

Characterization of ApuB, an Extracellular Type II Amylopullulanase from *Bifidobacterium breve* UCC2003[∇]

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The *apuB* gene of *Bifidobacterium breve* UCC2003 was shown to encode an extracellular amylopullulanase. ApuB is composed of a distinct N-terminally located α -amylase-containing domain which hydrolyzes α -1,4-glycosidic linkages in starch and related polysaccharides and a C-terminally located pullulanase-containing domain which hydrolyzes α -1,6 linkages in pullulan, allowing the classification of this enzyme as a bifunctional class II pullulanase. A knockout mutation of the *apuB* gene in *B. breve* UCC2003 rendered the resulting mutant incapable of growth in medium containing starch, amylopectin, glycogen, or pullulan as the sole carbon and energy source, confirming the crucial physiological role of this gene in starch metabolism.

The study of the human gut microbiota in the context of health maintenance or improvement has in recent years enjoyed an exponentially increasing interest (reviewed in references 35 and 58). It is known that several pathogenic bacteria can cause acute gastroenteritis, while representatives of other bacterial genera, such as the lactobacilli, have the potential to provide protection against infection (11). It has also become apparent that elements of the colonic microbiota are capable of influencing the incidence and severity of gastrointestinal diseases and disorders, such as ulcerative colitis, bowel cancer, and pseudomembranous colitis (41). There have also been many studies focusing on prebiotics, which are “selectively fermented food ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well being and health” (33; see also references 28 and 42 for reviews). Carbohydrates that have been shown to exert prebiotic effects include those from whole-grain wheat, fructooligosaccharides, galactooligosaccharides, and lactulose (12, 17, 20, 33). The development of functional foods containing prebiotics and/or probiotics which can change the composition or activity of the microbiota such that it prevents or ameliorates gastrointestinal disorders is a key goal for both the pharma and food industries. Achieving this goal may be facilitated by the availability of an increasing number of genome sequences of gastrointestinal microbes (1, 9, 10, 26, 40, 48, 49), since the genomic data should allow the selection of novel, perhaps more-selective prebiotics and would also be pivotal in attaining a fundamental understanding of the probiotic effect.

Although starch and related compounds are digested and absorbed in the stomach and small intestine, a significant fraction that is resistant to digestion may represent valuable carbon and energy sources for colonic bacteria, such as bifidobac-

teria, and which thus may act as prebiotics (57). Plant cells and seeds are a rich source of starch where it is deposited as granules in the cytoplasm. Starch is composed of two high-molecular-weight components: amylose, which comprises 15 to 25% of starch, is a linear polymer consisting of α -1,4-linked glucopyranose, while the predominant component, amylopectin, is a branched polymer containing, in addition to α -1,4-glycosidic linkages, α -1,6-linked branch points occurring every 17 to 26 glucose units (18).

Glycogen is the storage form of glucose in animals and humans and plays a role which is analogous to the function of starch/amylopectin in plants. Glycogen is synthesized and stored mainly in the liver and muscles. Structurally, glycogen is a branching polymer consisting of chains of glucose units connected by α -1,4 linkages with branch points that are formed by α -1,6 linkages that occur at intervals of 10 to 13 glucose units.

Pullulan is a fermentation product of the yeast *Aureobasidium pullulans* that has a starch-like structure in that it is an α -glucan. Pullulan has a relatively simple structure of three α -1,4-linked glucose molecules that act as the repeated subunit and create a linear polymer through α -1,6 linkages on the terminal glucose of each subunit (31).

Starch degradation in most organisms proceeds via the combined action of amylases (EC 3.2.1.1, EC 3.2.1.2, and EC 3.2.1.3) and amylopullulanases (APU EC 3.2.1.41). Many of these amylolytic enzymes are industrially important for the liquefaction of starch and in saccharification processes. At present there is only limited knowledge available on the metabolism of starch and related α -glucans by bifidobacteria. Wang et al. (55) observed that bifidobacteria could efficiently utilize high-amylose maize starch granules and that bifidobacteria produced several starch-degrading enzymes of various molecular weights. Ji et al. (23) purified and characterized an extracellular amylase (AmyB) from *Bifidobacterium adolescentis* INT57, while Rhim et al. (43) have heterologously expressed AmyB from *B. adolescentis* INT57 in *Bifidobacterium longum* MG1. Ryan et al. (46) have reported on the screening of various bifidobacteria for α -amylase and/or pullulanase activity by investigating their ability to utilize starch, amylopectin, and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>E. coli</i> EC101	Cloning host; <i>repA</i> ⁺ Kan ^r	30
<i>L. lactis</i> strains		
NZ9000	MG1363, nisin-inducible overexpression host; <i>pepN::nisRK</i>	14
NZ9000-pNZ- α amy	NZ9000 containing pNZ- α amy	This study
NZ9000-pNZ-pull	NZ9000 containing pNZ-pull	This study
<i>B. breve</i> strains		
UCC2003	Isolate from nursing stool	37
UCC2003-apuB	pORI19-apuB insertion mutant of UCC2003	This study
Plasmids		
pNZ8048	Cm ^r ; nisin-inducible translational fusion vector	14
pNZ- α amy	Cm ^r ; pNZ8048 derivative containing translational fusion of α -amylase encoding DNA fragment to nisin-inducible promoter	This study
pNZ-pull	Cm ^r ; pNZ8048 derivative containing translational fusion of pullulanase encoding DNA fragment to nisin-inducible promoter on pNZ8048	This study
pTGB019	Cm ^r ; 6.5-kb derivative of pVE6007 harboring temperature-sensitive <i>repA</i> gene	L. Steidler and S. Neirinck, unpublished results
pORI19	Em ^r RepA ⁻ Ori ⁺ ; cloning vector	30
pORI19-apuB	Internal 1-kb fragment of <i>apuB</i> cloned in pORI19	This study

