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Cellodextrin Utilization by *Bifidobacterium breve* UCC2003▽†

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Cellodextrins, the incomplete hydrolysis products from insoluble cellulose, are accessible as a carbon source to certain members of the human gut microbiota, such as *Bifidobacterium breve* UCC2003. Transcription of the *cldEFGC* gene cluster of *B. breve* UCC2003 was shown to be induced upon growth on cellodextrins, implicating this cluster in the metabolism of these sugars. Phenotypic analysis of a *B. breve* UCC2003::*cldE* insertion mutant confirmed that the *cld* gene cluster is exclusively required for cellodextrin utilization by this commensal. Moreover, our results suggest that transcription of the *cld* cluster is controlled by a LacI-type regulator encoded by *cldR*, located immediately upstream of *cldE*. Gel mobility shift assays using purified $CldR_{His}$ (produced by the incorporation of a His_{12}-encoding sequence into the 3′ end of the *cldC* gene) indicate that the *cldEFGC* promoter is subject to negative control by $CldR_{His}$, which binds to two inverted repeats. Analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) of medium samples obtained during growth of *B. breve* UCC2003 on a mixture of cellodextrins revealed its ability to utilize cellobiose, cellotriose, cellotetraose, and cellopentaose, with cellotriose apparently representing the preferred substrate. The *cldC* gene of the *cld* operon of *B. breve* UCC2003 is, to the best of our knowledge, the first described bifidobacterial β-glucosidase exhibiting hydrolytic activity toward various cellodextrins.

One of the dominant bacterial groups in the human and mammalian intestinal microbiota is represented by members of the genus *Bifidobacterium* (47, 48), which are high-GC-content Gram-positive, non-spore-forming, "bifid"-shaped, anaerobic bacteria. Specific bifidobacterial strains have been implicated in promoting host health through one or more beneficial activities, which include prevention of diarrhea, reduction of cholesterol levels, symptom alleviation of inflammatory bowel disease or irritable bowel syndrome, immunomodulation, anticarcinogenicity, easing of lactose intolerance, improving mineral adsorption, and production of vitamins (12, 46). The mechanism(s) of probiotic action is largely unknown and thus merits further investigation of bifidobacteria, which includes research into its genomic content, genetics, biochemistry, and metabolism (52).

Bifidobacterial growth and/or metabolism in the human gastrointestinal tract can be selectively stimulated by various dietary compounds, particularly by so-called prebiotic carbohydrates (22, 35). A prebiotic has been defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health" (35). According to this definition, the potential prebiotic component must fulfill the following criteria: nondigestible by the host, fermentation by the intestinal microbiota, and selective stimulation of growth and activity of beneficial intestinal bacteria. Recent publications have proposed that fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soybean-derived oligosaccharides (SOS) and xylo-oligosaccharides (XOS) possess properties that match the criteria mentioned above (for a review on this topic, see reference 11). In this context it is important to note that more than 8% of the identified genes on bifidobacterial genomes have been predicted to be involved in carbohydrate metabolism, thus being indicative of extensive carbohydrate-degrading abilities (41, 51). Our model microorganism *Bifidobacterium breve* UCC2003 has previously been shown to encode a β-fructofuranosidase involved in the partial degradation of FOS (38), an extracellular amylopullanase capable of hydrolyzing α-1,4- and α-1,6-glucosidic linkages in starch, amylose, amylopectin, pullulan, and glycogen (31, 37), an endogalactanase (GalA) responsible for the utilization of galactan (32), and two α-glucosidases that can hydrolyze panose, isomaltose, isomaltotriose, trehalose, and palatinose (34). In addition, a fructose phosphoenolpyruvate phosphotransferase system (PEP-PTS) (24) and a ribose utilization system (33) have been identified in *B. breve* UCC2003 and characterized.

Humans and other single-stomach animals are incapable of metabolizing cellulose (36). However, human-derived intestinal microorganisms, mainly *Bacteroides* spp., have been shown to ferment dietary fiber components, including cellulose and hemicellulose (10, 54). Enzymatic hydrolytic degradation of cellulose requires the action of a combination of extracellular endo- and exoglucanases and β-glucosidases (4). In addition, the degradation of cellulose may also be performed by 6-phospho-β-glucosidases (20). β-Glucosidases, including those encoded by noncellulolytic microorganisms, play an important role in the degradation of plant-derived oligosaccharides, such as cellodextrins, by converting them to glucose (4). *Bifidobacterium adolescentis* Int-57 and *B. breve* 203 were reported to

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produce intracellular β-glucosidases with hydrolytic activity toward cellobiose (5, 29, 30). In the current study we describe the identification of a novel cellodextrin utilization (*cld*) operon of *B. breve* UCC2003. Furthermore, we present the characterization of the *cldC* gene, whose protein product is responsible for the hydrolysis of β-1,4 bonds present in cellodextrins, while we also describe how a LacI-type regulator encoded by *cldR* acts as a likely transcriptional repressor of the *cld* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Bifidobacteria were cultivated in reinforced clostridium medium (RCM) (Oxoid, Hampshire, England) or modified de Man, Rogosa, and Sharpe (mMRS) medium (7), made from first principles, supplemented with 0.05% (wt/vol) L-cysteine-HCl (Sigma-Aldrich, Steinhein, Germany) and 1% (wt/vol) of a particular carbohydrate solution as the main carbon and energy source. Strains were grown under anaerobic conditions in a modular atmosphere controlled system (Davidson & Hardy Ltd., Dublin, Ireland). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium (40), and *Lactococcus lactis* strains were grown at 30°C in M17 medium supplemented with 0.5% glucose (GM17). Where appropriate, media were supplemented with 5 μg ml^{-1} chloramphenicol (Cm), 50 μg ml^{-1} kanamycin (Km), 100 μg ml^{-1} erythromycin (Em), or 100 μg ml^{-1} ampicillin (Amp) for plasmid maintenance.

Cellodextrin preparation. Cellodextrins were prepared from microcrystalline cellulose (Avicel PH105; 20 μm) using the mixed-acid hydrolysis protocol essentially as described previously (57). Freeze-dried cellodextrin powder was dissolved in deionized water (0.25-g ml^{-1} final concentration). To remove salts and reduce the amount of glucose, this cellodextrin solution was dialyzed for 24 h against 1 liter of deionized water using cellulose tubing (molecular mass cutoff, 100 Da) (Spectra/Por; Spectrum Europe B.V., Netherlands). The quality and composition of the prepared cellodextrin mixture were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (see below). The final dialyzed solution was then used for growth experiments.

***B. breve* UCC2003 consumption of cellodextrin from mMRS.** A dialyzed cellodextrin solution was added to 3× concentrated mMRS medium (to obtain a final sugar concentration of 0.85%). *B. breve* UCC2003 growth in mMRS supplemented with cellodextrins and 0.05% (wt/vol) L-cysteine-HCl was monitored every hour for 9 h by measuring the optical density at 600 nm (OD_{600}). In addition, HPAEC-PAD analysis (see below) of filtered, cell-free growth medium samples taken at the same time points was performed to analyze cellodextrin consumption.

Bioinformatics. Sequence data were obtained from the genome annotation of the *B. breve* UCC2003 sequencing project (S. C. Leahy, M. O'Connell-Motherway, J. A. Moreno Muñoz, G. F. Fitzgerald, D. G. Higgins, and D. van Sinderen, unpublished results). Database searches were performed using the nonredundant sequence database accessible at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) with tBlastN, BlastX, and BlastP (1, 2). Sequence analysis was performed using DNASTAR; MapDraw and EditSeq were used for sequence analysis, and MegAlign was used to align multiple protein or DNA sequences (Madison, WI).

