Differential Metabolism of Exopolysaccharides from Probiotic Lactobacilli by the Human Gut Symbiont *Bacteroides thetaiotaomicron*

Alicia Lammerts van Bueren,[a] Aakanksha Saraf,[a] Eric C. Martens,[b] Lubbert Dijkhuizen[a]

Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Groningen, The Netherlands[a]; Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA[b]

Probiotic microorganisms are ingested as food or supplements and impart positive health benefits to consumers. Previous studies have indicated that probiotics transiently reside in the gastrointestinal tract and, in addition to modulating commensal species diversity, increase the expression of genes for carbohydrate metabolism in resident commensal bacterial species. In this study, it is demonstrated that the human gut commensal species *Bacteroides thetaiotaomicron* efficiently metabolizes fructan exopolysaccharide (EPS) synthesized by probiotic *Lactobacillus reuteri* strain 121 while only partially degrading reuteran and isomalto/malto-polysaccharide (IMMP) α-glucan EPS polymers. *B. thetaiotaomicron* metabolized these EPS molecules via the activation of enzymes and transport systems encoded by dedicated polysaccharide utilization loci specific for β-fructans and α-glucans. Reduced metabolism of reuteran and IMMP α-glucan EPS molecules may be due to reduced substrate binding by components of the starch utilization system (*sus*). This study reveals that microbial EPS substrates activate genes for carbohydrate metabolism in *B. thetaiotaomicron* and suggests that microbially derived carbohydrates provide a carbohydrate-rich reservoir for *B. thetaiotaomicron* nutrient acquisition in the gastrointestinal tract.

Trillions of bacteria inhabit the gut, imparting symbiotic effects that benefit the overall health and well-being of individuals (1). While the human genome has approximately 30,000 genes, the human microbiome contributes an additional 3 to 9 million gene products (termed the gut metagenome) that contribute to functionalities in human lifestyle (2). One prominent example is the human utilization of carbohydrates. Carbohydrates provide a substantial proportion of the daily energy intake of an individual. However, humans have the ability to metabolize only very few dietary carbohydrate compounds, such as lactose, starch, and sucrose, by enzymes either present in saliva (amylases) or anchored to the epithelial wall of the small intestine with glycosylphosphatidylinositol (GPI) anchors (such as invertases and lactases). All other carbohydrates that traverse the gastrointestinal (GI) tract have the potential to be metabolized by commensal gut bacteria, where it is estimated that gut microbiota provide an additional 30,000 enzymes which facilitate the breakdown of carbohydrates in the gut (3).

Approximately 30% of resident human gut microbiota are from the *Bacteriodetes* phylum. These Gram-negative, obligate anaerobes are well armed with a repertoire of carbohydrate-degrading enzymes for harvesting carbohydrate nutritional resources. One species, *Bacteroides thetaiotaomicron*, devotes approximately 18% of its genome to carbohydrate foraging (4). Discrete polysaccharide utilization loci (PULs) within its genome are upregulated in response to carbohydrates dependent on the source. Most carbohydrate sources for *B. thetaiotaomicron* nutrient acquisition originate from dietary sources (such as nondigestible plant polysaccharides) or from the mucosal layer lining the gastrointestinal tract (mucins and mucopolysaccharides) (5). The ability of *B. thetaiotaomicron* to efficiently utilize a wide range of carbohydrates makes this organism well suited to inhabit the dynamic environment of the gastrointestinal tract.

Probiotic bacteria can be ingested without harm to an individual and provide a positive health benefit to the consumer (6). These strains most commonly are consumed by ingesting fermented milk products (FMPs) or are found at therapeutic levels by ingesting dried bacterial strains in capsular form. Ingesting probiotic bacteria and FMPs is correlated with creating balanced gut homeostasis by altering gut microbial composition, altering bacterial metabolic activity, and producing metabolites that directly affect host intestinal epithelium (7). Therefore, consuming probiotics is one strategy used to treat microbiome-associated diseases (8, 9).

Lactic acid bacteria (LAB) are a diverse group of probiotic microorganisms that reside in the upper GI tract and have immunomodulatory properties (10). Additionally, they are among the most abundantly consumed bacteria, as they are commonly used in food production for meats, beverages, pickling agents, and FMPs, producing flavor and textural features while reducing food spoilage. LAB produce lactic acid from fermentation of carbohydrates, which leads to acidification of the surrounding environment, and synthesize extensive exopolysaccharides (EPS) that promote their adhesion and growth in the gastrointestinal tract (11) and offer protection from pathogenic bacteria (12). The most widely known example is EPS from *Streptococcus mutans* that synthesizes a sticky biofilm called mutan, which aids in its colonization of the oral cavity (13). *S. mutans* and the majority of other

LAB genera, such as *Leuconostoc* and *Lactobacillus*, synthesize β-fructan and α-glucan EPS polymers, which protect the bacteria from immune detection and aid in attachment of bacteria to the mucosal lining of the intestine (14, 15). These homopolysaccharide EPS molecules are derived from dietary sucrose and are synthesized mainly in the oral cavity and small intestine, where there is an abundance of dietary sucrose present. LAB employ cell surface-associated transglycosidases using sucrose as the donor substrate to synthesize these extensive β-fructan and α-glucan EPS polymers (16, 17).