pullulan. Of the bifidobacterial strains examined, five *B. breve* strains were identified that could utilize starch and pullulan as primary carbohydrate sources. These activities were found to be both inducible and extracellular, as well as consistent with pullulanase type II (amylopullulanase) activity.

This research reports on the characterization and mutagenesis of *apuB*, encoding an extracellular amylopullulanase, that was identified on the genome of *Bifidobacterium breve* UCC2003.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Bifidobacterium breve* UCC2003 was routinely cultured in reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). Carbohydrate utilization by *B. breve* was examined in de Man Rogosa and Sharpe medium (MRS) prepared from first principles (13). Prior to inoculation, the MRS was supplemented with cysteine HCl (0.05%) and a carbohydrate source (1%). The carbohydrates used (purchased from Sigma-Aldrich Ireland Ltd., Dublin, Ireland) were starch (derived from potato), amylopectin (derived from potato), glycogen (derived from oyster), pullulan (from *Aureobasidium pullulans*), and glucose. Bifidobacteria cultures were incubated at 37°C (unless otherwise stated) under anaerobic conditions which were maintained by using an Anaerocult oxygen-depleting system (Merck, Darmstadt, Germany) in an aerobic chamber. *Escherichia coli* was cultured in Luria Bertani broth (LB) (47) at 37°C with agitation. *Lactococcus lactis* strains were cultivated in M17 broth containing 0.5% glucose (50) at 30°C. The growth medium contained chloramphenicol (Cm; 5 μ g ml⁻¹ for *L. lactis* and 2 μ g ml⁻¹ for *B. breve*), erythromycin (Em; 100 μ g ml⁻¹ for *E. coli* and 1 μ g ml⁻¹ for *B. breve*), or kanamycin (50 μ g ml⁻¹ for *E. coli*) when required for plasmid maintenance. Recombinant *E. coli* cells containing pORI19 were selected on LB agar containing Em and supplemented with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 40 μ g ml⁻¹) and 1 mM IPTG (isopropyl- β -D-galactopyranoside).

Nucleotide sequence analysis. Sequence data were obtained from the Artemis-mediated (45) genome annotations of the *B. breve* UCC2003 sequencing project (S. Leahy, M. O'Connell Motherway, J. Moreno Munoz, G. F. Fitzgerald, D. Higgins, and D. van Sinderen, unpublished data). Database searches were performed by using nonredundant sequences accessible at the National Center for Biotechnology Information internet site (<http://www.ncbi.nlm.nih.gov>) using BLAST (2, 3). Sequence alignments were performed by using the Clustal method of the MEGALIGN program of the DNASTAR software package (DNASTAR,

Madison, WI). The biological software program SignalP (<http://www.cbs.dtu.dk/services/SignalP>) was used to predict the presence and precise location of the secretion signal in the ApuB protein (6). Screening for the cell wall anchoring motif LPXTG was performed manually.

DNA manipulations. Chromosomal DNA was isolated from *B. breve* UCC2003 as previously described (38). Minipreparation of plasmid DNA from *E. coli* or *L. lactis* was achieved by using a Qiaprep spin plasmid miniprep kit (Qiagen GmbH, Hilden, Germany). For *L. lactis*, an initial lysis step was incorporated into the plasmid isolation procedure, and cells were resuspended in lysis buffer supplemented with lysozyme (30 mg ml⁻¹) and incubated at 37°C for 30 min. The procedures for DNA manipulations were performed essentially as described by Sambrook et al. (47). Restriction endonucleases, shrimp alkaline phosphatase, and T4 DNA ligase were obtained from Roche Diagnostics and used according to the supplier's instructions. (Roche Diagnostics, Bell Lane, East Sussex, United Kingdom). The synthetic single-stranded oligonucleotide primers used in this study were synthesized by MWG Biotech AG (Ebersberg, Germany). Standard PCRs were performed using Taq PCR mastermix (Qiagen), while high-fidelity PCR was achieved by using KOD polymerase (Novagen, Darmstadt, Germany). *B. breve* colony PCRs were performed according to standard procedures with the addition of 2 units of mutanolysin to each PCR, while an initial cell lysis step of 37°C for 30 min was incorporated into the PCR conditions. PCR fragments were purified by using a Qiagen PCR purification kit (Qiagen). Electroporation of plasmid DNA into *E. coli* was performed as described by Sambrook et al. (47) and into *L. lactis* as described by Wells et al. (56). Electrotransformation of *B. breve* UCC2003 with pTGB019 and, subsequently, pORI19-apuB was performed as described by Mazé et al. (37). Southern transfer and hybridization were performed according to standard procedures using an ECL gene hybridization and detection system (GE Healthcare, United Kingdom).