DNA manipulations. Large-scale preparation of chromosomal DNA from *Bifidobacterium* spp. was performed as described previously (24). Plasmid DNA was obtained from *L. lactis* NZ9000 using the QIAprep Spin Plasmid Miniprep kit (Qiagen GmbH, Hilden, Germany) as described previously (34).

QRT-PCR. Differential expression of genes was confirmed by real-time quantitative RT-PCR (QRT-PCR). *De novo* cDNAs were prepared as described previously (50). All primers were designed using the Universal ProbeLibrary Assay Design Center (Roche Applied Science) based on Primers 3 Plus (49). The resulting primer-probe pairs were tested for efficiency, and only sets yielding an efficiency of 2 using a log fold standard dilution curve evaluated using the Roche 480 software 1.5. were used for subsequent quantitative transcriptional experiments. Primer sequences are presented in Table S2 in the supplemental material. The *rnpA* gene (encoding RNase P) was used as a housekeeping gene with a presumed constitutive level of transcription to correct for variability in the initial amount of total RNA. Each amplification reaction mixture contained 1 μl of 6.7-fold-diluted cDNA, 10 μl of the 2× FastStart TaqMan Probe Master (Roche), 900 nM each primer, and 250 nM probe mix and was brought to a total volume of 20 μl by the addition of RNase-free water. All QRT-PCRs were performed in triplicate by means of a LightCycler 480 system (Roche) instrument using 384-well plates. Thermal cycling conditions were as recommended by the manufacturer (Roche). The $2^{-\Delta\Delta CT}$ method (19) was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Pooled cDNA from all samples was used as a reference calibrator for analysis of differential gene expression. Results were calculated from at least two independent RNA extractions. The QRT-PCR expression data, following $2^{-\Delta\Delta CT}$, analysis were subjected to a Mann-Whitney *t* test to compare all groups using GraphPad Prism 4 software (GraphPad Software, CA). Data are represented by mean ± standard error of the mean (SEM). *P* values of <0.05 were considered significant. The statistical analysis was performed blind to the origin of the data.

Construction of a *B. breve* UCC2003::*cldE* insertion mutant. The procedure by which the *cldE* insertion mutant was constructed was essentially as described previously (32), the specifics of which are outlined below. An internal 636-bp fragment of *cldE* (from base 163 to 799 of the 1,307-bp *cldE* coding region) was amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template and primer pair pOCldEFw and pOCldERv (see Table S2 in the supplemental material) and cloned into integration plasmid pORI19 (18) harboring the tetracycline resistance cassette, *tetW*, cloned from pAM5 (3). Potential mutants harboring pORI19-tet-cldE integrated into the *cldE* gene were confirmed by colony PCR using primer combinations tetWFw and tetWRv to detect the expected presence of the *tetW* gene and cldR-Fw (complement to the start of the *cldR* gene) and pORI19Fv to confirm the presumed chromosomal integration of the pORI19-tet-cldE in the *cldE* gene.

EMSA. DNA fragments representing different portions of the *cldE* promoter region were prepared by PCR using IRD800-labeled primers pairs (MWG Biotech) (see Table S1 in the supplemental material). Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (13). In all cases, the binding reactions were carried out in a final volume of 20 μl in the presence of poly[d(I-C)] in binding buffer (20 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 1 mM EDTA, 100 mM KCl, 10% glycerol). Various amounts of purified $CldR_{His}$ (produced by the incorporation of a His_{12}-encoding sequence into the 3′ end of the *cldC* gene) ranging from 0.08 nM to 0.01 μM and probe (0.1 pmol) were mixed on ice and subsequently incubated for 15 min at 37°C. Samples were loaded onto a 6% nondenaturing polyacrylamide (PAA) gel prepared in TAE buffer (40 mM Tris acetate [pH 8.0], 2 mM EDTA) and run in a 0.5× to 2.0× gradient of TAE at 100 V for 60 min in a mini-Protean II system (Bio-Rad Laboratories, Richmond, CA). Signals were detected using the Odyssey infrared imaging system (Li-Cor Biosciences UK Ltd., Cambridge, United Kingdom) and captured using the supplied software Odyssey V3.0.

Primer extension analysis. Total RNA was isolated from *B. breve* UCC2003, which was grown in mMRS supplemented with 1% cellobiose or a cellodextrin mixture to early exponential phase, using the Macaloid method (17). RNA samples were treated with RNase-free DNase (Ambion). The 5′ ends of the RNA transcripts were determined by primer extension as previously described (50). The generated cDNA (0.5 μl) was mixed with 0.3 μl loading buffer (Li-Cor Blue stop solution), followed by separation on a 6.5% Li-Cor gel matrix KB Plus and comparison with the products of a sequencing reaction (employing the Thermo Sequenase primer cycle sequencing kit [Amersham]) using the same primer as that employed for the primer extension (see Table S2 in the supplemental material) and using a PCR product encompassing the *cldE* promoter region as a template. Signal detection was performed by means of a Li-Cor sequencing instrument (Li-Cor Biosciences).

Plasmid construction and overexpression of $CldC_{His}$ and $CldR_{His}$. The entire coding region of *cldC* (gene locus Bbr_0109) and *cldR* (gene locus Bbr_0105) was amplified by PCR with genomic DNA from *B. breve* UCC2003 serving as a template and primer pairs CldCFw and CldCRv for *cldC* and CldRFw and CldRRv for *cldR* (see Table S2 in the supplemental material). For CldC the primers allowed the incorporation of a His_{12}-encoding sequence into the 3′ end of the *cldC* gene (here designated $cldC_{His}$). PCRs were performed using a PTC-200 Peltier thermal cycler (Bio-Sciences, Dublin, Ireland), *Taq* PCR master mix, and ProofStart DNA polymerase (Qiagen, GmbH, Hilden, Germany). The amplified 1.0-kb $cldC_{His}$-encompassing PCR fragment was restricted with SacI and XbaI and ligated into plasmid pNZ8150 (26) cut with the same enzymes, while the amplified *cldR*-encompassing PCR product was restricted with NcoI and BglII and ligated to similarly digested pQE60. The ligation mixture of the $cldC_{His}$-containing PCR fragment and pNZ8150 was introduced into *L. lactis* NZ9000 by electroporation, and resulting transformants were selected based on Cm resistance. The ligation mixture of the $cldR_{His}$-containing PCR fragment and pQE60 was introduced into *E. coli* XL1-Blue cells by electroporation, with subsequent transformant selection based on Amp and Tc resistance. The expected plasmid content of a number of transformants was verified by sequencing

to ensure the genetic integrity of the cloned recombinant *cldC* and *cldR* genes. In each case one transformant was selected for further use and designated pNZcldC for CldC overexpression and pQE60CldR for CldR overexpression.

