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Screening for and Identification of Starch-, Amylopectin-, and Pullulan-Degrading Activities in Bifidobacterial Strains

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Forty-two bifidobacterial strains were screened for α-amylase and/or pullulanase activity by investigating their capacities to utilize starch, amylopectin, or pullulan. Of the 42 bifidobacterial strains tested, 19 were capable of degrading potato starch. Of these 19 strains, 11 were able to degrade starch and amylopectin, as well as pullulan. These 11 strains, which were shown to produce extracellular starch-degrading activities, included 5 strains of *Bifidobacterium breve*, 1 *B. dentium* strain, 1 *B. infantis* strain, 3 strains of *B. pseudolongum*, and 1 strain of *B. thermophilum*. Quantitative and qualitative enzyme activities were determined by measuring the concentrations of released reducing sugars and by high-performance thin-layer chromatography, respectively. These analyses confirmed both the inducible nature and the extracellular nature of the starch- and pullulan-degrading enzyme activities and showed that the five *B. breve* strains produced an activity that is consistent with type II pullulanase (amylopullulanase) activity, while the remaining six strains produced an activity with properties that resemble those of type III pullulan hydrolase.

Starch is ubiquitous and is an easily accessible source of energy. It is composed exclusively of α-glucopyranose units that are linked to each other by α-1,4- or α-1,6-glucosidic bonds. The two high-molecular-weight components of starch are α-amylose (representing a 15 to 25% weight fraction of starch), which is a linear polymer composed exclusively of α-1,4-linked glucopyranose residues, and amylopectin (representing a 75 to 85% weight fraction of starch), which is also an α-1,4-linked glucopyranose polymer but in addition contains α-1,6-glycosidic linkages representing branch points occurring at every 17 to 26 residues (45). α-Amylose chains, which are not soluble in water but form hydrated micelles, are polydisperse, and their molecular masses vary from hundreds to thousands of kilodaltons. The molecular mass of amylopectin may be as high as 100,000 kDa, and in solution such a polymer is present in colloidal or micellar forms (6, 16). Pullulan is a linear polymer of maltotriose units (with two internal α-1,4-glucosidic linkages) that are joined by α-1,6-glucosidic bonds. It is a polysaccharide synthesized by the fungus *Aureobasidium pullulans* when it is grown on glucose- or sucrose-containing media (15). The pullulan molecule is neutral, and its molecular mass ranges from 1,500 to 810,000 kDa (15). Because of the complex structures of starch, amylopectin, and pullulan, bacteria that use these substrates as carbon and energy sources require an appropriate combination of enzymes for depolymerization to oligo- and monosaccharides.

Several amylolytic enzymes, such as α-amylase (EC 3.2.1.1; glycosyl hydrolase family 13), β-amylase (EC 3.2.1.2; glycosyl hydrolase family 14), and glucoamylase (EC 3.2.1.3; glycosyl hydrolase family 15), with different specificities can contribute to starch degradation (3). These enzymes, all of which hydrolyze α-1,4-glucosidic bonds, are capable of amylose degradation, yielding glucose, maltose, maltotriose, and other oligosaccharides. However, in the absence of a "debranching" enzyme capable of hydrolyzing α-1,6-glucosidic bonds, amylopectin degradation is incomplete. The α-1,6 bonds in amylopectin and pullulan are hydrolyzed by so-called pullulanases (5), which are enzymes belonging to glycosyl hydrolase family 57 that are widely distributed in nature. Over the last decade a variety of pullulolytic enzymes with different substrate specificities have been characterized (15); these enzymes are produced by a wide variety of microorganisms, many of which are thermophilic and mesophilic bacteria (22, 24, 33).

It is known that starches are a major carbohydrate source in the human colon. Some starches escape complete digestion due to their size and molecular conformation during passage through the human small intestine; these compounds are referred to as resistant starches and arrive in the colon as fermentable carbohydrate sources for intestinal bacteria (47). Some examples of these resistant starches are the granular starches synthesized by a number of food plants (14). In animal models, inclusion of resistant starches in the diet has been shown to increase the population of bifidobacteria in the intestinal tract, although essentially nothing is known about the assumed enzyme complement responsible for this metabolic activity (9, 28).

The aim of this study was to investigate and characterize different amylolytic and pullulytic activities in different bifidobacteria by screening a number of different bifidobacterial species to determine their capacities to utilize starch, amylopectin, and pullulan as sole carbon and energy sources. Data concerning the identification of novel saccharolytic activities in bifidobacteria which are consistent with amylopullulanase (type II pullulanase) and type III pullulan hydrolase enzyme activities are presented below.

MATERIALS AND METHODS

Bacterial strains. Bifidobacterial strains (Table 1) were obtained from the Japan Collection of Microorganisms (JCM) (Wako, Japan), the National Col-

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TABLE 1. Bacterial fermentation of starch (potato), amylopectin (potato), and pullulan

Species	Strain	Fermentation of:		
		Starch (potato)	Amylopectin (potato)	Pullulan
B. adolescentis	CIP 64.60	+	−	−
B. adolescentis	CIP 64.61	+	−	−
B. adolescentis	NCFB 2229	−	−	−
B. animalis	DSM 10140	−	−	−
B. animalis	DSM 20104	−	−	−
B. animalis	JCM 20097	−	−	−
B. animalis	DSM 20105	−	−	−
B. animalis	JCM 7117	−	−	−
B. asteroides	DSM 20431	−	−	−
B. bifidum	JCM 7002	−	−	−
B. bifidum	JCM 7003	−	−	−
B. bifidum	NCIMB 8810	−	−	−
B. bifidum	CIP 64.65	−	−	−
B. bifidum	CCUG 17358	−	−	−
B. breve	JCM 7019[a]	+	+	+
B. breve	CCUG 43878[a]	+	+	+
B. breve	CCUG 34405[a]	+	+	+
B. breve	UCC 2003[a,b]	+	+	+
B. breve	NCFB 2258[a]	+	+	+
B. dentium	NCFB 2243[a]	+	+	+
B. globosum	JCM 5820	+	+	−
B. globosum	JCM 7092	+	+	−
B. indicum	DSM 20214	−	−	−
B. infantis	CCUG 45868[a]	+	+	+
B. infantis	CCUG 36569	−	−	−
B. infantis	NCDO 2205	−	−	−
B. longum	JCM 7050	+	+	−
B. longum	JCM 7052	−	−	−
B. longum	JCM 7053	−	−	−
B. longum	JCM 7055	−	−	−
B. longum	JCM 7056	−	−	−
B. longum	NCIMB 8809	+	+	−
B. longum	CIP 64.63	+	+	−
B. longum	CCUG 15137	−	−	−
B. longum	CCUG 30698	−	−	−
B. longum/*B. infantis*	CCUG 18157	+	−	−
B. magnum	DSM 20222	−	−	−
B. pseudocatulatum	NCIMB 8811	−	−	−
B. pseudolongum	NCIMB 2244[a]	+	+	+
B. pseudolongum	DSM 20095[a]	+	+	+
B. pseudolongum subsp. *globosum*	DSM 20092[a]	+	+	+
B. thermophilum	JCM 7027[a]	+	+	+

[a] Bifidobacterial strain which fermented all three carbon sources.
[b] *B. breve* UCC 2003 is also known as NCIMB 8807.

lection of Industrial and Marine Bacteria (NCIMB) (Aberdeen, Scotland), the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) (Braunschweig, Germany), the Collection de l'Institut Pasteur (CIP) (Paris, France), the Culture Collection of the University of Goteborg (CCUG) (Department of Clinical Bacteriology, Goteborg, Sweden), and the National Collection of Food Bacteria (NCFB) (Reading, United Kingdom).