Cloning of the α -amylase- and pullulanase-encoding domains of *apuB* in pNZ8048. DNA fragments encompassing the α -amylase- or pullulanase-encoding domain of *apuB* were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using KOD DNA polymerase and primer combinations ApuAf and ApuAr or ApuPf and ApuPr (Table 2), respectively. NcoI and XbaI restriction sites were incorporated at the 5' ends of the forward and reverse primers, respectively. In addition, an in-frame His₁₀-encoding sequence was incorporated into each of the forward primers to facilitate protein purification using a Ni-nitrilotriacetic acid system (Qiagen). The amplicons, specifying Amy-His_{ApuB} and Pull-His_{ApuB}, were digested with NcoI and XbaI and ligated into the similarly digested nisin-inducible translational fusion plasmid pNZ8048 (Table 1). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation, and transformants selected based on Cm resistance. The plasmid

TABLE 2. Oligonucleotide primers used in this study

Purpose	Primer	Sequence ^a
Cloning of α -amylase fragment of <i>apuB</i> in pNZ8048	ApuAf	TGCATCCCATGGCCCATCACCATCACCATCACCA TCACCATCACACCGAGGCCAATGCACAG
	ApuAr	CTCGAATCTAGATTAGTAGGAGTCCCTCGCCGGTG
Cloning of pullulanase-encoding fragment of <i>apuB</i> in pNZ8048	ApuPf	TGCACGCCATGGCCCATCACCATCACCATCACCA TCACCATCACGCGACACGAGCCGCACCATC
	ApuPr	TGCGCATCTAGACTACTGTTCGGAGCCCTC
Cloning of internal fragment of <i>apuB</i> in pORI19	ApuBf	CTTGCGAAGCTTGGCGGCGACGGCTGGGTGAG
	ApuBr	GCGACGTCTAGACCTTCGGCGGGCTGTAGTGC
Amplification of 5.1-kb <i>apuB</i> fragment for use as probe in Southern hybridization	ApuF	ATGGCCGTACGACGATTAGCTC
	ApuR	CTGTTCGGAGCCCTCCTACTG
Colony PCR primer pair	Apu1 pORI19r	ATCGCCATCGATTCCAAACCAAC GATTAAGTTGGGTAAGCCG

^a The sequences in bold correspond to restriction sites.

content of a number of Cm^r transformants was screened by restriction analysis, and the integrity of positively identified clones was verified by sequencing.

Protein overproduction and purification. An amount of 400 ml of M17 broth supplemented with 0.5% glucose was inoculated with a 2% inoculum of a particular *L. lactis* strain, followed by incubation at 30°C until an optical density at 600 nm of 0.5 was reached, at which point protein overexpression was induced by the addition of purified nisin (5 ng ml⁻¹) and cultures were incubated at 30°C for 90 min. Cells were harvested by centrifugation, washed, and concentrated 40-fold in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole [pH 8.0]). Cell extracts were prepared by using 106- μ m glass beads and a Mini BeadBeater-8 cell disrupter (Biospec Products, Bartlesville, Oklahoma). After homogenization, the glass beads and cell debris were sedimented by centrifugation and the supernatant containing the cytoplasmic fractions retained. Protein purification from the cytoplasmic fraction was performed by using Ni-nitrilotriacetic acid matrices in accordance with the manufacturer's instructions (Qiagen). Elution fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described by Laemmli (29), on a 12.5% polyacrylamide gel. After electrophoresis, the gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Rainbow-prestained low-molecular-weight protein markers (New England Biolabs, Herfordshire, United Kingdom) were used to estimate the molecular weight of the purified proteins.

HPTLC analysis. High-performance thin-layer chromatography (HPTLC) allowed the qualitative determination of the breakdown products of starch, amylopectin, or pullulan following hydrolysis by the purified α -amylase, pullulanase, or cell extract of *B. breve* UCC2003. The purified α -amylase, pullulanase, cells, or cell extracts of *B. breve* UCC2003 were incubated with starch, amylopectin, glycogen, or pullulan in 50 mM phosphate buffer, pH 6.0, at 37°C for 72 h. An aliquot of the reaction mixture was spotted onto a silica gel 60 plate (10 by 10 cm; Merck) with a Nanomat 4 (Camag, Switzerland). The chromatogram was developed with a butanol-acetic acid-water (5:4:1, vol/vol/vol) solvent system in a horizontal developing chamber. Ascending development was repeated twice at room temperature. The plate was allowed to dry in a fume hood and then developed by spraying evenly with 20% (vol/vol) sulfuric acid in ethanol. The plate was dried and heated to 120°C for 10 min to visualize sugar-representing spots. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were purchased from Sigma, and a mixture of these sugars was used as a standard marker for the HPTLC experiments.

Construction of pORI19-*apuB*. An internal 1-kb fragment of *apuB* was amplified by PCR using *B. breve* UCC2003 chromosomal DNA as template and the oligonucleotide primers ApuBf and ApuBr (Table 2). The 1-kb PCR product generated was cloned into pORI19, an Ori⁺ RepA⁻ integration plasmid (30), by using the unique HindIII and XbaI restriction sites that were incorporated into the primers and introduced into *E. coli* EC101 by electroporation. The expected structure of the recombinant plasmid, pORI19-*apuB*, was confirmed by restriction mapping prior to its introduction into *B. breve* harboring pTGB019 by

electrotransformation and subsequent selection on RCA plates supplemented with Em and Cm.