A 400-ml volume of GM17 was inoculated with 8 ml of an overnight culture of *L. lactis* NZ9000 cells harboring pNZcldC and incubated at 30°C. When the optical density at 600 nm had reached approximately 0.5, expression of the protein was induced by the addition of 800 μl of supernatant from an overnight culture of a nisin A-producing *L. lactis* NZ9700 culture (8). After 2 h of incubation, cells were harvested, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), and disrupted with glass beads in a Mini Bead Beater (Biospec Products, Bartlesville, OK). Cellular debris was removed by centrifugation. The $CldC_{His}$ enzyme was purified from the crude cell extract using a nickel-nitrilotriacetic acid column (Qiagen GmbH) according to the manufacturer's instructions (QIAexpressionist 06/2003). Overexpression of $CldR_{His}$ was achieved as described previously (34). Cell crude extract was subsequently analyzed for the presence of the overexpressed protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (34).

Determination of hydrolytic activities of $CldC_{His}$. Determination of the hydrolytic activity and substrate specificity of $CldC_{His}$ was performed using a previously described method (15). Enzymatic assays were performed at 37°C in a total volume of 450 μl of 20 mM MOPS (morpholinepropanesulfonic acid) (pH 7.0) with a 25 mM concentration of a particular carbohydrate substrate. All reactions were initiated by the addition of crude cell extract containing $CldC_{His}$ (50 μl) and terminated after 17 h of incubation by placing the reaction tubes at 100°C for 5 min followed by cooling on ice. Reaction products were analyzed by HPAEC-PAD or by high-performance thin-layer chromatography (HPTLC).

HPTLC and HPAEC-PAD analyses. HPTLC analysis was carried out as described previously (34) with some modifications. Following sample spotting, the chromatogram was developed 4-fold using a butanol-acetic acid-water (5:4:1, vol/vol/vol) solvent system in a horizontal developing chamber. The plate was allowed to air dry in a fume hood and was then sprayed evenly with 20% (vol/vol) sulfuric acid in ethanol, air dried again, and finally heated at 120°C for 10 min to visualize the carbohydrate spots.

HPAEC-PAD analysis was performed using a Dionex ICS-3000 system equipped with a CarboPac PA-100 analytical exchange column (250 mm by 4 mm) and a pulsed electrochemical detector in the pulsed amperometric detection (PAD) mode as previously described (34) with the following modifications. The elution was performed at a constant flow rate of 1 ml min^{-1} at 30°C using the following linear gradient of sodium acetate in 100 mM NaOH: 0 to 5 min, 27.5 mM; 5 to 40 min, 0 to 275 mM; and 40 to 45 min, 275 mM. The injection volume was 10 μl. Reaction products were identified and quantified relative to standard carbohydrates.

Nucleotide sequence accession number. The nucleotide sequence of the *cld* cluster has been deposited in the GenBank database under accession number GQ329065.

RESULTS

Growth of *Bifidobacterium* strains on cellobiose. We were interested in the ability of bifidobacteria to grow on plant-derived carbohydrates. As part of a larger survey, we investigated if bifidobacteria are capable of growth on cellobiose, the smallest cellulose-derived cellodextrin. For this purpose, growth in modified Rogosa medium (mMRS) supplemented with glucose (as a positive control) or cellobiose was assessed for 36 different bifidobacterial strains, representing 11 bifidobacterial species, by measuring the OD_{600} following 24 h of anaerobic growth at 37°C (Fig. 1). All bifidobacterial strains grew well on glucose, reaching OD_{600} values in excess of 1. In contrast, just 4 out of the 36 bifidobacterial strains tested were able to reach an OD_{600} of higher than 1.0 when grown on cellobiose as the sole carbon source. All four of these strains belong to the *B. breve* species, including *B. breve* UCC2003. Eight strains were shown to grow rather poorly on cellobiose, with final OD_{600} values ranging from 0.3 to 0.7, while the remaining 24 bifidobacterial strains could not or could very poorly metabolize cellobiose (OD_{600} of <0.3). This indicates that only certain bifidobacterial strains (in particular *B. breve* strains) can selectively grow on cellobiose and that this sugar possibly represents a selective growth substrate for such strains.

Consumption of cellodextrins from mMRS medium by *B. breve* UCC2003. The results described above clearly showed that our model strain *B. breve* UCC2003 metabolizes cellobiose as its sole carbon source. To expand on this observation, we wanted to analyze the potential of *B. breve* UCC2003 to utilize higher-molecular-weight cellodextrins, i.e., cellotriose, cellotetraose, etc. For this purpose, a mixture of cellodextrins, obtained through partial acid hydrolysis of cellulose (see Materials and Methods), was included in mMRS as the main carbon source and the growth rate of *B. breve* UCC2003 was monitored as before, while HPAEC-PAD analysis was performed in order to determine the presence and quantity of each cellodextrin component in the culture supernatant at different time points (Fig. 2). The final OD_{600} of *B. breve* UCC2003 grown in mMRS with cellobiose was higher than that with cellodextrins, due to the higher concentration of the carbon source (1% cellobiose versus 0.85% cellodextrins). Nevertheless, this bacterium achieved similar growth rates in mMRS containing the cellodextrin mixture or cellobiose as a sole carbon source (Fig. 2A). It should be noted that the cellodextrin mixture did contain some residual glucose that was consumed by *B. breve* UCC2003 first before any cellodextrin metabolism was observed (Fig. 2B and C). The HPAEC-PAD analysis clearly demonstrates that *B. breve* UCC2003 metabolizes various different cellodextrins but does not utilize cellohexaose (C_6), celloheptaose (C_7), and probably cellodextrins with a higher degree of polymerization (Fig. 2B). Furthermore, from this analysis it appears that cellotriose (C_3) represented the preferred substrate, as it was consumed faster than the other cellodextrins present in the growth medium (0.23 mg ml^{-1} h^{-1} at between 4 and 6 h of growth). Cellopentaose (C_5) was the least preferred substrate, as it was consumed by *B. breve* UCC2003 at a relatively low rate, 0.03 mg ml^{-1} h^{-1}, and only when the shorter cellodextrins were nearly exhausted from the medium (Fig. 2C). Cellobiose and cellotetraose were consumed at similar rates (0.12 and 0.14 mg ml^{-1} h^{-1}, respectively).

Genome response of *B. breve* UCC2003 to growth on cellobiose and cellodextrins. Preliminary microarray experiments had shown that transcription of the adjacent *cldE*, *cldF*, *cldG*, and *cldC* genes was upregulated when *B. breve* UCC2003 was grown in cellobiose compared when it was grown in glucose (data not shown). In order to further investigate these preliminary observations, QRT-PCR experiments were performed to measure the transcription levels of these genes when *B. breve* UCC2003 was grown on cellobiose or cellodextrins and to compare these to their transcription levels when grown on glucose. For this purpose, total RNA was isolated from *B. breve* UCC2003 cultures grown on cellobiose, cellodextrins, or glucose (see Materials and Methods). The cultures were harvested at the time points that ensured that *B. breve* UCC2003 was metabolizing cellobiose or cellodextrins (as opposed to the residual glucose present in the cellodextrin preparation). Analysis of the QRT-PCR data obtained from two independent biological replicates indeed clearly showed that the expression of the adjacent *cldE*, *cldF*, *cldG*, and *cldC* genes was significantly upregulated (fold change of >3.0; $P < 0.001$) in *B. breve*