Several studies have investigated the effects of probiotics on altering gut microbial composition and how this may contribute to the health of an individual (7, 18–21). One area of particular interest is the observation that ingesting probiotic bacteria alters the gene expression profile and metabolic output of commensal bacteria, and the changes in metabolic output correlate with health benefits in an individual (20). Research by McNulty et al. showed that monozygotic twins and gnotobiotic mice administered common probiotic strains from FMPs exhibited little change in bacterial composition over time; however, gene expression profiles in commensal bacterial strains were significantly altered (20). Most significant increases were observed for genes involved in carbohydrate metabolism and transport, specifically starch and sucrose metabolism pathways, including levanases (EC 3.2.1.65), pectinesterases (EC 3.1.1.11), and cellobiose phosphorylase (EC 2.4.1.20). The researchers could not establish what exactly caused the effect of increased carbohydrate metabolism, but they determined that the effect was rapid and consistent during probiotic supplementation.

As probiotic LAB strains transit the GI tract, commensal microbiota will be exposed to LAB-synthesized carbohydrate-based EPS molecules which would be available for fermentation. If EPS is a fermentable substrate, its presence would cause an upregulation of genes required for metabolism of these carbohydrate compounds. Therefore, the hypothesis in this study posits that human gut commensal bacteria have the ability to degrade LAB EPS by activating and producing genome-encoded carbohydrate-active enzymes (CAZymes) and transport systems with activity directed toward β-fructan and α-glucan EPS substrates, specifically genes encoding levanases and starch-metabolizing CAZymes, leading to the metabolism of LAB EPS molecules by gut commensal microbes.

To test this hypothesis, different preparations of EPS derived from *Lactobacillus reuteri* 121 (termed LrEPS and isomalto/malto-polysaccharide [IMMP]) were synthesized and purified. Purified EPS was provided as a sole carbon source to the human saccharolytic bacterial symbiont *Bacteroides thetaiotaomicron* to see whether bacteria can metabolize LAB EPS products and, furthermore, what molecular mechanisms are involved in EPS breakdown. Results show that *B. thetaiotaomicron* completely metabolized LAB-derived β-fructan EPS, as would be predicted from previous work on selective levan metabolism by this species (22), and partially degraded α-glucan EPS. Furthermore, EPS degradation was associated with the production of enzymes and transport proteins encoded by polysaccharide utilization loci (PULs) in the *B. thetaiotaomicron* genome, namely, PULs for fructan and starch catabolism (22–24). Although *L. reuteri* α-glucan EPS triggered the production of starch and dextran PUL-associated components, *B. thetaiotaomicron* was unable to completely degrade α-glucan EPS polymers. To our knowledge, this is the first study to demonstrate that *B. thetaiotaomicron* activates PULs in response to LAB-derived EPS molecules. These results provide a plausible explanation for the observed upregulation of carbohydrate metabolism genes in commensal gut bacterial species in the presence of probiotic bacteria. Furthermore, these findings suggest a mechanism for EPS metabolism by members of gut microbial species that may transiently or persistently colonize the gut.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. *Bacteroides thetaiotaomicron* VPI-5482 was purchased from the ATCC. A *B. thetaiotaomicron* starch deletion mutant (BtΔ3702) and β-fructan deletion mutant (BtΔ1760) were provided by E. C. Martens. *Lactobacillus reuteri* strain 121 and *L. reuteri* strain 35-5 were from TNO Quality of Life, Zeist, The Netherlands. All medium components were purchased from Sigma (Zwijndrecht, Netherlands) or as mentioned.

Purification of EPS components. *L. reuteri* EPS was purified as described in reference 25 using the following procedure. *L. reuteri* 121 and the 35-5 mutant were grown on MRS agar containing either 10% sucrose (to obtain levan plus reuteran EPS) or 10% maltodextrin (to obtain IMMP) and incubated anaerobically in anaerobic jars (Oxoid) containing a GasPak (BD) for 48 h. After growth, plates were scraped of colonies and resuspended in deionized water. Tubes were stirred for 2 h at room temperature to release EPS into solution. After 2 h, cells were pelleted and supernatants were collected, to which 3 volumes of ice-cold 100% ethanol were added and stirred overnight at 4°C to allow precipitation of EPS polysaccharides. The precipitated material was pelleted, dried, and dissolved in deionized water. Ten percent trichloroacetic acid (TCA) was added to each preparation to precipitate out any contaminating proteins. After 1 h of incubation with 10% TCA at room temperature, precipitated material was pelleted and supernatants containing EPS were dialyzed into deionized water for 3 days with 3,500-molecular-weight-cutoff (MWCO) tubing (Thermo Scientific). After dialysis, polysaccharides were freeze-dried and stored under desiccating conditions until further use.