RESULTS

Identification and analysis of a *B. breve* UCC2003 amylopullulanase-encoding gene. A gene designated *apuB*, predicted to encode amylopullulanase activity, was identified from the annotation of the genome sequence of *B. breve* UCC2003. (S. Leahy, M. O'Connell Motherway, J. Moreno Munoz, G. F. Fitzgerald, D. Higgins, and D. van Sinderen, unpublished results). *apuB* is located upstream of *dnaK* (53) and downstream from and in the opposite orientation to a gene predicted to encode an LacI-type transcriptional regulator (Fig. 1A). At the protein level, the predicted product of *apuB* displays significant similarity (67%) to predicted amylopullulanases encoded on the genomes of *Bifidobacterium adolescentis* strains L2-32 (gi: 154487452; GenBank accession number AAXD0000000) and ATCC 15703 (gi:119025726; GenBank accession number AP009256). The *apuB* gene is 5,127 bp in length and corresponded to a deduced protein of 1,708 amino acids with an estimated molecular mass of 182.36 kDa. Based on the presence of a signal sequence of 34 amino acids with a corresponding cleavage site located between Ala³⁴ and Asp³⁵, ApuB is predicted to represent a secreted protein. The C-terminally located LPNTG sequence suggests that ApuB can be covalently linked to the peptidoglycan at the carboxy terminus via the LPXTG motif (51). The molecular mass of the mature extracellular, cell wall-anchored ApuB (from Asp³⁵ to Thr¹⁶⁷⁰) is expected to be 174.98 kDa. Individual domains representing α -amylase and pullulanase activity are predicted to be located in the amino-terminal portion and carboxy-terminal portion, respectively, of ApuB. Highly conserved regions were found when the amino acid sequence of ApuB was aligned with sequences of other characterized amylopullulanases (Fig. 1A) (22). For example, two copies of four highly conserved sequences designated I, II, III, and IV characteristic of the active site of amylolytic enzymes (7) were evident (Fig. 1B). The first of these is located between amino acids Asp¹³³ and Asp³²⁸ in