Verification of *Bifidobacterium* species by PCR performed with 16S rRNA gene-targeted primers for *Bifidobacterium* spp. The 16S rRNA primers used for detection of *Bifidobacterium* species were primers g-Bifid-F (5′-CTCCTGGAA ACGGGTGG-3′) and g-Bifid-R (5′-GGTGTTCTTCCCGATATCTACA-3′) (32). PCR and the subsequent sequence analysis of the amplicons obtained were performed as described below.

DNA manipulations. Small-scale bifidobacterial total DNA was prepared as described previously (39). Purified DNA was obtained by cesium chloride ultracentrifugation (44). PCRs were performed using the *Taq* polymerase template PCR system (Promega, Southampton, United Kingdom) in accordance with the manufacturer's instructions and a Primus thermal cycler (MWG-Biotech AG, Ebersberg, Germany). Sequencing was performed by MWG-Biotech AG (Ebersberg, Germany).

Growth substrates. Starch (potato), amylopectin (potato), and pullulan were all purchased from Sigma (Dorset, United Kingdom).

Growth conditions. The bacterial strains listed in Table 1 were individually subcultured from stocks stored at −20°C in 20 ml of basal medium (BM) supplemented with 1% glucose. BM contained (per liter) 10 g Trypticase peptone, 2.5 g yeast extract, 3 g tryptose, 3 g K_2HPO_4, 3 g KH_2PO_4, 2 g triammonium citrate, 0.3 g pyruvic acid, 1 ml Tween 80, 0.574 g $MgSO_4 \cdot 7H_2O$, 0.12 g $MnSO_4 \cdot 7H_2O$, and 0.034 g $FeSO_4 \cdot 7H_2O$. After autoclaving BM was supplemented with 0.05% (wt/vol) filter-sterilized cysteine-HCl, and strains were grown at 37°C under anaerobic conditions maintained using an Anaerocult oxygen-depleting system (Merck, Darmstadt, Germany) in an anaerobic chamber. Aliquots (1%) from overnight cultures were inoculated into 20 ml of BM supplemented with 1% starch, amylopectin, or pullulan. BM without an added carbon source and BM with 1% glucose were used as negative and positive controls, respectively. Cultures were incubated anaerobically at 37°C for 48 h, and the optical density at 600 nm was determined at 0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h following dilution into fresh BM.

Starch hydrolysis on agar plates. After 24 h of incubation in BM broth with various substrates as described above, bacterial cells were separated from the growth medium by centrifugation at 960 × *g* for 15 min at 4°C. Cell-free supernatant was then obtained by filtration (pore diameter, 0.45 μm; Minisart; Sartorius, Surrey, United Kingdom) to remove any remaining cells and kept on ice, while bacterial cell pellets were gently washed twice with water and resuspended in 0.05 M potassium phosphate buffer, pH 6.0. Cells were broken by subjecting a cell suspension to physical disruption using acid-washed beads (425 to 600 μm; Sigma) and a bead beater for 3 min with 1-min intervals on ice. Samples were centrifuged at 1,920 × *g* for 10 min. Each resulting crude cell extract was transferred into a fresh tube and stored on ice. Ten-microliter portions of the cell-free supernatant or crude cell extract of each bacterial strain grown in a specific medium were transferred into individual wells in a petri dish containing 30 ml of BM agar supplemented with 1% starch, 1% amylopectin, or 1% pullulan. The plates were incubated in an anaerobic chamber for 2 days at 37°C, after which they were flooded with iodine to visualize clearing zones around the wells, which was indicative of substrate-degrading enzyme activity. A control consisting of uninoculated medium was also transferred into a well of each plate.

Determination of the activity of starch-degrading enzymes. Bifidobacterial strains were grown on BM supplemented with 1% starch, amylopectin, or pullulan. After incubation for 48 h at 37°C in anaerobic conditions, the cultures were inoculated into 10 ml of BM broth supplemented with the relevant substrate (1%) and grown to an optical density at 600 nm of 0.6. Cell-free supernatants and crude cell extracts were obtained as described above. α-Amylase or pullulanase activity was determined using an adaptation of the dinitrosalicylic acid assay (5), which measures the amount of released reducing sugars. One milliliter of crude cell extract or cell-free supernatant was added to 1 ml of 0.2% substrate in 0.05 M acetate buffer (pH 6.0). The mixture was then incubated at 37°C for 60 min. α-Amylase or pullulanase activity was expressed as μmol reducing sugar released h^{-1} mg protein $^{-1}$. Protein concentrations were determined using the Bradford method (10).

DNA sequencing and bioinformatics analysis. The sequence of a predicted bifidobacterial amylopullulanase was obtained from the preliminary genome sequence of *Bifidobacterium breve* UCC 2003 (S. Leahy, M. O'Connell-Motherway, J. A. Moreno-Munoz, G. F. Fitzgerald, D. Higgins, and D. van Sinderen, unpublished data). Sequence data assembly and analysis were performed with DNAStar software (DNAStar, Madison, Wis.). Database searches were performed with nonredundant sequences at the NCBI website (http://www.ncbi.nlm.nih.gov) using the available BLAST tools (1, 2). Sequence alignment was performed by using the Clustal method of the Megalign program of the DNASTAR software package, and conserved domains I and IV in α-amylase and pullulanases were identified on the basis of the results (Fig. 1 and 2, respectively) (4, 5, 12, 15). The following primer combinations designed from these conserved regions for the α-amylase and pullulanase regions were used in PCRs: for amplification of part of the α-amylase-encoding genes, Amy-for (5′-GCCGAC TCGGTCATCAACCACACCACC-3′) and Amy-rev (5′-ATCCCAGTTGGTC ACCCACACTGCGGC-3′); and for amplification of part of the pullulanase-encoding genes, Pul-for (5′-GATGGATGTGGTCTACAACCACGTCTAC-3′) and Pul-rev (5′-TTGTCGTGGATCTCGACGTACTGCACC-3′). Chromosomal DNAs isolated from *B. bifidum* JCM 7003 and *B. longum* JCM 7055 (strains which cannot grow on starch [Table 1]) were used as (negative) controls in these PCRs.