NMR analysis. Nuclear magnetic resonance (NMR) was carried out as described previously (25). Samples were exchanged twice with 200 μl of 99.9 atom% D$_2$O (Cambridge Isotope Laboratories Ltd., United Kingdom) with intermediate lyophilization and finally dissolved in 650 μl of 99.9 atom% D$_2$O containing 25 ppm acetone as an internal standard (δ1H 2.225). One-dimensional 600-MHz ^{1}H NMR spectra were recorded on a Varian Inova 600 spectrometer (NMR Department, University of Groningen, The Netherlands), collecting 32 transients of 16,000 complex data points, using a 5,000-Hz spectral width. The residual HOD (chemical shift of H$_2$O) peak was suppressed using a WET1D suppression pulse. All spectra were processed using MestReNova 5.3 (Mestrelabs Research SL, Santiago de Compostella, Spain), using Whittaker Smoother baseline correction.

B. thetaiotaomicron growth experiments. *B. thetaiotaomicron* growth was carried out as described previously (26). Briefly, overnight cultures of *B. thetaiotaomicron* were grown at 37°C under anaerobic conditions. The following day, 1 ml of *B. thetaiotaomicron* overnight culture was prepared in a carbon-limited minimally defined medium of 100 mM KH$_2$PO$_4$ (pH 7.2), 15 mM NaCl, 8.5 mM (NH$_4$)$_2$SO$_4$, 4 mM L-cysteine, 1.9 μM hematin, 200 μM L-histidine, 100 nM MgCl$_2$, 1.4 nM FeSO$_4$ · 7 H$_2$O, 50 μM CaCl$_2$, 1 μg/ml vitamin K$_3$, 5 ng/ml vitamin B$_{12}$, and individual carbon sources (0.5%, wt/vol). Growth curves were obtained by incubating microtiter plates at 37°C, and the optical density at 600 nm (OD$_{600}$) was recorded at 30-min intervals.

To obtain culture supernatants for analysis of carbohydrate activity, *B. thetaiotaomicron* cells were prepared in the same carbon-limited minimal media as that for the microtiter plates. One milliliter of bacterial cell suspension was added to 1 ml of 10 mg/ml carbohydrate solution (final concentration of 5 mg/ml) in a sterilized glass test tube with a metal cap, placed in an anaerobic jar (Oxoid) with a GasPak (BD), and placed in a 37°C incubator for 48 h.

After growth, cultures of *B. thetaiotaomicron* in carbohydrate-defined media were centrifuged to obtain supernatants which subsequently were filtered through a 0.2-μm-pore-size syringe filter (Millipore). One hundred μl of culture supernatant was added to 100 μl of a 10 mg/ml solution of carbohydrate (35-5 EPS reuteran, wild-type LrEPS, or IMMP) in deionized water and incubated at 37°C overnight to allow the enzyme reactions to proceed.

qPCR of PUL genes. Isolation of RNA, synthesis of cDNA, and quantitative PCR (qPCR) measurements were carried out as described previously (27). Values reported are the fold difference compared to that of the glucose control RNA and were normalized against 16S gene expression. Primers used for quantification of PUL activation were directed against *susC*-like genes for each designated PUL and are listed in Table S1 in the supplemental material.

TLC. Thin-layer chromatography (TLC) of carbohydrate products was completed as described in the literature (28) Briefly, 2 μl of each reaction mixture was spotted, dried, and then subsequently spotted again on a silica gel 60 plate (Millipore). The solvent system used was 3:1:1 isopropanol, ethylacetate, and deionized water, and plates were run in a TLC jar for approximately 4 to 5 h. Plates were removed from the jar and dried, and spots were visualized by staining with 20% sulfuric acid plus 5% orcinol in methanol and heated at 110°C for half an hour. Plates were scanned and figures prepared using Adobe Photoshop. For enzymatic degradation of reuteran, 5 U of each enzyme was added to 100 μl of 1% reuteran solution in phosphate-buffered saline (PBS) and incubated for 1 h at appropriate temperatures. Products were observed by TLC as mentioned above.

HPAEC-PAD. The oligosaccharides produced by *B. thetaiotaomicron* culture supernatants were analyzed by high-pH anion-exchange chromatography on a Dionex DX500 work station equipped with an ED40 pulsed amperometric detection system (HPAEC-PAD). The oligosaccharides were separated on a CarboPac PA-1 column (250 by 5 mm; Dionex) by using a linear gradient of 10 to 240 mM sodium acetate in 100 mM NaOH (1 ml/min).