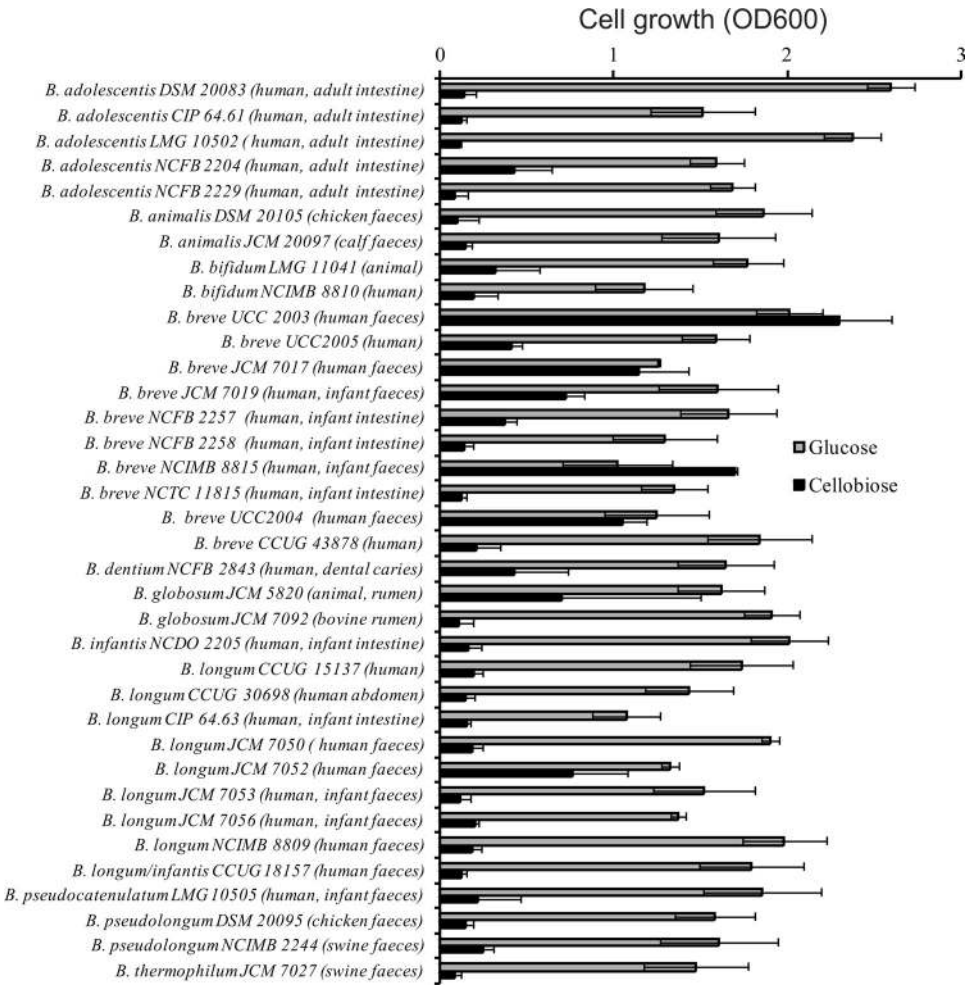
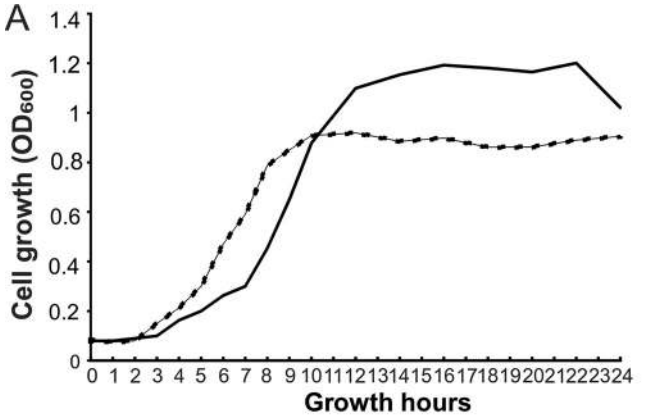
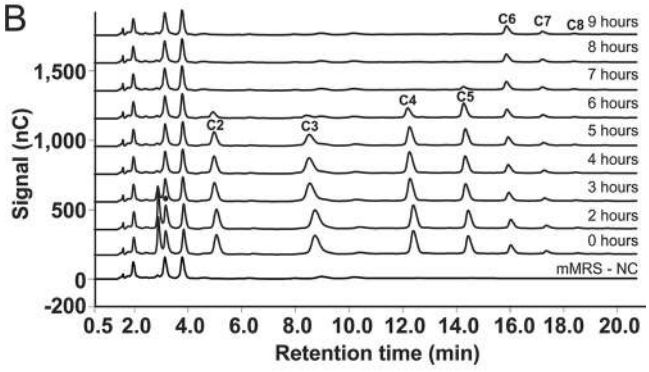


FIG. 1. Final optical density (OD_{600}) values obtained following 24 h of growth of 36 different bifidobacterial strains in modified MRS containing 0.01 g ml^{-1} glucose or cellobiose as the sole carbon source. The results presented are mean values and SEMs obtained from three separate experiments.

UCC2003 cultures grown on cellobiose or cellodextrins relative to cultures grown on glucose (Table 1). These results implicate the *cldEFGC* gene cluster in cellobiose/cellodextrin metabolism in *B. breve* UCC2003.

Genetic organization of the putative cellodextrin utilization operon. Our presumption, based on the QRT-PCR analysis, that the *cld* gene cluster is involved in utilization of cellodextrins was supported by the high level of sequence similarity between *cldEFGC* and predicted and/or proven cellodextrin-specific metabolic genes from a wide variety of bacteria. The genetic organization of the *cld* gene cluster on the chromosome of *B. breve* UCC2003 and its comparison to similar clusters found in other bacteria are schematically displayed in Fig. 3. Due to its adjacent location (Fig. 3) and similarity to LacI-type transcriptional repressors, the protein product of the *cldR* gene was suspected to represent the regulator of the *cldEFGC* operon of *B. breve* UCC2003 (see also below). The *cldE*, *cldF*, and *cldG* genes are predicted to encode a putative cellodextrin-binding protein and two cellodextrin permease proteins, respectively, which together are presumed to specify the cellodextrin uptake system of *B. breve* UCC2003. A β-glucosidase, which is involved in cellodextrin hydrolysis, is encoded by the *cldC* gene (see below). The gene order of the *B. breve* UCC2003 *cld* gene cluster is similar to that of the *cld* cluster in *Streptomyces reticuli* (*cldREFGbglC*) that was previously characterized (42), with the only difference being that the *cldR* orientation is opposite from that of the *cdlEFGC* genes. In the case of the *Thermobifida fusca bgl* operon, which is involved in cellodextrin degradation (45), the gene specifying the transcriptional regulator (*celR*) in this microorganism is situated downstream of the ABC transporters and β-glucosidase-encoding genes. Comparative genome analysis showed that the *B. breve* UCC2003 *cld* operon is most similar to the similarly organized putative *cld* gene clusters in the *Bifidobacterium dentium* ATCC 27678 and *B. dentium* Bd1 genomes (Fig. 3), although the suspected *cldR* homolog (locus tag, *BIFDEN_00681*) of *B. dentium* ATCC27678 and *cldC* homolog (locus tag, BDP_0124) of *B. dentium* Bd1 are not linked to the *cld* operon. A predicted β-glucosidase-encoding gene is present in the genomes of the sequenced *B. longum* subsp. *infantis* ATCC 15697 (50% identical to the *B. breve* UCC2003 CldC protein) and *Bifidobacterium animalis* subsp. *lactis* HN019 (49% identical to