HPTLC analysis. For qualitative determination of saccharolytic breakdown products produced by the different bifidobacterial strains, cell-free supernatants

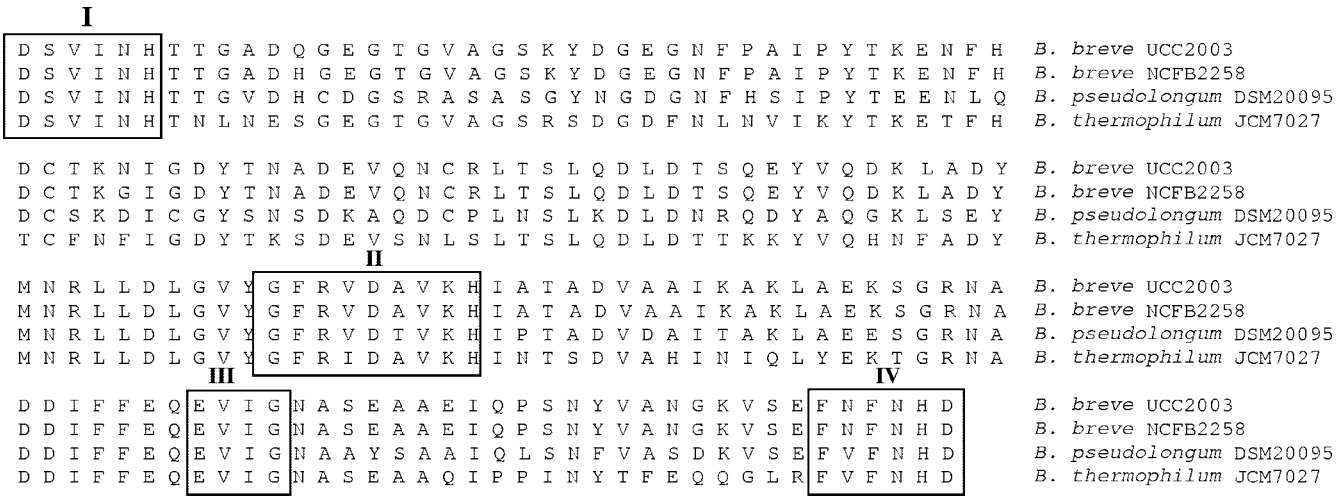

FIG. 1. Identification of the four conserved regions in the predicted amylase section derived from the various bifidobacterial strains used in this study. The four regions indicated by roman numerals and boxes are sequence motifs that were identified.

were incubated with 1% starch, amylopectin, or pullulan in 50 mM phosphate buffer (pH 6.0) at 37°C for 60 h. The samples were analyzed by high-performance thin-layer chromatography (HPTLC) as follows. An aliquot (0.5 μl) of the reaction products of a sample was spotted onto a Silica Gel 60 plate (10 by 10 cm; Merck) using a Nanomat 4 device (Camag, Switzerland). The chromatogram was developed with a butanol-acetic acid-water (5:5:3, vol/vol/vol) solvent system in a horizontal developing chamber. Ascending development was repeated twice at room temperature. The plate was allowed to air dry in a hood and then developed by spraying it evenly with 20% (vol/vol) sulfuric acid in ethanol. The plate was dried and heated at 120°C for 10 min to visualize the sugar-containing spots. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were purchased from Sigma, and a mixture of these sugars was used as molecular weight standards. Further analysis of the specificity of the observed enzymatic activity with the substrate pullulan was performed by adding α-glucosidase from *Saccharomyces cerevisiae* (Sigma) to each predigested reaction mixture, followed by incubation at pH 6.8 at 37°C for 1 h and subsequent analysis by HPTLC as described above.

Nucleotide sequence accession numbers. The sequence of the *apuB* gene from *B. breve* UCC 2003 and the sequence segments from the other bifidobacterial strains have been deposited in the GenBank database under the following accession numbers: *B. breve* UCC 2003 *apuB*, DQ022105; *B. breve* NCFB 2258 *amy*, DQ341116; *B. breve* NCFB 2258 *pul*, DQ341119; *B. pseudolongum* DSM 20095 *amy*, DQ341117; *B. pseudolongum* DSM 20095 *pul*, DQ341120; *B. thermophilum* JCM 7027 *amy*, DQ341118; and *B. thermophilum* JCM 7027 *pul*, DQ341121.