Proteomic analysis of culture supernatants. The proteins in the supernatants were precipitated using cold trichloroacetic acid and washed 3 times with ice-cold acetone. Proteins were redissolved in 50 mM ammonium bicarbonate (pH 8), chemically reduced by adding 5 μl of 5 mM tris(2-carboxyethyl)phosphine, and alkylated by adding 5 μl of 100 mM iodoacetamide at room temperature for 30 min. To incorporate proteins into a gel directly in the Eppendorf vial, 18.5 μl of acrylamide-bisacrylamide solution (40%, vol/vol, 29:1), 2.5 μl of 10% (wt/vol) ammonium persulfate, and 1 μl of 100% N,N,N',N'-tetramethylethylenediamine (TEMED) was applied to the protein solution and polymerized in 30 min. The resulting gel was cut into small pieces and washed several times with 1 ml of 50 mM ammonium bicarbonate containing 50% (vol/vol) acetonitrile. The gel samples were further dehydrated with 100% acetonitrile. Proteolytic digestion then was performed with trypsin in 50 mM ammonium bicarbonate with incubation overnight at 37°C. The peptide mixture was acidified by adding 5 μl formic acid and extracted. Samples were analyzed by nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) on an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) interfaced on-line with an LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Peptide mixtures were loaded onto a trapping microcolumn (inner diameter [i.d.], 5 mm by 300 μm) packed with C_{18} PepMAP100 5-μm particles (Dionex) in 0.1% formic acid at a flow rate of 20 μl/min. After loading and washing for 5 min, peptides were back-flush eluted onto a nanocolumn (15 cm by 75 μm i.d.) packed with C_{18} PepMAP100 3-μm particles (Dionex). The following mobile-phase gradient was delivered at a flow rate of 300 nl/min: 2 to 50% solvent B for 60 min, 50 to 90% B for 7 min, 90% B for 10 min, and back to 2% B for 5 min. Solvent A was H_2O-acetonitrile (100:0, vol/vol) with 0.1% formic acid, and solvent B was H_2O-acetonitrile (0:100, vol/vol) with 0.1% formic acid.

Peptides were infused into the mass spectrometer via a dynamic nanos-pray probe (Thermo Electron Corp.) with a stainless steel emitter (Proxeon, Odense, Denmark). The typical spray voltage was 1.8 kV with no sheath and auxiliary gas flow; the ion transfer tube temperature was 200°C. The mass spectrometer was operated in data-dependent mode. The automated gain control (AGC) was set to 5×10^5 charges and 1×10^4 charges for MS/MS on the linear ion trap analyzer. The data-dependent acquisition (DDA) cycle consisted of the survey scan within *m/z* 300 to 1,300 on the Orbitrap analyzer with target mass resolution of 60,000 (full width at half maximum [FWHM] at *m/z* 400), followed by MS/MS fragmentation of the five most intense precursor ions under the relative collision energy of 35% in the linear trap. Singly charged ions were excluded from MS/MS experiments, and the *m/z* values of fragmented precursor ions were dynamically excluded for a further 60 s. The ion selection threshold for triggering MS/MS experiments was set to 500 counts.

The software PEAKS Studio (version 7) was applied to the spectra generated by the LTQ Orbitrap XL to search against either the protein sequence database UniProtKB/Trembl of the UniProt Knowledgebase (UniProtKB), limited to protein sequences of *Bacteroides thetaiotaomicron*, or the Swiss-Prot database. Searching for the fixed-modification carbamidomethylation of cysteine and the variable posttranslational modifications, oxidation of methionine was done with a maximum of 5 posttranslational modifications per peptide at a parent mass error tolerance of 10 ppm. The false discovery rate was set at 0.1%.

SusD binding activity assay. SusD protein was purified as described previously (26). SusD was buffer exchanged into 100 mM sodium bicarbonate buffer and labeled with fluorescein isothiocyanate (FITC). Labeled SusD was isolated and buffer exchanged into 20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM $CaCl_2$ (BB) buffer solution and concentrated with an Amicon Centricon device with a 10,000-MWCO membrane to a concentration of ~20 mg/ml. The control protein, *Thermotoga maritima* CBM41 (*Tm*CBM41), was prepared as previously described (29) and FITC labeled. Membranes for carbohydrate macroarrays were prepared in-house based on previously published procedures (29). Briefly, 1 μl of a 1% (wt/vol) carbohydrate solution was spotted onto a nitrocellulose membrane grid and allowed to dry for 1 h at room temperature. After macroarrays were prepared, membranes were blocked in BB plus 0.5% Tween 20 (BBT) and 1% bovine serum albumin (BSA) for 1 h at room temperature. After blocking, membranes were washed 3× and placed in BB plus 200 μg of corresponding protein at a final concentration of 40 μg/ml and incubated overnight at 4°C with continuous agitation. The following day, membranes were washed 3× with BBT and visualized on a Chemidoc with fluorescein filters (Thermo Scientific).