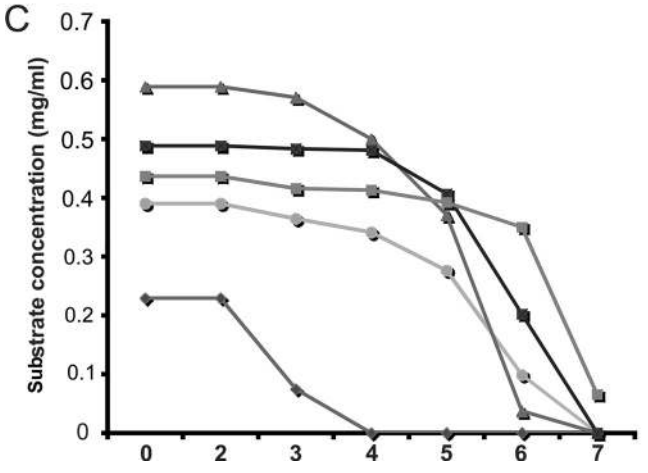


FIG. 2. (A) Growth profile of *B. breve* UCC2003 in mMRS containing 0.01 g ml^{-1} cellobiose (solid line) or 0.0085 g ml^{-1} cellodextrin mixture (broken line). (B) HPAEC-PAD chromatograms monitoring cellodextrin consumption by *B. breve* UCC2003 from mMRS medium, which was used as a negative-control sample (mMRS-NC). Other chromatograms are labeled with the culture growth time points. A black filled circle indicates the peak that corresponds to glucose. The C_2 to C_8 peaks indicate cellodextrin molecules with the corresponding degree of polymerization (DP) (ranging from cellobiose [DP = 2] up to cello-octaose [DP = 8]). (C) Cellodextrin consumption by *B. breve* UCC2003 from mMRS medium as represented by quantified amounts of individual cellodextrins present in the culture medium as calculated from HPAEC-PAD data (shown in panel B). Diamonds, glucose; circles, cellobiose; triangles, cellotriose; dark squares, cellotetraose; gray squares, cellopentaose.

TABLE 1. Effect of cellobiose or a cellodextrin mixture on the transcription of *cld* genes from *B. breve* UCC2003

Locus tag (gene)	Putative function	Expression ratio quantified by QRT-PCR[a]	
		Cellobiose	Cellodextrins
Bbr_0106 (*cldE*)	Cellodextrin-binding protein	69.94	104.58
Bbr_0107 (*cldF*)	Cellodextrin transport system permease protein	74.23	97.32
Bbr_0108 (*cldG*)	Cellodextrin transport system permease protein	90.56	110.54
Bbr_0109 (*cldC*)	β-Glucosidase	58.52	79.80

[a] cDNA templates were derived from RNA samples of *B. breve* UCC2003 culture grown on cellobiose, cellodextrins, or glucose (as a comparator).

the CldC protein) (Fig. 3; Table 2), but the ABC transporter- and regulator-encoding genes appear to be absent from the immediate vicinity of each of these putative *cldC* homologs (data not shown). In addition, CldC encoded by *B. breve* UCC2003 exhibits 48% identity to the β-glucosidase BglC protein encoded by *B. breve* 203, but the *B. breve* 203 genome sequence is not available and we therefore cannot comment on the operon organization (29).

Disruption of the *cldE* gene in *B. breve* UCC2003. In order to establish if disruption of the *cldEFGC* operon in *B. breve* UCC2003 would result in loss of this strain's ability to metabolize cellodextrins, a *cldE* insertion mutant was generated (Fig. 4A). To investigate the expected phenotype of the *B. breve* UCC2003::*cldE* insertion mutant strain, both the wild-type and the insertion mutant strains were analyzed for their ability to grow in mMRS supplemented with cellobiose, the cellodextrin mixture, or glucose (positive control) as the sole carbon source (Fig. 4B).

As expected, and in contrast to the case for the wild type, the *B. breve* UCC2003::*cldE* insertion mutant was shown to be incapable of growth on cellobiose or cellodextrins as its sole carbon source. Although the *cldE* disruption in *B. breve* UCC2003::*cldE* is likely to have a polar effect on the transcription of the downstream genes of the *cld* operon, this nevertheless demonstrates that (elements of) the *cld* gene cluster is exclusively required for cellodextrin metabolism in *B. breve* UCC2003.

β-Glucosidic properties of $CldC_{His}$. In order to characterize the CldC protein, comparative sequence analysis was performed. The *cldC* gene (1,401 bp) encodes a protein of 466 amino acids (molecular mass, ~52.13 kDa). BLASTP and multiple-sequence alignments showed that CldC displays significant similarity to putative or proven β-glucosidases from *Micromonospora* sp. strain ATCC 39149, *Jonesia denitrificans* DSM 20603, *B. dentium* ATCC 27678, *B. animalis* subsp. *lactis* HN019, various *Streptomyces* spp., and *T. fusca* XY. All these β-glucosidases are members of glycosyl hydrolase family 1 (GH1) (14), and CldC is therefore assigned to this family (Table 2). Multiple-sequence alignment of these putative and/or proven representatives of β-glucosidases and other annotated GH1 members revealed a number of conservative amino acids (Fig. 5). The characteristic conserved glutamate residues of the