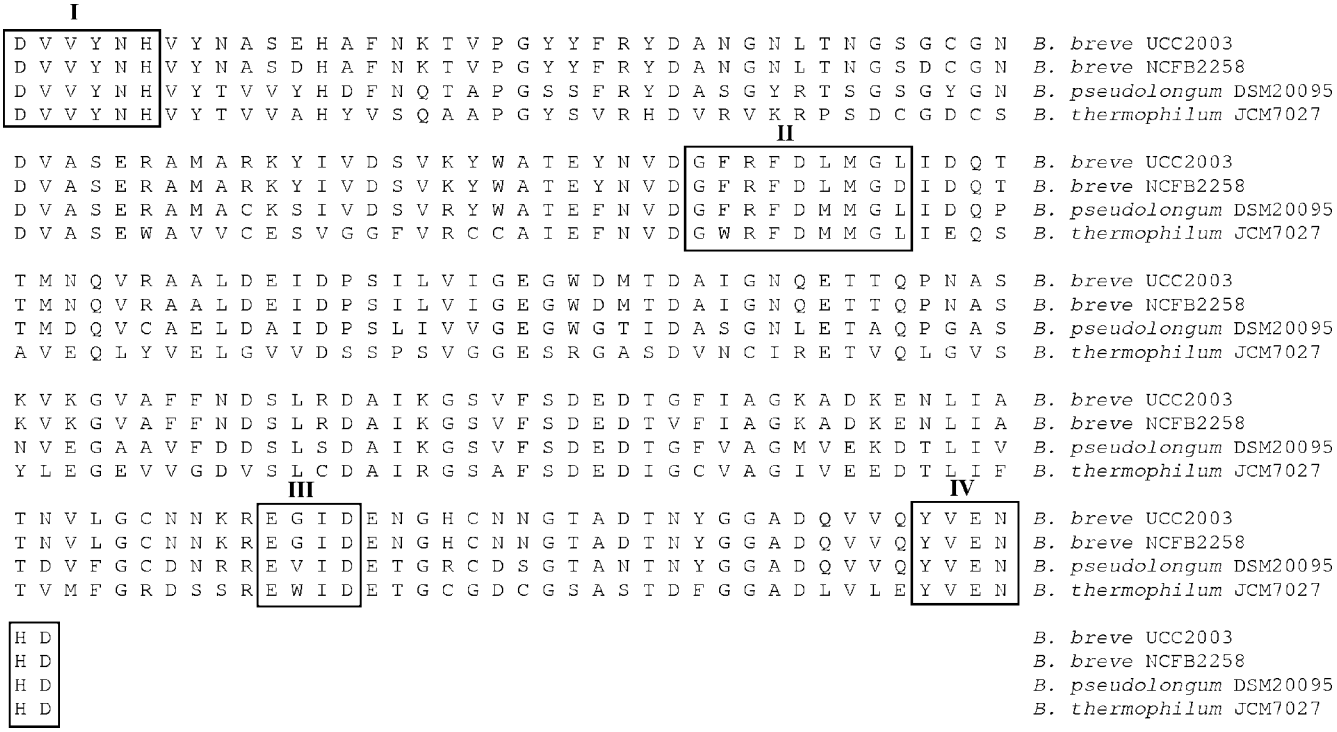

FIG. 2. Identification of the four conserved regions in the predicted pullulanase section derived from the various bifidobacterial strains used in this study. The four regions indicated by roman numerals and boxes are sequence motifs that were identified.

RESULTS

Screening of bifidobacterial strains for growth on various starch-type substrates. Each of the 46 bifidobacterial strains was shown to be capable of growth in the basal medium containing 1% glucose, whereas growth was not observed in basal medium without a hexose-based carbon source, demonstrating that this medium was carbon and energy limiting for these strains. Of the 42 bifidobacterial strains tested, 19 were capable of growth on potato starch (Table 1); 11 of these 19 strains were able to grow on both amylopectin (isolated from potato) and pullulan (Table 1). These 11 strains included all 5 strains of *B. breve* tested, 1 *B. dentium* strain, 1 *B. infantis* strain, 3 strains of *B. pseudolongum*, and 1 strain of *B. thermophilum*. The species identities of these 11 strains were confirmed by determining the sequence of a relevant section of the 16S rRNA-encoding DNA (data not shown). Based on the results described above, these 11 strains were selected for further investigation.

Pullulan and amylopectin hydrolysis on agar plates. The 11 bifidobacterial strains selected were examined for amylolytic activity produced intracellularly and/or extracellularly using a plate assay, as described in Materials and Methods. Distinct clearing zones indicative of substrate hydrolysis were observed for all 11 strains tested but only with samples that represented the extracellular (cell-free supernatant) fraction of a given strain. This implies that the amylopectin- and pullulan-degrading activities produced by these 11 bifidobacterial strains are secreted into the growth medium.

Activities of amylolytic and pullulytic enzymes. The specific activities of the amylolytic and pullulytic enzymes identified with individual substrates were quantified for the various bacterial strains grown for 48 h in the basal medium containing glucose (negative control) (data not shown), starch, amylopectin, and pullulan (Table 2). The enzyme activity was measured by determining the amount of released reducing sugars following incubation of crude cell extract or cell-free supernatant of a strain with a substrate. As expected, no appreciable amylolytic or pullulytic activity was detected in cell-free supernatants from strains that had been grown in glucose or in any of the crude cell extracts irrespective of the growth conditions. In contrast, such activities were detected in cell-free supernatants obtained from the 11 starch-degrading bifidobacterial cultures that had been grown in starch, amylopectin, or pullulan. These results are in agreement with the results obtained from the plate assays in which substrate hydrolysis was examined, and they are also fully compatible with the growth capabilities of these strains on starch and related substrates. We therefore concluded that the amylolytic and pullulytic activities of the 11 strains are extracellular and that the production of these activities is regulated by and is dependent on the presence of starch, amylopectin, or pullulan in the growth medium of the bifidobacterial cultures used.

Identification of hydrolysis products. In order to investigate possible differences in the enzymatic activities produced by the 11 bifidobacterial strains, the degradation products of starch, amylopectin, or pullulan were analyzed by HPTLC following incubation with the cell-free supernatant of a bifidobacterial strain (Fig. 3).

The cell-free supernatants obtained from all 11 strains were capable of hydrolyzing starch (Fig. 3A) and amylopectin (Fig. 3B), although considerable variation was observed between the various strains in terms of the degradation products of the hydrolysis reactions. For example, *B. pseudolongum* DSM 20095 appeared to completely degrade amylopectin and starch into glucose, whereas *B. pseudolongum* NCIMB 2244, *B. pseudolongum* subsp. *globosum* DSM 20092, and *B. thermophilum* JCM 7027 produced maltose and glucose from these substrates. Nevertheless, it is clear that all of the strains are capable of producing extracellular activities that can hydrolyze both α-1,4- and α-1,6-glucosidic linkages. The amylolytic activities present in the cell-free supernatants of the five *B. breve* strains were capable of hydrolyzing starch and amylopectin into a number of oligosaccharides, and the majority of the products were in the maltotriose to maltohexaose range. *B. breve* JCM 7019 and *B. breve* NCFB 2258 also produced maltose as the smallest hydrolyzed product.

The *B. breve* strains hydrolyzed pullulan and generated mainly a trisaccharide, either maltotriose or panose, while strains CCUG 43878 and CCUG 34405 also produced maltohexaose as a minor side product, which could have been the result of incomplete digestion (Fig. 3C). Maltotriose possesses two α-1,4-glucosidic linkages, while panose possesses one α-1,4-glucosidic linkage and one α-1,6-glucosidic linkage. If maltotriose is incubated with α-glucosidase (which hydrolyzes only α-1,4 linkages), maltotriose is degraded to glucose, whereas if panose is incubated with α-glucosidase, two products are formed, isomaltose (which is composed of two glucose molecules joined by an α-1,6-glucosidic linkage) and glucose. During secondary incubation of the primary degradation products obtained from pullulan (catalyzed by the extracellular activity produced by any of the five *B. breve* strains) with a commercial α-glucosidase, only glucose was formed, which demonstrated that the pullulytic enzymes produced by the *B. breve* strains degraded this polymer into maltotriose (Fig. 4A). These results indicate that the enzyme (or enzymes) produced by the *B. breve* strains act on pullulan as a true pullulanase by means of α-amylase activity. If this activity is represented by a single enzyme, it can be classified as an amylopullulanase (type

TABLE 2. Activities of starch-degrading enzymes in culture supernatant extracts from 11 bacterial strains grown in basal media containing various carbon sources