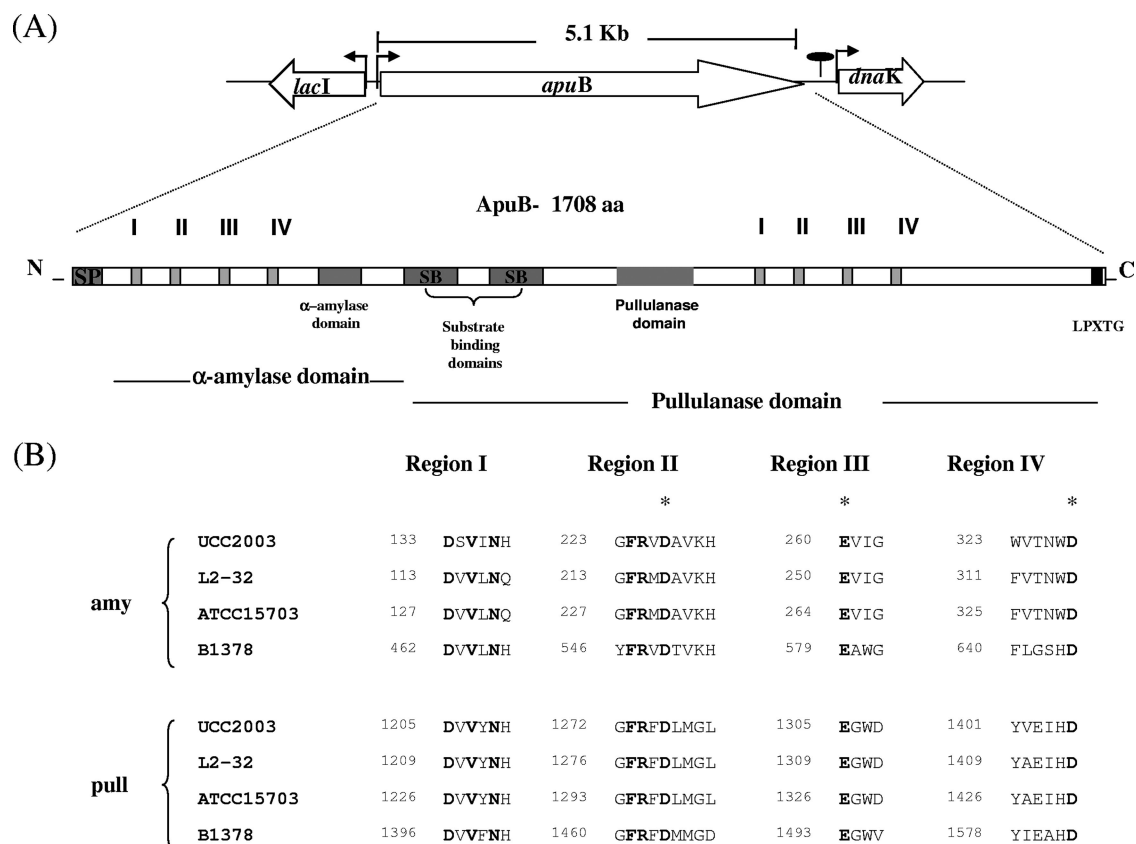


FIG. 1. (A) Schematic representation of *apuB* and surrounding genes. *ApuB* is encoded by a single open reading frame of 5,127 bp, producing a protein of 1,708 amino acids which includes a signal sequence of 34 amino acids (SP). The α -amylase and pullulanase domains are located in the amino-terminal and carboxy-terminal portion, respectively. Within the protein, four regions highly conserved in α -amylase-like proteins were identified. In addition, specific α -amylase and pullulanase domains were identified. Two copies of a domain (SB) rich in aromatic amino acids were identified between the α -amylase and pullulanase domains. These domains are believed to be involved in substrate binding. aa, amino acids. (B) Two copies of the four regions highly conserved among α -amylases, pullulanases, and amylopullulanases were identified in *ApuB* and in amylopullulanases from *B. adolescentis* strains L2-32 and ATCC 15703. The amino acids in bold are conserved among all amyolytic enzymes, while the putative catalytic amino acids are denoted by asterisks. The sequence of the well-characterized alkaline amylopullulanase from *Bacillus* sp. KSM-1378 (GenBank accession no. D78258) is included.

the predicted α -amylase domain, while the second set is located between amino acid positions Asp¹²⁰⁵ and Asp¹⁴⁰⁶ of the putative pullulanase domain. Based on the similarity with characterized α -amylases, pullulanases, and amylopullulanases, the catalytic residues of the presumed α -amylase domain (and of the pullulanase domain) of *ApuB* were predicted to be Glu²⁶⁰ (Glu¹³⁰⁵) in conserved region III, Asp²²⁷ (Asp¹²⁷⁶) in conserved region II, and Asp³²⁸ (Asp¹⁴⁰⁶) in conserved region IV. Between the predicted α -amylase and pullulanase domains, two copies of a conserved substrate binding domain were identified; the first extends from Thr⁶³² to Asp⁷³⁶ and the second from Gln⁷⁴⁴ to Asn⁸⁵⁷, and both are rich in aromatic amino acids (34). The identification of amylase and pullulanase domains, together with the presence of two apparently independently operating active sites, would indicate that *ApuB* is a so-called type II bifunctional amylopullulanase (21) that functions in cell wall-associated/extracellular metabolism of starch and related polysaccharides by *B. breve* UCC2003.

***ApuB* represents a bifidobacterial amylopullulanase with dual specificity.** In order to verify that *apuB* encodes a type II bifunctional amylopullulanase, the predicted α -amylase- and

pullulanase-encoding domains of *apuB* were individually amplified by PCR and cloned in the nisin-inducible expression vector pNZ8048 to generate pNZ- α amy and pNZ-pull, respectively (See Materials and Methods). The prospective α -amylase- and pullulanase-encoding gene products were each over-expressed and purified from the soluble cell extract fraction of *L. lactis* NZ9000 harboring the recombinant plasmid pNZ- α amy or pNZ-pull by using metal chelate affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of Amy-His_{*ApuB*} and Pull-His_{*ApuB*} revealed, for each protein, a single band at an apparent molecular mass of approximately 70 kDa and 110 kDa, respectively, which is in agreement with the expected size calculated from the recombinant *apuB* gene sequence (results not shown). The end products formed by the hydrolysis of starch, amylopectin, glycogen, or pullulan following incubation with cells or cell-free supernatant of *B. breve* UCC2003 (representing the positive controls) or purified α -amylase (Amy-His_{*ApuB*}) or pullulanase (Pull-His_{*ApuB*}) were analyzed by HPTLC (Fig. 2). The results show that purified Amy-His_{*ApuB*} can liberate maltooligosaccharides, predominantly maltotriose and maltose, from carbo-

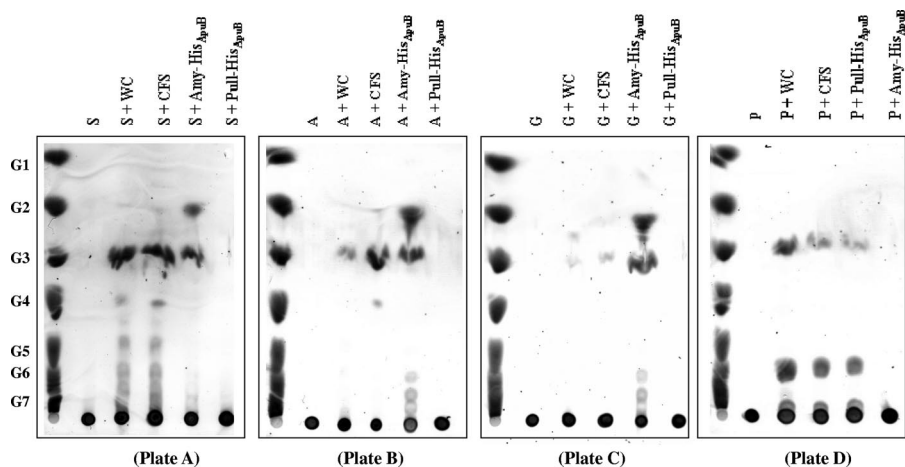


FIG. 2. HPTLC analysis of the reaction products generated by washed cells (WC) of *B. breve* UCC2003, cell-free supernatant (CFS) of *B. breve* UCC2003, or the purified α -amylase (Amy-His_{ApuB}) or pullulanase (Pull-His_{ApuB}) of ApuB following incubation with starch (S) (plate A), amylopectin (A) (plate B), glycogen (G) (plate C) or pullulan (P) (plate D). The standards glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7) are included in each HPTLC.