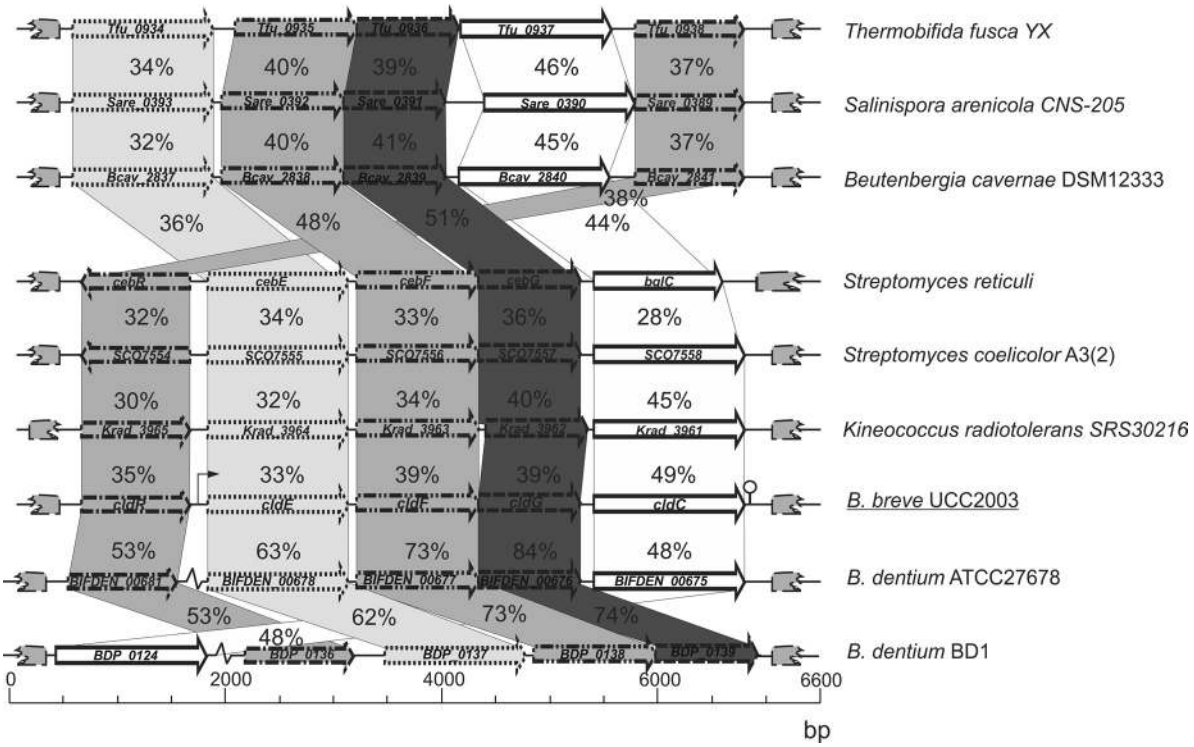


FIG. 3. Comparison of the *cld* locus of *B. breve* UCC2003 with corresponding (putative or proven) cellodextrin/cellobiose utilization loci from other bacteria. Each solid arrow indicates an open reading frame (ORF). The length of the arrow is proportional to the length of the predicted ORF, and the gene locus name, which is indicative of its putative function, is indicated within the arrow. Orthologs are marked with the same shade, while the amino acid identity of each predicted protein is indicated as a percentage relative to its equivalent protein encoded by *B. breve* UCC2003. The bent arrow indicates the *B. breve* UCC2003 *cld* promoter; the lollipop sign designates the putative *rho*-independent terminator.

GH1 family, present in the so-called NEP and ENG motifs, were found in all aligned sequences and correspond to a catalytically active nucleophile and acid-base catalyst, respectively (53).

In order to demonstrate the suspected β-glucosidase activity of CldC from *B. breve* UCC2003 and its involvement in cellobiose/cellodextrin degradation, we cloned *clcC* and overexpressed it as a His-tagged version, designated $CldC_{His}$ (see Materials and Methods). Crude cell extract containing $CldC_{His}$ displayed hydrolytic activity against cellobiose, cellodextrins, 2-nitrophenyl-β-D-glucopyranoside, 2-nitrophenyl-β-D-cellobioside, and 4-nitrophenyl-β-D-cellobioside (results not shown). Under the same experimental conditions, we showed that crude cell extract from *L. lactis* NZ9000 harboring pNZ8150 (negative control) did not exhibit any hydrolytic activity toward the above-mentioned carbohydrates. These results clearly suggest that, consistent with the results from our comparative sequence analysis and the *B. breve* UCC2003::*cldE* mutant

TABLE 2. Similarity of CldC from *B. breve* UCC2003 to proven and annotated β-glucosidases in other bacterial strains

Bacterial strain	Locus tag or gene	Annotated or proven function	NCBI reference no.	Identity (%) to CldC
Micromonospora sp. strain ATCC 39149	MCAG_00986	β-Glucosidase	ZP_04604729	51
Jonesia denitrificans DSM 20603	JdenDRAFT_1772	Broad-specificity cellobiase	ZP_0386834	50
B. dentium ATCC 27678	BIFDEN_00695	Hypothetical protein BIFDEN_00695	ZP_02917416	50
B. dentium ATCC 27678	BIFDEN_00675	Hypothetical protein BIFDEN_00675	ZP_02917396	13
B. dentium BD1	BDP_0124	β-Glucosidase	YP_003359633.1	48
B. breve 203		β-D-Glucosidase	BAA19881	48
B. animalis subsp. *lactis* HN019	BIFLAC_00409	β-Glucosidase	ZP_02963645	49
B. longum subsp. *infantis* ATCC 15697	Blon_1905	β-Glucosidase	YP_002323355	50
B. animalis subsp. *lactis* DSM 10140	Balat_0151	Putative β-glucosidase	YP_002969174	47
Kineococcus radiotolerans SRS30216	Krad_3961	β-Glucosidase	YP_001363688	48
Thermobifida fusca YX	Tfu_0937	β-Galactosidase	YP_288998	44
Streptomyces coelicolor A3(2)	SCO2798	Cellobiose hydrolase	NP_627028	47
Streptomyces reticuli	*bglC*	β-Glucosidase	AJ009797	24
Salinispora arenicola CNS-205	Sare_0390	β-Glucosidase	YP_001535310	47
Beutenbergia cavernae DSM 12333	Bcav_2840	β-Galactosidase	YP_002882847	26

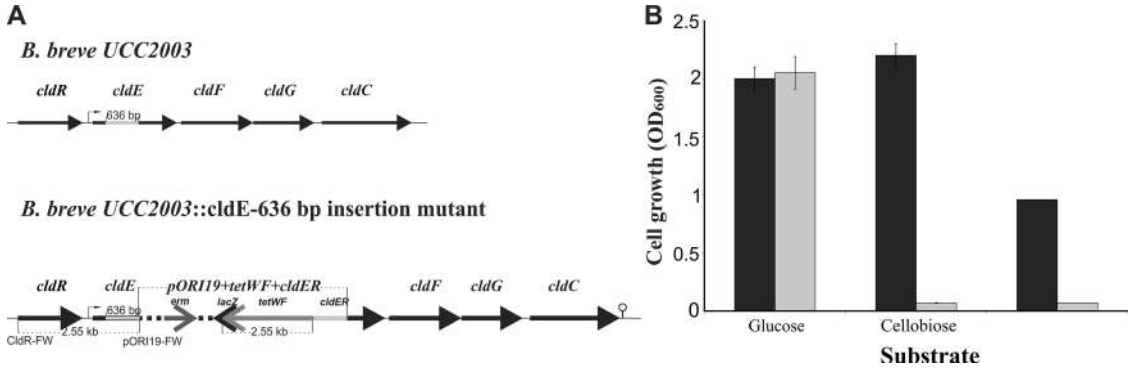


FIG. 4. (A) Schematic representation of the *cld* region of the *B. breve* UCC2003 and *B. breve* UCC2003::*cldE* chromosomes. Chromosomal DNA is represented by a thin line, the *cldE* gene is represented by a blank arrow, and the internal *cldE* fragment used for homologous recombination to obtain the insertion mutant is indicated by a solid light gray line. Segments of the integrated plasmid are indicated by solid medium gray (*tetW* gene), solid (*lacZ* gene), and boxed black and solid dark gray (*erm* gene) lines. (B) Growth profiles of wild-type *B. breve* UCC2003 (dark gray) and the *B. breve* UCC2003::*cldE* insertion mutant (light gray) in mMRS supplemented with glucose, cellobiose, or a mixture of cellodextrins.

analysis, $CldC_{His}$ represents a β-glucosidase being responsible for the observed carbohydrate-degrading activity, although it is theoretically possible that (expression of) $CldC_{His}$ somehow activates a silent β-glucosidase in its lactococcal expression host. We could not exclude the latter possibility, because for some unknown reason the $CldC_{His}$ protein completely lost its hydrolytic activity upon purification (results not shown). Under our experimental conditions, the $CldC_{His}$-containing cell extract was not capable of hydrolyzing sucrose, lactose, maltose, 1-kestose, and raffinose; however, all cellodextrins present in the reaction mixture (from C_2 to C_9) were completely converted into glucose (results not shown). The results obtained therefore show that crude extract containing $CldC_{His}$ is capable of specifically cleaving the β-1,4-linkage present in cellodextrins and that it does not possess hydrolytic activity toward other tested glycosidic linkages.

