—a glucose-inducible, pore-forming protein from the outer membrane of *Pseudomonas aeruginosa*.

FEMS Microbiol Lett 8, 105–109.

Harvey, A. J., Hrmova, M., De Gori, R., Varghese, J. N. & Fincher, G. B. (2000). Comparative modeling of the three-dimensional structures of family 3 glycoside hydrolases. *Proteins* 41, 257–269.

Hirsch, P. (1986). Microbial life at extremely low nutrient levels. *Adv Space Res* 6, 287–298.

Hottes, A. K., Meewan, M., Yang, D., Arana, N., Romero, P., McAdams, H. H. & Stephens, C. (2004). Transcriptional profiling of *Caulobacter crescentus* during growth on complex and minimal media. *J Bacteriol* 186, 1448–1461.

Hrmova, M. & Fincher, G. B. (1993). Purification and properties of three (1→3)- β -D-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*). *Biochem J* 289, 453–461.

Hrmova, M., MacGregor, E. A., Biely, P., Stewart, R. J. & Fincher, G. B. (1998). Substrate binding and catalytic mechanism of a barley β -D-glucosidase/(1,4)- β -D-glucan exohydrolase. *J Biol Chem* 273, 11134–11143.

Ireland, M. M., Karty, J. A., Quardokus, E. M., Reilly, J. P. & Brun, Y. V. (2002). Proteomic analysis of the *Caulobacter crescentus* stalk indicates competence for nutrient uptake. *Mol Microbiol* 45, 1029–1041.

Iwamoto, R. & Imanaga, Y. (1991). Direct evidence of the Entner-Doudoroff pathway operating in the metabolism of D-glucosamine in bacteria. *J Biochem* 109, 66–69.

Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H. & Krogh, A. (2003). Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* 12, 1652–1662.

Keen, N. T., Boyd, C. & Henrissat, B. (1996). Cloning and characterization of a xylanase gene from corn strains of *Erwinia chrysanthemi*. *Mol Plant Microbe Interact* 9, 651–657.

Kolbert, C. P. & Persing, D. H. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Curr Opin Microbiol* 2, 299–305.

Kunst, A., Draeger, B. & Ziegenhorn, J. (1984). Colorimetric methods with glucose oxidase and peroxidase. In *Methods of Enzymatic Analysis*, 3rd edn, pp. 178–185. Edited by Bergmeyer, H. U. . Weinheim: Verlag Chemie.

Larsen, B. S. & Biedermann, K. (1993). Isolation and characterization of the outer membrane proteins of *Serratia marcescens* W225. *Anal Biochem* 214, 212–221.

Laub, M. T., Shapiro, L. & McAdams, H. H. (2007). Systems biology of *Caulobacter*. *Annu Rev Genet* 41, 429–441.

Lee, J. E. & Ahn, T. I. (2000). Periplasmic localization of a GroES homologue in *Escherichia coli* transformed with groESx cloned from *Legionella*-like endosymbionts in *Amoeba proteus*. *Res Microbiol* 151, 605–618.

Lessie, T. G. & Phibbs, P. V. Jr. (1984). Alternative pathways of carbohydrate utilization in pseudomonads. *Annu Rev Microbiol* 38, 359–388.

Li, Y., Irwin, D. C. & Wilson, D. B. (2007). Processivity, substrate binding, and mechanism of cellulose hydrolysis by *Thermobifida fusca* Cel9A. *Appl Environ Microbiol* 73, 3165–3172.

Lohmiller, S., Hantke, K., Patzer, S. I. & Braun, V. (2008). TonB-dependent maltose transport by *Caulobacter crescentus*. *Microbiology* 154, 1748–1754.

Lyman, E. S., Li, B. & Renganathan, V. (1995). Purification and characterization of a cellulose-binding β -glucosidase from cellulose-degrading cultures of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 61, 2976–2980.

Mašková, H. P., Vasilyeva, L. V., Kofroňová, O. & Kunc, F. (1988). Microflora participating in the decomposition of carboxymethyl cellulose continuously added to the soil. *Folia Microbiol (Prague)* 33, 482–490.

Matsushita, K., Shinagawa, E. & Ameyama, M. (1982). D-Gluconate dehydrogenase from bacteria, 2-keto-D-gluconate-yielding, membrane-bound. *Methods Enzymol* 89, 187–193.

Neeser, J. R. & Schweizer, T. F. (1988). *Analysis of Carbohydrates as O-Alkyloxime Derivatives by Gas-Liquid Chromatography*. Boca Raton, FL: CRC Press.

Neu, H. C. & Heppel, L. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J Biol Chem* 240, 3685–3692.

Neugebauer, H., Herrmann, C., Kammer, W., Schwarz, G., Nordheim, A. & Braun, V. (2005). ExbBD-dependent transport of maltodextrins through the novel MalA protein across the outer membrane of *Caulobacter crescentus*. *J Bacteriol* 187, 8300–8311.

Nierman, W. C., Feldblyum, T. V., Laub, M. T., Paulsen, I. T., Nelson, K. E., Eisen, J. A., Heidelberg, J. F., Alley, M. R. & Ohta, N. & other authors (2001). Complete genome sequence of *Caulobacter crescentus*. *Proc Natl Acad Sci U S A* 98, 4136–4141.

Nikaido, H. (1994). Porins and specific diffusion channels in bacterial outer membranes. *J Biol Chem* 269, 3905–3908.

Ohashi-Kunihiro, S., Yohda, M., Masaki, H. & Machida, M. (2007). A novel vector for positive selection of inserts harboring an open reading frame by translational coupling. *Biotechniques* 43, 751–752, 754.