Species	Strain	Activities (μmol reduced sugar produced h^{-1} mg of $protein^{-1}$) with the following carbon sources[a]:		
		Starch (potato)	Amylopectin (potato)	Pullulan
B. breve	UCC 2003	1.22 ± 0.5	1.87 ± 0.2	2.04 ± 0.4
B. breve	NCFB 2258	1.10 ± 0.2	1.76 ± 0.6	2.13 ± 0.4
B. breve	CCUG 34405	1.35 ± 0.2	1.47 ± 0.1	1.84 ± 0.2
B. breve	CCUG 43878	1.22 ± 0.3	1.76 ± 0.4	1.95 ± 0.1
B. breve	JCM 7019	0.95 ± 0.5	1.15 ± 0.2	1.42 ± 0.3
B. infantis	CCUG 45868	1.13 ± 0.3	1.36 ± 0.3	1.78 ± 0.6
B. dentium	NCFB 2243	0.99 ± 0.3	1.48 ± 0.6	1.73 ± 0.1
B. pseudolongum	NCIMB 2244	1.15 ± 0.4	1.75 ± 0.6	0.88 ± 0.7
B. pseudolongum	DSM 20095	0.96 ± 0.4	1.59 ± 0.3	2.35 ± 0.2
B. pseudolongum subsp. *globosum*	DSM 20092	0.84 ± 0.1	1.38 ± 0.2	2.15 ± 0.3
B. thermophilum	JCM 7027	0.95 ± 0.5	1.41 ± 0.2	2.43 ± 0.3

[a] The data are means ± standard deviations for supernatants containing the different forms of starch.

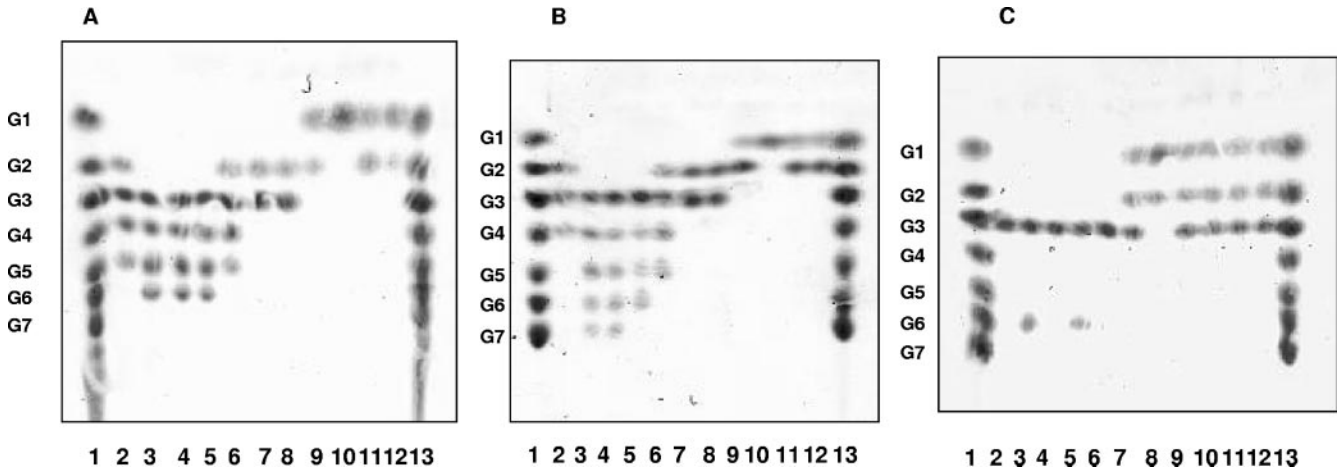

FIG. 3. HPTLC identification of the hydrolysis products obtained from starch (A), amylopectin (B), and pullulan (C) following incubation with the cell-free supernatants of 11 bifidobacterial strains. Lanes 1 and 13 contained standards, including glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentose (G5), maltohexaose (G6), and maltoheptaose (G7). Lane 2, *B. breve* JCM 7019; lane 3, *B. breve* CCUG 43878; lane 4, *B. breve* UCC 2003; lane 5, *B. breve* CCUG 34405; lane 6, *B. breve* NCFB 2258; lane 7, *B. dentium* NCFB 2243; lane 8, *B. infantis* CCUG 45868; lane 9, *B. pseudolongum* NCIMB 2244; lane 10, *B. pseudolongum* DSM 20095; lane 11, *B. pseudolongum* subsp. *globosum* DSM 20092; lane 12, *B. thermophilum* JCM 7027.

II pullulanase), which attacks α-1,6-glucosidic linkages in pullulan and amylopectin but also hydrolyzes α-1,4-glucosidic linkages in polysaccharides other than pullulan.

Using pullulan as a substrate for the activities in the cell-free supernatants of the remaining six *Bifidobacterium* strains resulted in the formation of different products, including maltotriose or panose or a mixture of the two, isomaltose, and glucose (Fig. 3C), indicating that these strains have different pullulytic specificities than the *B. breve* strains. In the case of *B. infantis* CCUG 45868, only two reaction products, isomaltose and glucose, were observed. Secondary incubation with α-glucosidase from *S. cerevisiae* resulted in two products, confirming the results described above (Fig. 4B). Overall, these six strains also produced extracellular activities that could hydrolyze both α-1,4- and α-1,6-glucosidic linkages. If such activities were present in a single enzyme, it would correspond to type III pullulan hydrolase. The latter enzyme is known to hydrolyze α-1,4- as well as α-1,6-glucosidic linkages in pullulan, forming maltotriose, panose, and maltose, and is also able to degrade starch and amylopectin (7, 15, 38).

Detection of putative α-amylase and pullulanase genes using PCR. Preliminary sequence analysis of the genome of