Transcriptional analysis of the *cld* gene cluster. In *B. breve* UCC2003, the *cldE*, *cldF*, *cldG*, and *cldC* genes (Fig. 6A) are presumed to be expressed as a single transcript, based on their similar expression patterns as determined from the microarray

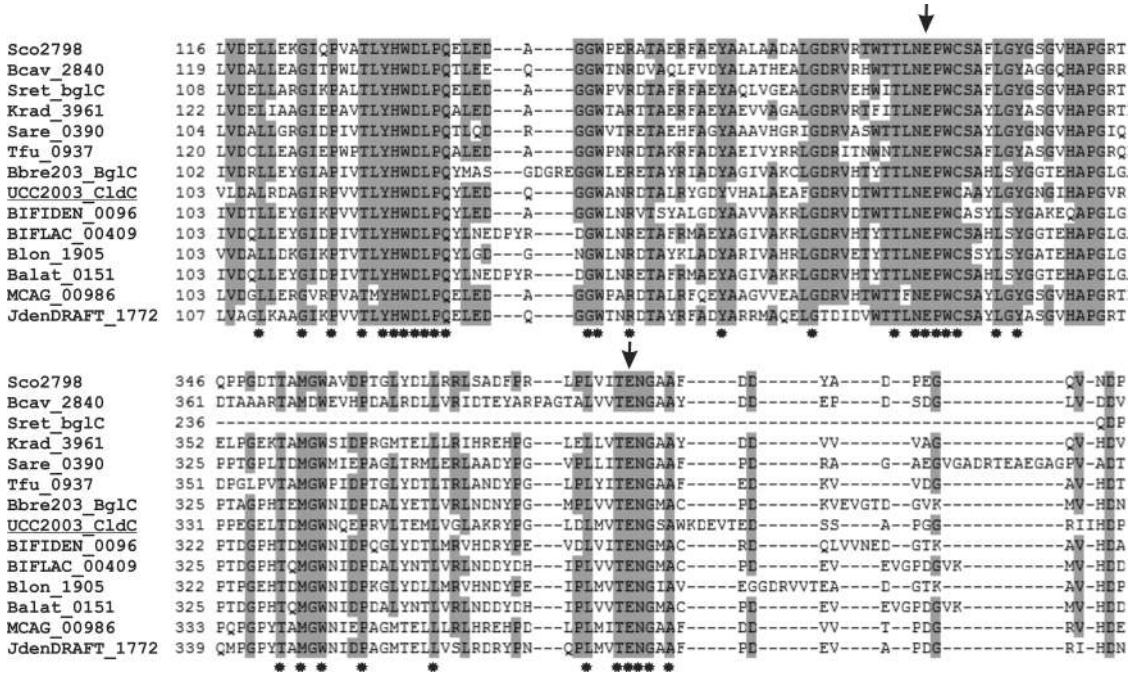


FIG. 5. Multiple-sequence alignment between CldC proteins from *B. breve* UCC2003 and 13 putative or proven β-glucosidases from different bacterial strains using CLUSTAL W (48). The sequence labeled UCC2003_CldC corresponds to CldC encoded by *B. breve* UCC2003. Other labels indicate locus tags of genes that encode putative GH1 family members from different bacteria (for details, see Table 2). Conserved residues are shaded based on conservation in 60% or more of the sequenced used. Fully conserved residues are indicated by an asterisk below the alignment. The arrows above the sequence point at the conserved glutamate residues that have been identified as an acid-base catalyst (glutamic acid 165 in UCC2003) and an active-site nucleophile (glutamic acid 368 in UCC2003) (57).

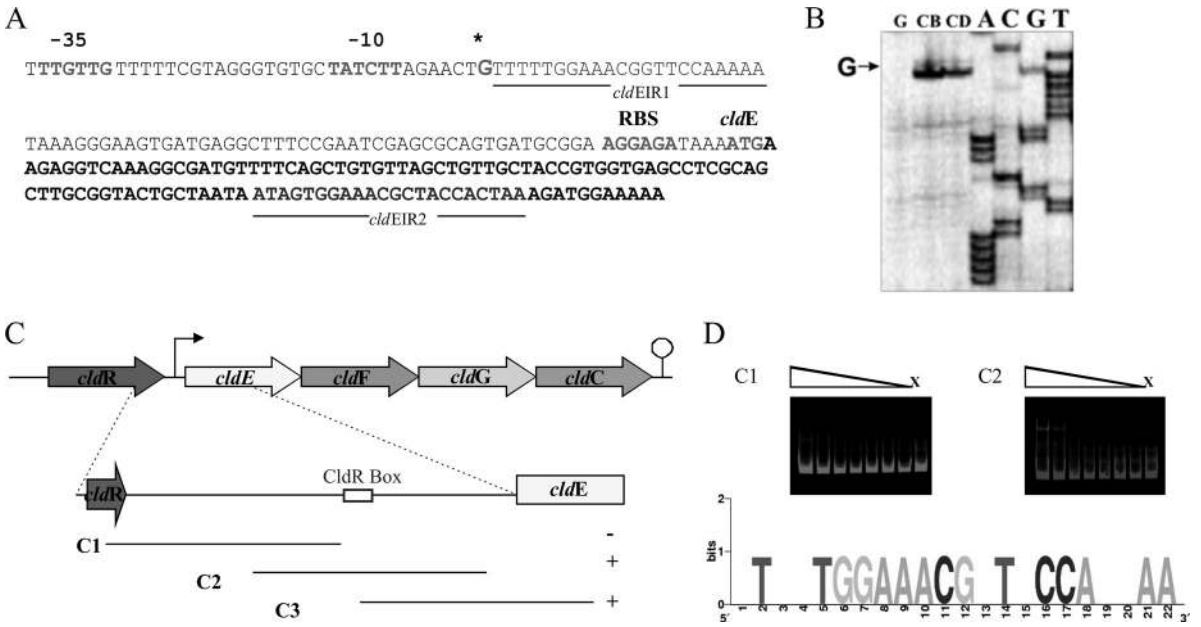


FIG. 6. (A) Schematic representation of the *B. breve* UCC2003 *cldE* promoter region and partial coding sequence. Boldface and underlining indicate the −10 and −35 hexamers (as deduced from the primer extension results shown in panel B) and ribosomal binding site (RBS); the transcriptional start site is indicated by an asterisk. The CldR binding inverted repeat sequences cldEir1 and cldEIR2 are underlined. (B) Primer extension results. CB, cellobiose; CD, cellodextrins. (C) Representation of the *B. breve* UCC2003 cellodextrinase operon and DNA fragments used in EMSAs for the *cldE* promoter region. Plus and minus signs indicate whether or not CldR was able to bind to the particular DNA fragment, respectively. (D) Weblogo representation of predicted binding sequences of CldR together with EMSA demonstrating binding of CldR to the C2 fragment.

analysis and RT-PCR experiments. Additional RT-PCR experiments confirmed the suspected operon structure, as they showed that amplification products were generated from cDNA (generated from total RNA of cellobiose-grown *B. breve* UCC2003) using various primer combinations that spanned the individual genes of the *cld* gene cluster (data not shown). Furthermore, the only predicted Rho-independent terminator structure in the DNA region that harbors the very tightly organized *cldEFGC* genes is present downstream of the *cldC* gene (Fig. 6C). The transcriptional start site of the presumed *cldEFGC* operon was determined by primer extension analysis (see Materials and Methods) and was shown to be located 79 bp upstream of the predicted *cldE* start codon (Fig. 6A and B) and 6 bp downstream of sequences resembling consensus −10 and −35 sequences of a vegetative promoter (Fig. 6A). In addition, the TG sequence upstream of the presumed −10 sequence has previously been shown to act as an enhancer element for transcription in other bacteria (23).