For testing binding to insoluble starch, 10 mg of insoluble wheat starch was washed 3× with BBT and resuspended in 1 ml of BB. Two hundred micrograms of FITC-labeled SusD or *Tm*CBM41 was added to individual tubes and incubated at room temperature for 1 h with continuous agitation. After 1 h, Eppendorf tubes containing starch and bound protein were centrifuged to pellet the starch. The starch pellet was thoroughly washed 3× with BBT. It was resuspended in a 100-μl final volume of BB, and 5 μl of the suspension was spotted on a nitrocellulose membrane, dried, and visualized.

SusG activity. Enzymatic reactions were carried out as described previously (28). Reuteran, IMMP, amylopectin, and insoluble amylose were prepared at a concentration of 10 mg/ml in 20 mM Tris, pH 8.0, 150 mM NaCl. Reactions were carried out in 20-μl volumes, where 2 μl (approximately 50 μg) of SusG was added to 18 μl of each carbohydrate solution. Reaction mixtures were incubated at 37°C for 4 h and analyzed by TLC as described above.

RESULTS

***B. thetaiotaomicron* metabolized *L. reuteri* β-fructan exopolysaccharides.** EPS was purified from the probiotic strain *Lactobacillus reuteri* 121, which synthesizes a thick EPS layer composed of the fructan polymer levan (β-2,6-fructan) and the α-glucan polymer reuteran from a sucrose donor substrate and IMMP
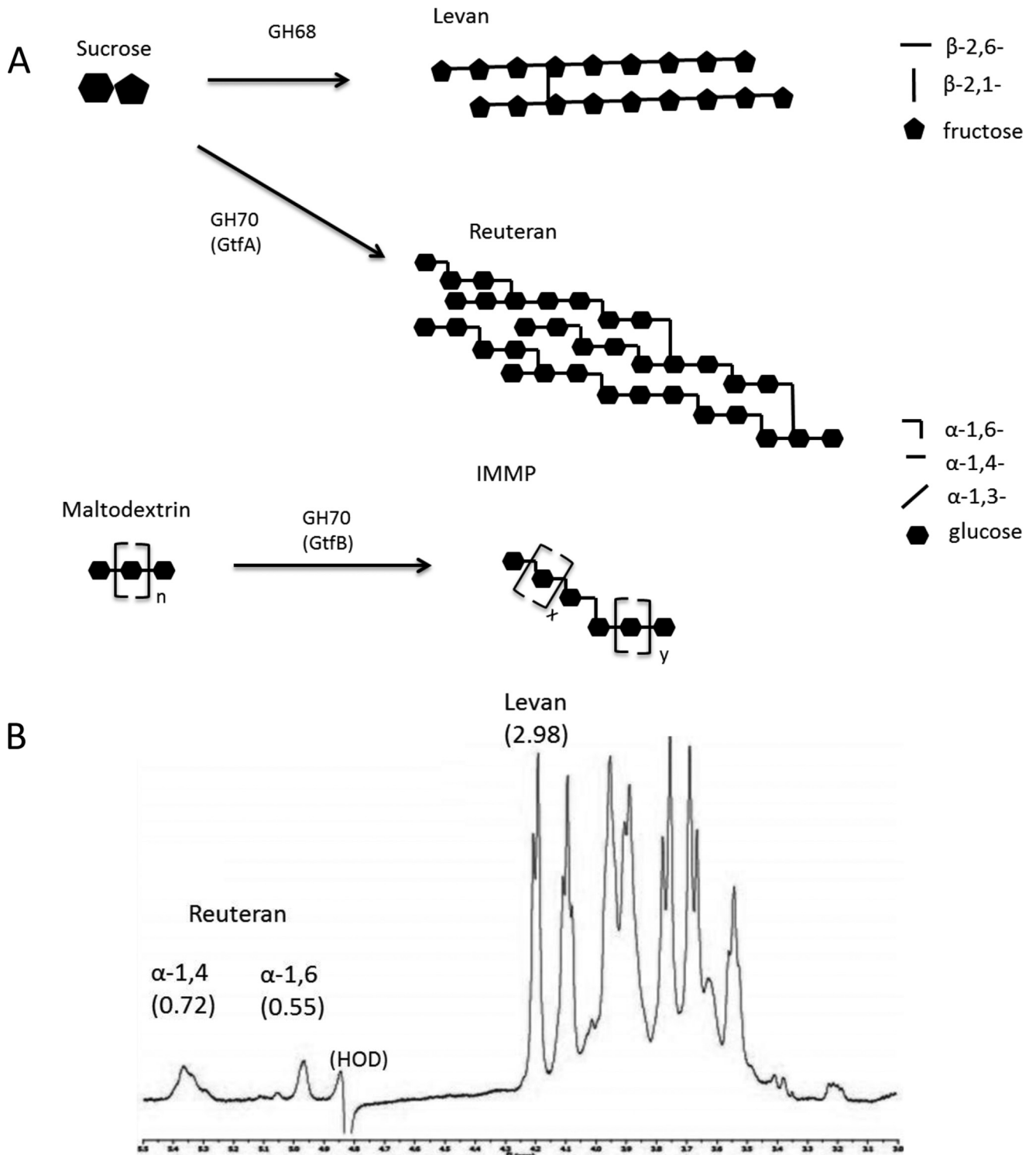
FIG 1 (A) Graphical representation of β-fructan levan and α-glucans reuteran and IMMP exopolysaccharides produced by *Lactobacillus reuteri* 121 via transglycosylation reactions using glycoside hydrolase family 68 (GH68) or GH70 enzymes (www.cazy.org [39]). (B) NMR analysis of LrEPS products indicating the abundance of monosaccharides present as ratios of each peak representing the anomeric carbon present.