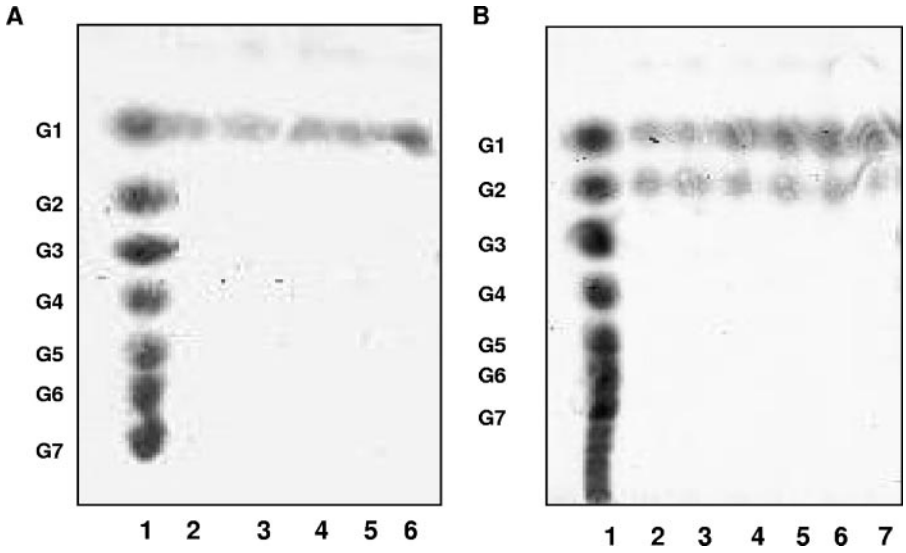

FIG. 4. HPTLC identification of the hydrolysis products obtained from pullulan as a result of incubation with the cell-free supernatants of 11 bifidobacterial strains (see Fig. 3), followed by a secondary incubation with α-glucosidase from *S. cerevisiae*. Lane 1 contained standards, including glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentose (G5), maltohexaose (G6), and maltoheptaose (G7). (A) Lane 2, *B. breve* JCM 7019; lane 3, *B. breve* CCUG 43878; lane 4, *B. breve* UCC 2003; lane 5, *B. breve* CCUG 34405; lane 6, *B. breve* NCFB 2258. (B) Lane 2, *B. infantis* CCUG 45868; lane 3, *B. dentium* NCFB 2243; lane 4, *B. pseudolongum* NCIMB 2244; lane 5, *B. pseudolongum* DSM 20095; lane 6, *B. pseudolongum* subsp. *globosum* DSM 20092; lane 7, *B. thermophilum* JCM 7027.

B. breve UCC 2003 indicated that this organism produces a bifunctional amylopullulanase, a finding which has been confirmed by functional analysis (S. M. Ryan, M. O'Connell-Motherway, G. F. Fitzgerald, and D. van Sinderen, unpublished results). On the basis of a comparative analysis of the *B. breve* amylopullulanase gene and homologous genes from other organisms, two different primer sets were designed on the basis of two of the four conserved regions (region I and region IV) found in both amylase and pullulanase enzymes (34). Amplification products were obtained using chromosomal DNA from each of the 11 bifidobacterial strains. Amplification products from four strains were sequenced, and each of the conserved domains present in amylolytic enzymes (37) could be identified in the individual PCR products sequenced. The deduced amino acid alignment revealed a high level of amino acid conservation in the four conserved regions of the four strains (Fig. 1 and 2). Comparative analysis showed that the PCR products obtained using a primer combination that was based on the α-amylase conserved domain exhibited significant similarity to the relevant section of various α-amylases. The highest levels of similarity were the levels of similarity to a secreted α-amylase from *Streptomyces coelicolor* A3 (2) (level of identity, 41%; accession no. CAB88153.1), α-amylase from *Bacillus* sp. (level of identity, 43%; accession no. BAA22082.1), and α-amylase from *Streptomyces avermitilis* MA-4680 (level of identity, 43%; accession no. BAC73693.1). The PCR products that were obtained using a primer combination based on two conserved domains of known pullulanases all contained a contiguous open reading frame which predicted a protein with significant similarity to corresponding regions of known pullulanases. The highest levels of similarity were the levels of similarity to a thermostable pullulanase from *Lactobacillus acidophilus* NCFM (level of identity, 47%; accession no. YP_194553.1) and pullulanases from *Bacillus cereus* strains ATCC 14579 and ATCC 10987 (level of identity, 44%; accession no. NP_832487.1 and NP_979065.1). No amplification products were obtained following a PCR using chromosomal DNA isolated from *B. bifidum* JCM 7003 or *B. longum* JCM 7055, neither of which exhibit starch-degrading activity.

DISCUSSION

In this study a number of bifidobacterial strains were shown to produce enzymes capable of degrading starch, amylopectin, and pullulan. The utilization of different forms of starch by bifidobacteria is consistent with the results of other studies which have demonstrated that some forms of resistant starch in the diet have the capacity to sustain good growth of indigenous bifidobacteria in the colon of rats (46) or pigs (11).

There are also indications from animal models that resistant starch reduces serum cholesterol and has significant beneficial implications for people with certain forms of diabetes (13, 20, 21). However, the consequences of ingestion of resistant starch in humans and its effect on the colonic microbiota are largely unexplored. Also, little is known about the ability of resistant starch to act as a bifidogenic factor, despite the possible advantage that it is broken down slowly, giving this substrate an increased likelihood of being available as a carbon source to the colonic microflora, unlike shorter-chain sugars, which may be broken down more rapidly.

A range of human intestinal bacteria can ferment soluble starch; the most numerically dominant of these commensal bacteria are members of the genera *Bacteroides*, *Fusobacterium*, *Butyrivibrio*, and *Bifidobacterium* (47). The genus *Bifidobacterium* was shown to be the principal amylose-degrading group, and starch-degrading activities were detected in the cell-bound fraction. This fraction produced starch-utilizing bands with different molecular weights, apparently representing a number of enzymes (47). In contrast, our findings indicate that under the conditions which we used, the starch-degrading activities of 11 *Bifidobacterium* species are extracellular. The reason for this difference in enzymatic location is not clear but may be related to the different growth conditions.

Although there is experimental evidence that bifidobacteria can utilize starch, there has been only one report that described purification of an extracellular α-amylase (27). Several genera of amylolytic bacteria, one of which was *Bifidobacterium*, were isolated from human feces by Macfarlane and Englyst (30). These workers used soluble starch as the sole carbon source in selective agar plates. Other reports have described purification of α-galactosidases and β-galactosidases, none of which were able to degrade starch, although they were shown to digest the breakdown products of starch, such as maltose and isomaltose (26, 42, 43).

If certain carbohydrates, such as starch, amylopectin, and pullulan, are metabolized by particular probiotic bifidobacteria, then diets containing prebiotic substrates may specifically select for such beneficial bacteria. Investigation and identification of the enzymes involved in the utilization of these sugars are therefore of great interest, especially when prebiotic compounds for bifidobacteria are defined. Bifidobacteria rapidly colonize the intestinal tract of newly born infants and become the predominant organisms in the colon. Studies have shown that the number of bifidobacteria gradually decreases with age and that the composition of bifidobacterial species also changes (23, 35). *B. infantis* and *B. breve* are commonly found in infant feces, while *B. adolescentis* and the *B. catenulatum* group appear to be most common in adults (18). The factors involved which change during weaning of an infant include the addition of nonmilk foods to the diet and the continued development of intestinal function. As weaning begins, infants are exposed for the first time to different complex, mostly plant-derived carbohydrates, such as starch, that are not present in milk. A significant proportion of ingested starch escapes digestion and enters the colon (17, 40) because of the lack of chewing ability and because the intestine of the neonate is immature and the infant has low levels of salivary and pancreatic amylase, which reach adult levels between 6 months and 1 year (36, 41). The ability to degrade starch could therefore explain why *B. breve* is frequently encountered in the gut flora of infants. To our knowledge, detection of pullulan-degrading enzymes in bifidobacteria has not been reported previously, and pullulan may therefore represent a novel prebiotic.