CldR binds to the *cldE* promoter region. The presence of *cldR*, encoding a putative LacI-type regulator within the cellodextrin gene cluster, suggests that this gene is involved in the transcriptional regulation of the cellodextrin gene cluster, as was obvious from the QRT-PCR data (Table 1). In order to establish if CldR is capable of direct interaction with specific operators within the *cldE* promoter region, we first cloned the *cldR* gene in the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible vector pQE60 with the introduction of a His tag-encoding sequence to facilitate subsequent protein purification. The purified CldR protein was then used to perform electrophoretic mobility shift assays (EMSAs), which clearly demonstrate that the CldR protein can form a complex with IRD700-labeled DNA fragments encompassing a presumed CldR-binding sequence that is located within the *cldE* promoter region (Fig. 6C). Further delineation of this CldR-binding sequence or CldR box revealed the presence of two inverted repeats, the first located directly downstream of the transcription start site and the second present within the *cldE* coding sequence (Fig. 6C and D). These CldR box sequences are thus presumed to represent operator sequences for the CldR protein and to act as *cis* elements in the transcriptional regulation of the *cldEFGC* operon.

DISCUSSION

Life on Earth is to a significant degree dependent on the generation of plant biomass through photosynthesis. Cellulose, the most abundant component of plant biomass, which in nature is almost exclusively found in plant cell walls (21), cannot be metabolized by humans due to the absence of cellulose-degrading enzymes. Nevertheless, a number of cellulolytic bacteria have been identified in the human gastrointestinal tract and shown to extracellularly hydrolyze cellulose to soluble cellodextrins, which in turn may be utilized by noncellulolytic human gut bacteria with β-glucosidic activity (6, 9, 16, 54). This was further substantiated by a recent study which demonstrated that β-glucosidases are widespread among colonic bacteria (25). Interestingly, bifidobacterial strains are reported to exhibit the highest β-glucosidic activity among the colonic microbiota (6), and several bifidobacterial β-glucosidases involved in the utilization of cellobiose and/or

other carbohydrate substances have been described and characterized (5, 29, 30, 39, 56).

The current report describes the genetic identification and characterization of the cellodextrin utilization cluster, *cldEFGC* and its regulator *cldR*, carried by *B. breve* UCC2003. The insertion mutant *B. breve* UCC2003::*cldE* confirmed that the *cld* gene cluster is exclusively responsible for cellodextrin metabolism by *B. breve* UCC2003. Although *B. breve* 203 was previously shown to metabolize cellobiose (30), this is, to the best of our knowledge, the first report to demonstrate that particular bifidobacterial strains can utilize a range of cellodextrins and that a specific gene cluster, *cldEFGC*, is required for its metabolism.

The upregulation of *cldEFGC* transcription when *B. breve* UCC2003 was grown in mMRS containing a mixture of cellodextrins was significantly higher than that for growth on cellobiose only. Therefore, these results suggest that cellobiose is less efficient in derepression of the *cldR* repressor of *B. breve* UCC2003 *cld* operon than the cellodextrins. Even though various ruminal cellulolytic bacteria display similar growth behavior, these latter degrade cellodextrins usually by a phospho-dependent reaction by phosphorolytic cleavage, generating glucose-1-phosphate as an end product (44, 55).

Comparative sequence analysis of gene clusters for cellodextrin utilization from other bacteria (e.g., *Kineococcus radiotolerans*, *Streptomyces coelicolor*, *Beutenbergia cavernae*, *Salinispora arenicola*, and *T. fusca*) shows that they are generally organized in a similar genetic fashion as the *B. breve* UCC2003 *cld* gene cluster, with the exception of *B. dentium*, where the regulator-encoding gene (in *B. dentium* ATCC 72678) and putative β-glucosidase-encoding gene (in *B. dentium* Bd1) appear to be disconnected from the rest of the gene cluster. The results obtained from comparative sequence and genome analyses indicate that just two other bifidobacterial strains, in addition to strain UCC2003, possess the complete *cld* operon (i.e., strains JCM7017 and NCIMB8815), consistent with the finding that these strains are also capable of cellobiose metabolism. In contrast, *B. breve* JCM7019 is able to metabolize cellobiose but does not appear to contain the *cld* cluster, which suggests that this bacterium may use an alternative cellodextrin utilization system which is not homologous to the one identified in *B. breve* UCC2003. This could be the one found in *B. breve* 203, which is significantly different in sequence (Table 2) (39).

From the results presented here we have obtained convincing evidence to conclude that the *B. breve* UCC2003 CldC protein represents a β-glucosidase that exhibits exclusive hydrolytic activity toward cellobiose and higher-molecular-weight cellodextrins, cleaving the β-1→4-glucosidic bond between glucose molecules in these carbohydrates. The presence of β-glucosidases in bifidobacteria has been reported previously for *B. breve* 203 (29, 30), *B. adolescentis* Int-57 (5), and *Bifidobacterium* sp. strain SEN (56). The β-glucosidase from *Bifidobacterium* sp. strain SEN did not exhibit hydrolytic activity against cellobiose, while both *B. breve* 203 and *B. adolescentis* Int-57 produced β-glucosidase activity with the ability to hydrolyze cellobiose. Furthermore, seven different *B. bifidum* strains, two *B. infantis* strains, and two *B. breve* strains were demonstrated to have specific β-glucosidase activities (28). However, none of these enzymes were examined for their ability to degrade cellodextrins with a higher degree of polymerization.

A specific ABC transporter system specified by the *cldEFG* genes is presumed to be involved in cellodextrin internalization. Interestingly, no gene encoding an ATP-binding protein is present in this gene cluster, suggesting that *B. breve* UCC2003 may use a general ATP-binding protein whose transcription is not under cellobiose/cellodextrin control. A similar scenario was observed in *S. reticuli*, which contains the *msiK* gene, encoding an ABC transporter-type ATP-binding protein involved in both cellobiose and maltose transport (43). Our results (Fig. 2B and C) suggest that the cellodextrin ABC transporter is capable of transporting cellodextrins with a degree of polymerization (DP) ranging from 2 to 5, which is consistent with data from a similar study of *Clostridium thermocellus* (27). Interestingly, the crude cell extract containing the $CldC_{His}$ enzyme is capable of hydrolyzing cellodextrins with a higher DP (up to 9 [results not shown]), although they are not utilized by *B. breve* UCC2003. This observation suggests that the *B. breve* UCC2003 ABC uptake system for cellodextrins limits the ability of this microorganism to metabolize higher-molecular-weight cellodextrins, although this presumption needs to be confirmed by further experimental work.

Insoluble plant fiber consisting mostly of plant cell wall polysaccharides, including cellulose, is one of the major components of the everyday human diet. The complex microbial community of the human colon is capable of utilization of insoluble cellulose by producing solubilized end products, i.e., cellodextrins, that become accessible to other microorganisms of the human colon microbiota (for a review, see reference 10). This, to the best of our knowledge, represents the first in-depth study describing a bifidobacterial gene cluster responsible for cellodextrin utilization. The ability of *B. breve* UCC2003 to metabolize cellobiose/cellodextrins may provide a competitive advantage to assist this strain to colonize, adapt to, and persist in the human intestinal environment.

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