hydrates such as starch, amylopectin, and glycogen (Fig. 2, plates A, B, and C). Pull-His_{ApuB} liberates predominantly maltotriose and polymers of maltotriose from pullulan (Fig. 2, plate D). Pull-His_{ApuB} did not hydrolyze starch, amylopectin, or glycogen to short-chain maltooligosaccharides. In addition, no disaccharide or monosaccharide hydrolysis products, indicative of α -1,4-glucosidic activity, were detected after the incubation of Pull-His_{ApuB} with pullulan. Similar end products were produced with all substrates when a cell-free supernatant or cells of *B. breve* UCC2003 were used, thereby indicating that some of the protein is cell bound while some is released from the cell into the culture medium. Long-chain maltooligosaccharides may have been produced by the hydrolysis of the α -1,6-glucosidic bonds in amylopectin, glycogen, and pullulan by Pull-His_{ApuB}; however, the HPTLC system employed in this study does not allow the visualization of such long oligosaccharides. Collectively, these results demonstrate that Amy-His_{ApuB} is an α -amylase exhibiting α -1,4-glucosidase activity while Pull-His_{ApuB} is a true pullulanase, only hydrolyzing the α -1,6-glucosidic linkages in pullulan. Furthermore, these data illustrate that the extracellular amylopullulanase, encoded by *apuB* of *B. breve* UCC2003, is capable of hydrolyzing α -1,4 and α -1,6 linkages at different active sites and can therefore be classified as a so-called type II bifunctional amylopullulanase (15, 21).

Disruption of the *apuB* gene in *B. breve* UCC2003. In order to determine whether ApuB is essential for the metabolism of starch and related polysaccharides by *B. breve* UCC2003, we tested the functionality of the plasmid integration strategy described by Law et al. (30) for the creation of an *apuB* gene knockout. The *apuB* disruption through plasmid integration in *B. breve* UCC2003 was achieved by using a strategy essentially as described for *L. lactis* (30), although with some modifications. pTGB019, a 6.5-kb derivative of the lactococcal temperature-sensitive plasmid pVE6007, was used as it exhibited a higher level of segregational instability compared to the smaller pVE6007 plasmid (M. O'Connell Motherway, unpublished data). The loss of pTGB019 from *B. breve* upon growth at elevated temperature is attributed

to the combination of the temperature sensitivity and segregational instability of this plasmid. *B. breve* UCC2003 harboring pTGB019 was transformed with pORI19-*apuB* (see Materials and Methods) and plated on RCA supplemented with Em and Cm followed by incubation at 33°C under anaerobic conditions. Em^r Cm^r transformants carrying both pTGB019 and pORI19-*apuB* were cultured once overnight at 33°C in RCM broth. Subsequently, cells were passaged for 50 generations at 42°C, with selection for pORI19-*apuB* only, thus allowing integration into the chromosome upon the loss of pTGB019. Following this temperature-induced enrichment, serial dilutions of the culture were plated onto RCA with Em selection and incubated overnight at 42°C.

Screening for the loss of pTGB019 (Cm^s colonies) was performed by replica plating individual colonies onto RCA-Em and RCA-Cm with overnight incubation at 33°C. Potential *apuB* gene disruption isolates exhibited an Em^r Cm^s phenotype and were verified by colony PCR analysis using a forward primer upstream of the region of integration and a reverse primer based on pORI19 (Apu1 and pORI19r) (Table 2). An expected PCR product of 1.7 kb was obtained in some cases, indicating that integration had occurred (data not shown). In order to unequivocally prove that the disruption of *apuB* was the result of the integration of pORI19-*apuB*, Southern hybridization was performed using HindIII-digested genomic DNA and the 5.1-kb PCR fragment encompassing *apuB* as a probe. HindIII was selected for the genomic digests as there are no corresponding restriction sites within the *apuB* gene sequence (Fig. 3A). The *apuB* fragment probe hybridized to a 9-kb fragment of strain UCC2003 genomic DNA, while in the suspected UCC2003-*apuB* mutant strains, this band was absent and instead two hybridization signals of 5 kb and 7 kb were observed. For one of the obtained UCC2003-*apuB* insertion mutants (Fig. 3B, lane 3), the *apuB* probe also hybridized to a 3.1-kb HindIII fragment. This hybridization signal indicated that duplication of pORI19-*apuB* had occurred after integration of the plasmid into the bacterial chromosome in this mutant strain. However, this hybridiza-