(isomalto/malto-polysaccharides) from maltodextrins (Fig. 1A). *L. reuteri* was chosen as our model LAB strain, as it is an endogenous mammalian gut strain more adapted to human colonization than other LAB species involved in food preparation, and much is

known on the structure of EPS produced by this strain (25, 30, 31). Additionally, it is used as a probiotic supplement, such as treatment of *Helicobacter pylori* infections and infant ailments such as colic (32, 33). We observed that wild-type *L. reuteri* grown in the
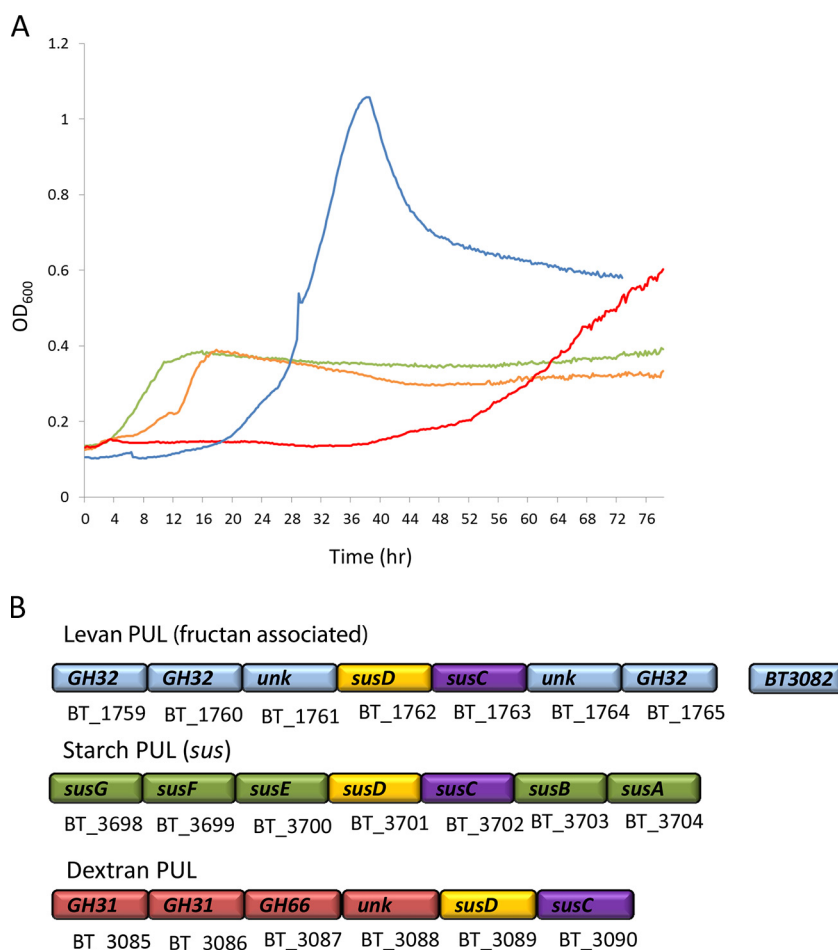
FIG 2 (A) Growth of *B. thetaiotaomicron* VPI-5482 and mutants derived on *Lactobacillus reuteri* EPS. Wild type, green; BtΔ3702 (*sus* mutant), yellow; BtΔ1763 (fructan-associated PUL mutant), red; and glucose control, blue. *B. thetaiotaomicron* and deletion mutants were grown under anaerobic conditions at 37°C in minimal growth media supplemented with wild-type LrEPS (levan plus reuteran) or glucose (control) at 5 mg/ml final concentration. Only one curve was carried out for each sample due to the limited availability of substrate, and results were verified by qPCR. (B) Graphical representation of *B. thetaiotaomicron* PULs mentioned in this study.