Orphan, V. J., Hinrichs, K. U., Ussler, W. III., Paull, C. K., Taylor, L. T., Sylva, S. P., Hayes, J. M. & Delong, E. F. (2001). Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. *Appl Environ Microbiol* 67, 1922–1934.

Peekhaus, N. & Conway, T. (1998). What's for dinner?: Entner–Doudoroff metabolism in *Escherichia coli*. *J Bacteriol* 180, 3495–3502.

Peekhaus, N., Tong, S., Reizer, J., Saier, M. H. Jr., Murray, E. & Conway, T. (1997). Characterization of a novel transporter family that includes multiple *Escherichia coli* gluconate transporters and their homologues. *FEMS Microbiol Lett* 147, 233–238.

Poindexter, J. S. (1964). Biological properties and classification of the *Caulobacter* group. *Bacteriol Rev* 28, 231–295.

Postle, K. & Larsen, R. A. (2007). TonB-dependent energy transduction between outer and cytoplasmic membranes. *Biometals* 20, 453–465.

Pradhan, P., Li, W. & Kaur, P. (2009). Translational coupling controls expression and function of the DrrAB drug efflux pump. *J Mol Biol* 385, 831–842.

Price, M. N., Arkin, A. P. & Alm, E. J. (2006). The life-cycle of operons. *PLoS Genet* 2, e96.

Riley, R. G. & Kolodziej, B. J. (1976). Pathway of glucose catabolism in *Caulobacter crescentus*. *Microbios* 16, 219–226.

Rojas-Escudero, E., Alarcón-Jiménez, A. L., Elizalde-Galván, P. & Rojo-Callejas, F. (2004). Optimization of carbohydrate silylation for gas chromatography. *J Chromatogr A* 1027, 117–120.

Sakon, J., Irwin, D., Wilson, D. B. & Karplus, P. A. (1997). Structure and mechanism of endo/exocellulase E4 from *Thermomonospora fusca*. *Nat Struct Biol* 4, 810–818.

Schauer, K., Rodionov, D. A. & de Reuse, H. (2008). New substrates for TonB-dependent transport: do we only see the ‘tip of the iceberg’? *Trends Biochem Sci* 33, 330–338.

Shen, H. B. & Chou, K. C. (2010). Gneg-mPLOC: a top-down strategy to enhance the quality of predicting subcellular localization of Gram-negative bacterial proteins. *J Theor Biol* 264, 326–333.

Shrivastava, R., Basu, B., Godbole, A., Mathew, M. K., Apte, S. K. & Phale, P. S. (2011). Repression of the glucose-inducible outer-membrane protein OprB during utilization of aromatic compounds and organic acids in *Pseudomonas putida* CSV86. *Microbiology* 157, 1531–1540.

Sigdel, T. K., Cilliers, R., Gursahaney, P. R. & Crowder, M. W. (2004). Fractionation of soluble proteins in *Escherichia coli* using DEAE-, SP-, and phenyl sepharose chromatographies. *J Biomol Tech* 15, 199–207.

Song, N., Cai, H. Y., Yan, Z. S. & Jiang, H. L. (2013). Cellulose degradation by one mesophilic strain *Caulobacter* sp. FMC1 under both aerobic and anaerobic conditions. *Bioresour Technol* 131, 281–287.

Stephens, C., Christen, B., Watanabe, K., Fuchs, T. & Jenal, U. (2007). Regulation of D-xylose metabolism in *Caulobacter crescentus* by a LacI-type repressor. *J Bacteriol* 189, 8828–8834.

Tamber, S. & Hancock, R. E. (2003). On the mechanism of solute uptake in *Pseudomonas*. *Front Biosci* 8, s472–s483.

Tokuda, H. (2009). Biogenesis of outer membranes in Gram-negative bacteria. *Biosci Biotechnol Biochem* 73, 465–473.

Tokuda, H. & Matsuyama, S. (2004). Sorting of lipoproteins to the outer membrane in *E. coli*. *Biochim Biophys Acta* 1694, IN1–IN9.

Tshala-Katumbay, D., Monterroso, V., Kayton, R., Lasarev, M., Sabri, M. & Spencer, P. (2009). Probing mechanisms of axonopathy. Part II: protein targets of 2,5-hexanedione, the neurotoxic metabolite of the aliphatic solvent n-hexane. *Toxicol Sci* 107, 482–489.

van den Berg, B. (2012). Structural basis for outer membrane sugar uptake in pseudomonads. *J Biol Chem* 287, 41044–41052.

Wu, X., Lei, L., Gong, S., Chen, D., Flores, R. & Zhong, G. (2011). The chlamydial periplasmic stress response serine protease cHtrA is secreted into host cell cytosol. *BMC Microbiol* 11, 87–102.

Wylie, J. L. & Worobec, E. A. (1995). The OprB porin plays a central role in carbohydrate uptake in *Pseudomonas aeruginosa*. *J Bacteriol* 177, 3021–3026.

Yip, V. L. & Withers, S. G. (2004). Nature's many mechanisms for the degradation of oligosaccharides. *Org Biomol Chem* 2, 2707–2713.

Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinalp, S. C. & Ester, M. & other authors (2010). PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26, 1608–1615.

Zhou, W., Irwin, D. C., Escovar-Kousen, J. & Wilson, D. B. (2004). Kinetic studies of *Thermobifida fusca* Cel9A active site mutant enzymes. *Biochemistry* 43, 9655–9663.