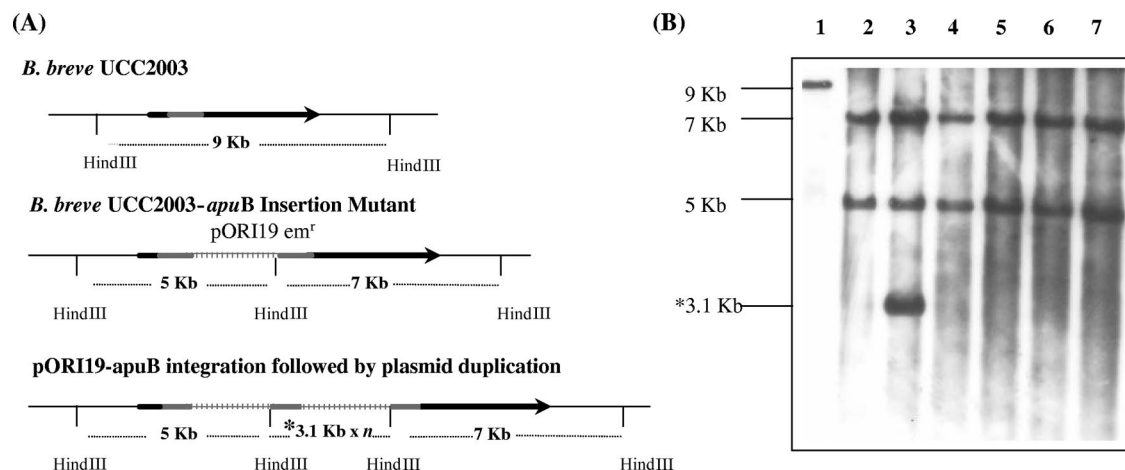


FIG. 3. (A) Schematic representation of the relevant regions of the *B. breve* UCC2003 and UCC2003-*apuB* chromosomes. Chromosomal DNA is represented by a thin line, the *apuB* gene is represented by a black arrow, the internal *apuB* fragment is indicated by a solid gray line, and pORI19 is indicated by a boxed line. HindIII sites relevant to the Southern hybridization analysis are indicated. (B) Southern hybridization analysis of HindIII chromosomal DNAs of *B. breve* UCC2003 (lane 1) and six representative *B. breve* UCC2003-*apuB* insertion mutants (lanes 2 to 7) obtained with pORI19-*apuB* integration. The sizes of the hybridizing fragments are indicated to the left of the panel. A PCR product of 5.1-kb encompassing *apuB* was used as a probe for the hybridization. “*3.1 Kb \times *n*” represents the duplication of pORI19-*apuB*, present in an unknown number of copies (*n*) on the chromosome.

tion profile does not identify the number of duplicated copies of the plasmid represented in the culture. The duplication of plasmid copies after the insertion of pORI-type plasmids has been reported previously for *L. lactis* and *Lactobacillus acidophilus* and has been attributed to recombinatory activity between flanking DNA regions of homology that result from Campbell-like integration (32, 44). The plasmid duplication is influenced by a number of factors that

include the antibiotic selection and the nature and location of the insertion event.

Analysis of the *B. breve* UCC2003-*apuB* insertion mutant phenotype. *B. breve* UCC2003 and *B. breve* UCC2003-*apuB* were analyzed for the ability to grow on starch, amylopectin, glycogen, or pullulan as the sole carbohydrate source (Fig. 4). *B. breve* UCC2003-*apuB* failed to grow on starch, amylopectin, glycogen, or pullulan in comparison to the growth of

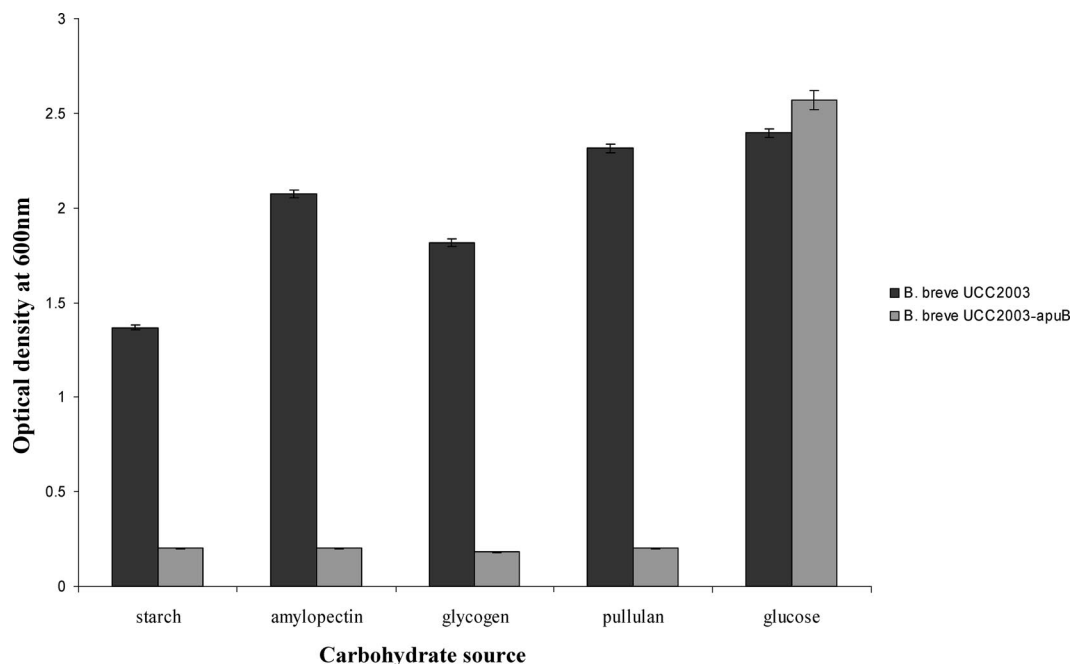


FIG. 4. Growth profile analysis of *B. breve* UCC2003 and *B. breve* UCC2003-*apuB* in modified Rogosa broth supplemented with starch, amylopectin, glycogen, pullulan, or glucose as indicated. Error bars show standard deviations.

the parent strain, while comparable growth of the parent and *apuB* mutant strains was observed when glucose was the sole carbohydrate source. These results indicate that the extracellular enzyme specified by *apuB* is the sole enzyme responsible for the breakdown of starch, amylopectin, glycogen, and pullulan by *B. breve* UCC2003.