presence of sucrose synthesized both levan and reuteran EPS molecules at a ratio of approximately 2.3:1, as calculated by integration of the area under the peak corresponding to the anomeric center for the monosaccharide (Fig. 1B), and produced a pure IMMP α-glucan EPS polymer when grown on maltodextrins (Fig. 1A) (31, 34). Thus, three forms of *L. reuteri* EPS were used for this study: LrEPS from *L. reuteri* 121 grown on sucrose, which consists of both levan and reuteran, reuteran only, which was produced by the mutant *L. reuteri* 121 strain 35-5 lacking the levansucrase enzyme grown on sucrose (25, 35), and IMMP from wild-type *L. reuteri* 121 grown on maltodextrins (31). *B. thetaiotaomicron* grown in the presence of 5 mg/ml LrEPS demonstrated only moderate growth on LrEPS (Fig. 2A, green line) compared to a 5 mg/ml glucose standard (Fig. 2A, blue line), suggesting that *B. thetaiotaomicron* was using only a portion of the EPS substrate. Because *B. thetaiotaomicron* contains dedicated PULs in its genome for the metabolism of β-fructans (β-2,6-linked fructose) (22), starch (*sus*) (α-1,4-linked glucose) (23), and dextran (α1,6-linked glucose) (Fig. 2B), we suspected that these PULs contribute to the observed bacterial growth. To determine the contribution of the fructan-associated and *sus* PULs to the degradation of

LrEPS, *B. thetaiotaomicron* fructan-associated PUL deletion mutant BtΔ1763 and *sus* deletion mutant BtΔ3702 were grown in the presence of LrEPS. BtΔ3702, which lacks the ability to degrade starch, showed a slightly delayed growth profile but reached wild-type growth levels after 16 h (Fig. 2A, yellow line). BtΔ1760, which lacks the ability to grow on levan, exhibited no growth on LrEPS after 16 h, while after 60 h it begins to show a slow increase in cell density (Fig. 2A, red line), implying that levan is the preferred EPS substrate contributing to bacterial growth. To further verify these results, quantification of *sus*, dextran-associated, and fructan-associated PUL activation was determined by qPCR to analyze expression levels of mRNA extracted from wild-type *B. thetaiotaomicron* grown in the presence of LrEPS. The level of the fructan-associated PUL was increased 19.2-fold ± 3.93-fold compared to the level for glucose, whereas there was a slight decrease in expression levels of *sus* and dextran-associated PUL (Table 1). Together, these results demonstrate that *B. thetaiotaomicron* activates the β-fructan PUL (BT1759-BT1765) for metabolism of LrEPS when a combination of levan and reuteran is present.

B. thetaiotaomicron partially metabolized reuteran and IMMP EPS. Since *B. thetaiotaomicron* is well equipped with PULs

TABLE 1 Major proteins identified from proteomic analysis of material from culture supernatants of *B. thetaiotaomicron*[a]

Protein category	Proteins identified with[b]:		
	LrEPS	Reuteran	IMMP
PUL-associated carbohydrate-active enzymes and binding proteins	Endo-2,6-β-fructanase GH32 BT1760 (fructan associated), 2,6-β-fructofuranosidase GH32 BT1759 (fructan associated), 2,6-β-fructofuranosidase GH32 BT3082 (fructan associated), outer membrane protein SusE BT3700 (*sus*), outer membrane protein SusF BT3699 (*sus*), and α-glucosidase GH97 BT2620	SusG amylase GH13 BT3698 (*sus*), SusB α-glucosidase GH97 BT3703 (*sus*), outer membrane protein SusF BT3699 (*sus*), and outer membrane protein SusE BT3700 (*sus*)	Endodextranase BT3087 (dextran associated), α-glucosidase BT3086 (dextran associated), SusE-F-like binding protein BT3088 (dextran associated), SusB α-glucosidase GH97 BT3703 (*sus*), SusA pullulanase BT3704 (*sus*), and α-glucosidase GH97 BT2620
Non-PUL-associated carbohydrate-active enzymes	α-Amylase GH57 BT4305 and 4,6-glucanotransferase BT4303		α-Glucosidase GH97 BT4581, α-amylase GH57 BT4305, and 4,6-glucanotransferase BT4303

[a] Proteins were identified from carbon-limited experiments with 5 mg/ml LrEPS, reuteran, or IMMP. PUL systems were identified based on the presence of both members of SusC-D pairs (values in parentheses are from qPCR results): the BT1762/BT1763 (fructan-associated) pair was present on LrEPS (19.2 ± 3.93) but not on reuteran or IMMP, the BT3089/BT3090 (dextran-associated) pair was present on IMMP but not on reuteran or LrEPS (0.54 ± 0.12), and the BT3701/BT3702 (*sus*) pair was present on LrEPS (0.49 ± 0.10), reuteran, and IMPP.