Bifidobacterium strains have been investigated for possible links between adhesion to starch granules and substrate utilization (14). For *B. adolescentis* VTT E-001561 and *B. pseudolongum* ATCC 25526, adhesion seemed to be specific for α-1,4-linked glucose sugars. However, not all bifidobacteria adhere to granular starch, and adhesion does not appear to be a

requirement for starch utilization by all strains in this genus (14). It is possible that physical association with starch may provide adherent bifidobacteria with a competitive advantage for utilization of resistant starch as a carbon and energy source in the human colon and that this could be exploited in the development of synbiotics which include both resistant starch and *Bifidobacterium*.

Pullulan can be hydrolyzed by five types of enzymes: (i) type I pullulanase, (ii) type II pullulanase, (iii) type I pullulan hydrolase (neopullulanase), (iv) type II pullulan hydrolase (isopullulanase), and (v) type III pullulan hydrolase, all of which can be identified by their end products (15). A large number of pullulanases have been isolated, particularly from thermophilic microorganisms (8, 15). Some of the type II pullulanases studied at a genetic level are the enzymes from *Thermoanaerobacter ethanolicus* 39E (31), *Thermoanaerobacterium thermosaccharoliticum* (19), *Desulfurococcus mucosus* (16), *Bacillus* sp. strain KSM-1378 (5), *Bacillus* sp. strain TS-23 (29), *Bacillus circulans* F-2 (25), *Bacillus* sp. strain DSM 405 (12), and *Clostridium thermohydrosulfuricum* (34). All of these pullulanases are secreted enzymes, which is in agreement with our findings.

An unusual pullulanase has been found recently in the anaerobic hyperthermophilic archaeon *Thermococcus aggregans* (38). This extremely thermolabile archaeal enzyme is unique since it is the only enzyme presently known that attacks α-1,4- as well as α-1,6-glucosidic linkages in pullulan. This enzyme is also able to degrade starch, amylase, and amylopectin, forming maltotriose and maltose as the main products (38). On the basis of this peculiar substrate specificity, it has been classified as a type III pullulan hydrolase. Similar pullulan degradation end products were detected for *B. infantis* CCUG 45868, *B. dentium* NCFB 2243, *B. pseudolongum* NCIMB 2244, *B. pseudolongum* DSM 20095, *B. pseudolongum* subsp. *globosum* DSM 20092, and *B. thermophilum* JCM 7027. This may suggest that these commensal bacteria produce this new type of pullulan-degrading activity, although at this point the possibility that the end products are the result of a combination of different pullulytic and amylolytic enzymes cannot be ruled out. In fact, sequences from the starch-degrading bifidobacteria *B. pseudolongum* DSM 20095 and *B. thermophilum* JCM 7027 are predicted to encode specific and separate pullulytic and amylolytic activities. This indicates that these organisms are different from *T. aggregans*.

In conclusion, we found that starch, amylopectin, and pullulan can be utilized by some, but not all, bifidobacteria and that these organisms produce a number of different enzymatic activities not previously identified in bifidobacteria. To increase our understanding of bifidobacterial starch metabolism, future work will involve characterization of the amylopullulanse gene and its locus in *B. breve* UCC 2003.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Antranikian, G.** 1990. Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. FEMS Microbiol. Lett. **75:**201–218.
4. **Ara, K., K. Igarashi, H. Hagihara, K. Sawada, T. Kobayashi, and S. Ito.** 1996. Separation of functional domains for the α-1,4 and α-1,6 hydrolytic activities of a *Bacillus* amylopullulanase by limited proteolysis with papain. Biosci. Biotechnol. Biochem. **60:**634–639.
5. **Ara, K., K. Saeki, K. Igarashi, M. Takaiwa, T. Uemura, H. Hagihara, S. Kawai, and S. Ito.** 1995. Purification and characterization of an alkaline amylopullulanase with both α-1,4 and α-1,6 hydrolytic activity from alkalophilic *Bacillus* sp. KSM-1378. Biochim. Biophys. Acta **1243:**315–324.
6. **Bertoldo, C., and G. Antranikian.** 2001. Amylolytic enzymes from hyperthermophiles. Methods Enzymol. **330:**269–289.
7. **Bertoldo, C., and G. Antranikian.** 2002. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Curr. Opin. Chem. Biol. **6:**151–160.
8. **Bertoldo, C., F. Duffner, P. L. Jorgensen, and G. Antranikian.** 1999. Pullulanase type I from *Fervidobacterium pennavorans* Ven5: cloning, sequencing, and expression of the gene and biochemical characterization of the recombinant enzyme. Appl. Environ. Microbiol. **65:**2084–2091.
9. **Bouhnik, Y., L. Raskine, G. Simoneau, E. Vicaut, C. Neut, B. Flourie, F. Brouns, and F. Bornet.** 2004. The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. Am. J. Clin. Nutr. **80:**1658–1664.
10. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
11. **Brown, I., M. Warhurst, J. Arcot, M. Playne, R. J. Illman, and D. L. Topping.** 1997. Fecal numbers of bifidobacteria are higher in pigs fed *Bifidobacterium longum* with a high amylose cornstarch than with a low amylose cornstarch. J. Nutr. **127:**1822–1827.
12. **Brunswick, J. M., C. T. Kelly, and W. M. Fogarty.** 1999. The amylopullulanase of *Bacillus* sp. DSM 405. Appl. Microbiol. Biotechnol. **51:**170–175.
13. **Cheng, H. H., and M. H. Lai.** 2000. Fermentation of resistant rice starch produces propionate reducing serum and hepatic cholesterol in rats. J. Nutr. **130:**1991–1995.
14. **Crittenden, R., A. Laitila, P. Forssell, J. Matto, M. Saarela, T. Mattila-Sandholm, and P. Myllarinen.** 2001. Adhesion of bifidobacteria to granular starch and its implications in probiotic technologies. Appl. Environ. Microbiol. **67:**3469–3475.
15. **Doman-Pytka, M., and J. Bardowski.** 2004. Pullulan degrading enzymes of bacterial origin. Crit. Rev. Microbiol. **30:**107–121.
16. **Duffner, F., C. Bertoldo, J. T. Andersen, K. Wagner, and G. Antranikian.** 2000. A new thermoactive pullulanase from *Desulfurococcus mucosus*: cloning, sequencing, purification, and characterization of the recombinant enzyme after expression in *Bacillus subtilis*. J. Bacteriol. **182:**6331–6338.
17. **Edwards, C. A., A. M. Parrett, S. E. Balmer, and B. A. Wharton.** 1994. Faecal short chain fatty acids in breast-fed and formula-fed babies. Acta Paediatr. Scand. **83:**459–462.
18. **Fuller, R.** 1989. Probiotics in man and animals. J. Appl. Bacteriol. **66:**365–378.
19. **Ganghofner, D., J. Kellermann, W. L. Staudenbauer, and K. Bronnenmeier.** 1998. Purification and properties of an amylopullulanase, a glucoamylase, and an alpha-glucosidase in the amylolytic enzyme system of *Thermoanaerobacterium thermosaccharolyticum*. Biosci. Biotechnol. Biochem. **62:**302–308.
20. **Han, K. H., M. Fukushima, T. Kato, M. Kojima, K. Ohba, K. Shimada, M. Sekikawa, and M. Nakano.** 2003. Enzyme-resistant fractions of beans lowered serum cholesterol and increased sterol excretions and hepatic mRNA levels in rats. Lipids **38:**919–924.
21. **Han, K. H., M. Sekikawa, K. Shimada, K. Sasaki, K. Ohba, and M. Fukushima.** 2004. Resistant starch fraction prepared from kintoki bean affects gene expression of genes associated with cholesterol metabolism in rats. Exp. Biol. Med. **229:**787–792.
22. **Henrissat, B.** 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **280:**309–316.
23. **Hopkins, M. J., R. Sharp, and G. T. Macfarlane.** 2002. Variation in human intestinal microbiota with age. Dig. Liver Dis. **34**(Suppl. 2)**:**S12–S18.
24. **Janecek, S., B. Svensson, and B. Henrissat.** 1997. Domain evolution in the alpha-amylase family. J. Mol. Evol. **45:**322–331.
25. **Kim, C. H.** 1995. Substrate specificity and detailed characterization of a bifunctional amylase-pullulanase enzyme from *Bacillus circulans* F-2 having two different active sites on one polypeptide. Eur. J. Biochem. **3:**687–693.
26. **Leder, S., W. Hartmeier, and S. P. Marx.** 1999. Alpha-galactosidase of *Bifidobacterium adolescentis* DSM 20083. Curr. Microbiol. **38:**101–106.
27. **Lee, S. K., Y. B. Kim, and G. E. Ji.** 1997. Note: purification of amylase secreted from *Bifidobacterium adolescentis*. J. Appl. Microbiol. **83:**267–272.