DISCUSSION

A gene, *apuB*, encoding an extracellular amylopullulanase, was identified on the genome of *B. breve* UCC2003. ApuB was shown to contain four highly conserved regions in two separate domains of the enzyme, each of which is proposed to contain an active center and a substrate binding site, a characteristic shared by other amylopullulanases (15, 16, 21). The N-terminally located α -amylase and C-terminally located pullulanase domains were independently overexpressed and purified. The α -amylase enzyme domain was shown to cleave α -1,4-glucosidic linkages in starch, amylopectin, and glycogen to produce maltooligosaccharides, while the pullulanase enzyme domain was shown to hydrolyze α -1,6-glucosidic linkages in pullulan to produce maltotriose and polymers of maltotriose (Fig. 2). Based on the end products formed following the hydrolysis of starch, amylopectin, glycogen, and pullulan by the enzymatic activity of the purified ApuB domains, the product of *apuB* can be classified as an amylopullulanase (4, 21), the first to be identified in the genus *Bifidobacterium*. Recent investigations of bifunctional type II pullulanases have led to a division of these enzymes into two distinct groups: those that hydrolyze both α -1,4- and α -1,6-glucosidic bonds using a single active center and substrate binding site and those, such as ApuB, that perform the two catalytic activities at two different sites within the same protein (5, 25).

The alkaline amylopullulanase from *Bacillus* sp. KSM-1378 exhibits different activities based on thermal stability, the pH stability profile, and inhibition by metals, indicating that the dual catalytic activities of the enzyme involved different active sites (25). The amylopullulanases from *Thermoanaerobium* sp. strain Tok-B1 (39) and *Bacillus circulans* F-2 (25) have also been suggested to contain separate active sites for the individual activities on the basis of the results of competitive kinetics studies with mixed substrates, namely, amylose and pullulan. In contrast, other reports unequivocally showed that a single active site is responsible for the hydrolyzing activities of certain amylopullulanases (24, 36). For example, in the amylopullulanase of *Thermoanaerobacter ethanolicus* 39E, the modification of one of the two conserved Asp residues by using site-directed mutagenesis was shown to lead to the loss of both α -amylase and pullulanase activities (36).

To establish if ApuB was the sole enzyme responsible for the metabolism of starch and related polysaccharides by *B. breve* UCC2003, an *apuB* gene disruption strain was created. To the best of our knowledge, gene inactivation via homologous recombination has not been reported yet for the genus *Bifidobacterium*. The ability to create gene disruptions/knockouts in *Bifidobacterium* has been a fundamental obstacle in attaining a full understanding of the probiotic effect (54). Here we successfully exploited and adapted the well-established lactococ-

cal mutagenesis system as described by Law et al. (30). The *B. breve* UCC2003-*apuB* insertion mutants generated in this study were no longer capable of growth on starch, amylopectin, glycogen, or pullulan due to the disruption of *apuB*. This research thus illustrates that ApuB is essential in the metabolism of starch and starch-like polysaccharides by *B. breve* UCC2003.

Ryan et al. (46) demonstrated that all *B. breve* strains screened appeared to possess amylopullulanase activity. From these results, it can be suggested that this enzyme may be characteristic of *B. breve* strains, and if so, this activity must have some relevance to the organism's activity in the gut. It has been reported that after the first week of life of a newborn baby, a flora rich in *Bifidobacterium* spp. is established in the baby's gut (19, 52). The ApuB enzyme may play an important ecological role by allowing this *B. breve* strain to remain competitive in an environment where food sources change. During weaning, nonmilk foods are added to the diet and infants are exposed for the first time to different complex carbohydrates. A significant proportion of the carbohydrate, e.g., starch, will escape digestion and enter the colon because of the infant's lack of chewing ability and low levels of salivary amylase activity and the immaturity of the infant's intestine (8, 27). Such resistant starch sources therefore represent excellent carbohydrate sources for those bacteria that can produce amylolytic enzymes.

This extracellular bifunctional enzyme encoded by *apuB* may be one of the first crucial players in a carbohydrate metabolic pathway in *B. breve*, hydrolyzing extracellular starch or long-chain maltooligosaccharides to produce shorter (chain length ranging between 2 and 6) maltooligosaccharides. Analogous to other starch-degrading microorganisms, the short-chain maltooligosaccharides produced are expected to be taken up by the bifidobacterial cell where they are further degraded to glucose. It will be interesting to determine how *B. breve* UCC2003 performs the latter functions, and future research will focus on this aspect of starch metabolism.

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