28. **Le Leu, R., I. Brown, Y. Hu, A. Bird, M. Jackson, A. Esterman, and G. Young.** 2005. Asymbiotic combination of resistant starch and *Bifidobacterium lactis* facilitates apoptotic deletion of carcinogen-damaged cells in rat colon. J. Nutr. **135:**996–1001.
29. **Lin, L. L., M. R. Tsau, and W. S. Chu.** 1994. General characteristics of thermostable amylopullulanase and amylases from the alkalophilic *Bacillus* sp. TS-23. Appl. Microbiol. Biotechnol. **42:**51.
30. **Macfarlane, G. T., and H. N. Englyst.** 1986. Starch utilization by the human large intestinal microflora. J. Appl. Bacteriol. **60:**195–201.
31. **Mathupala, S. P., S. E. Lowe, S. M. Podkovyrov, and J. G. Zeikus.** 1993. Sequencing of the amylopullulanase (*apu*) gene of *Thermoanaerobacter ethanolicus* 39E, and identification of the active site by site-directed mutagenesis. J. Biol. Chem. **268:**16332–16344.
32. **Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka.** 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. **70:**7220–7228.
33. **Matzke, J., A. Herrmann, E. Schneider, and E. P. Bakker.** 2000. Gene cloning, nucleotide sequence and biochemical properties of a cytoplasmic cyclomaltodextrinase (neopullulanase) from *Alicyclobacillus acidocaldarius*, reclassification of a group of enzymes. FEMS Microbiol. Lett. **183:**55–61.
34. **Melasniemi, H., M. Paloheimo, and L. Hemio.** 1990. Nucleotide sequence of the alpha-amylase-pullulanase gene from *Clostridium thermohydrosulfuricum*. J. Gen. Microbiol. **136:**447–454.
35. **Mitsuoka, T.** 1990. Bifidobacteria and their role in human health. J. Ind. Microbiol. **6:**263–268.
36. **Mobassaleh, M., R. K. Montgomery, J. A. Biller, and R. J. Grand.** 1985. Development of carbohydrate absorption in the fetus and neonate. Pediatrics **75:**160–166.
37. **Nakajima, R., I. T., and S. Aiba.** 1986. Comparison of amino acid sequences of eleven different alpha-amylases. Appl. Microbiol. Biotechnol. **23:**355–360.
38. **Niehaus, F., A. Peters, T. Groudieva, and G. Antranikian.** 2000. Cloning, expression and biochemical characterisation of a unique thermostable pullulan-hydrolysing enzyme from the hyperthermophilic archaeon *Thermococcus aggregans*. FEMS Microbiol. Lett. **190:**223–229.
39. **O'Riordan, K.** 1998. Studies on antimicrobial activity and genetic diversity of *Bifidobacterium* species: molecular characterization of a 5.75 kb plasmid and a chromosomally encoded *recA* gene homologue from *Bifidobacterium breve*. Ph.D. thesis. National University of Ireland, Cork, Cork, Ireland.
40. **Parrett, A. M., S. Khanna, and C. A. Edwards.** 2000. Excretion of faecal starch and fat in breast fed and formula fed infants during weaning. Proc. Nutr. Soc. **59:**64A.
41. **Rossiter, M. A., J. A. Barrownman, and B. A. Wharton.** 1974. Amylase content of mixed saliva in children. Acta Paediatr. Scand. **63:**389–392.
42. **Roy, D., L. Blanchette, L. Savoie, P. Ward, and P. Chevalier.** 1992. Alpha- and beta-galactosidase properties of *Bifidobacterium infantis*. Milchwissenschaft **47:**18–21.
43. **Sakai, K., Tachiki, T., Kumagai, H., and T. Tochikura.** 1987. Hydrolysis of alpha-D-galactosyl oligosaccharides in soy by alpha-D-galactosidase of *Bifidobacterium breve* 203. Agric. Biol. Chem. **51:**315.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Satyanarayana, T., S. M. Noorwez, S. Kumar, J. L. Rao, M. Ezhilvannan, and P. Kaur.** 2004. Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. Biochem. Soc. Trans. **32:**276–278.
46. **Silvi, S., C. J. Rumney, A. Cresci, and I. R. Rowland.** 1999. Resistant starch modifies gut microflora and microbial metabolism in human flora-associated rats inoculated with faeces from Italian and UK donors. J. Appl. Microbiol. **86:**521–530.
47. **Wang, X., P. L. Conway, I. L. Brown, and A. J. Evans.** 1999. In vitro utilization of amylopectin and high-amylose maize (Amylomaize) starch granules by human colonic bacteria. Appl. Environ. Microbiol. **65:**4848–4854.