[b] Proteins and enzymes are identified and compared to the glucose controls (a full list can be found in the supplemental material) and correlated with their associated PULs, which are identified in parentheses (4).

for the degradation of starch and dextran, it was surprising to see that reuteran from the LrEPS mixture was not being metabolized. Therefore, independent *B. thetaiotaomicron* culture experiments were performed to test for bacterial growth on the α-glucan EPS substrates reuteran and IMMP separately. Compared to *B. thetaiotaomicron* cultures of LrEPS (2.3:1 levan-reuteran) (Fig. 3, green line), reuteran substrate showed a biphasic growth profile with a slight increase in growth after 16 h and an increase up to an OD_{600} of 0.4 after 52 h (Fig. 3, red line), while IMMP as a substrate showed very little increase in growth after 72 h (change in OD_{600} of 0.12 ± 0.09) (Fig. 3, purple line). Although *B. thetaiotaomicron* can effectively degrade starch, a polymer of amylose (α-1,4-glucan) and amylopectin (α-1,4-α-1,6-mixed glucan), it was difficult for *B. thetaiotaomicron* to metabolize α-glucan EPS substrates containing the same types of glucan linkages found in starch.

The identification and activity of *B. thetaiotaomicron* CAZymes induced by LrEPS, reuteran, and IMMP. Previous observations from the seminal work of the Anderson and Salyers laboratory on starch degradation by *B. thetaiotaomicron* showed that PUL-activated CAZymes can be found in culture supernatants (23). Therefore, to further characterize the activity of CAZymes produced by *B. thetaiotaomicron* in the presence of LrEPS, fresh *B. thetaiotaomicron* culture supernatants from whole LrEPS, reuteran, and IMMP growth experiments were incubated with EPS molecules, and the products of EPS degradation by CAZymes present in culture supernatants were analyzed by thin-layer chromatography and HPAEC-PAD. Enzymes present in culture supernatants from wild-type LrEPS cultures completely degraded levan into its monosaccharide component, fructose, and small amounts of fructose disaccharide (Fig. 4 red line; also see the inset, lane 1) but showed no activity on reuteran (Fig. 4, inset, lane 2). Culture supernatants then were analyzed for the presence of PUL-encoded proteins using proteomics (see Materials and Methods). Results from proteomic analysis of LrEPS culture supernatants showed that almost all extracellular enzymatic components of the fructan-associated PUL were present (Table 1 and Fig. 2B) (see File S1 in the supplemental material for a full listing of

proteins identified in culture supernatants), which accounts for the observed degradation of levan in LrEPS.

Culture supernatants from *B. thetaiotaomicron* growth on reuteran and IMMP also were tested for enzymatic activity on pure reuteran or IMMP substrates. The activity of reuteran culture supernatants shows that small amounts of glucose and maltose were produced (Fig. 5, lane 1), which is not observed in the glucose control supernatant (Fig. 5, lane 3), while IMMP culture supernatant activity on IMMP showed the same general product profile (Fig. 5, lane 2). Proteomic analysis of reuteran culture supernatants identified several components from the *sus* starch utilization PUL (Table 1 and Fig. 2B), including the transporter component SusD and extracellular amylase SusG, suggesting that the partial growth phenotype exhibited by *B. thetaiotaomicron* on reuteran is due to *sus* PUL-associated enzymes. Culture supernatants from IMMP growth revealed that enzymes from the *sus* operon were present, whereas additional enzymes from the dextran-associated PUL BT3085-BT3090 also were present (Table 1 and Fig. 2B; also see File S1 in the supplemental material for a full listing). Additional enzymes found in the IMMP supernatant include endodextranase BT3087 (GH66) and α-glucosidase II BT3086 (GH31) (Table 1). These results suggest that *sus* and dextran-associated PUL-encoded α-glucan-specific CAZymes are produced by *B. thetaiotaomicron* in the presence of LAB α-glucan EPS, implying that other factors, such as substrate recognition or enzymatic activity, prevent the full degradation and metabolism of these substrates.

Role of *sus*-encoded transport component SusD and surface-associated α-amylase SusG on α-glucan EPS metabolism. The extracellular α-amylase SusG and transport component SusD were identified in *B. thetaiotaomicron* culture supernatant grown in reuteran only (Table 1). To further elucidate the effects of *sus* system components on reuteran digestion by *B. thetaiotaomicron*, purified SusG and SusD were tested for their activity on reuteran. SusG is the surface-associated α-amylase from the *sus* system involved in the breakdown of starch into smaller glucooligosaccharides (28). SusG was tested for its degradation properties on
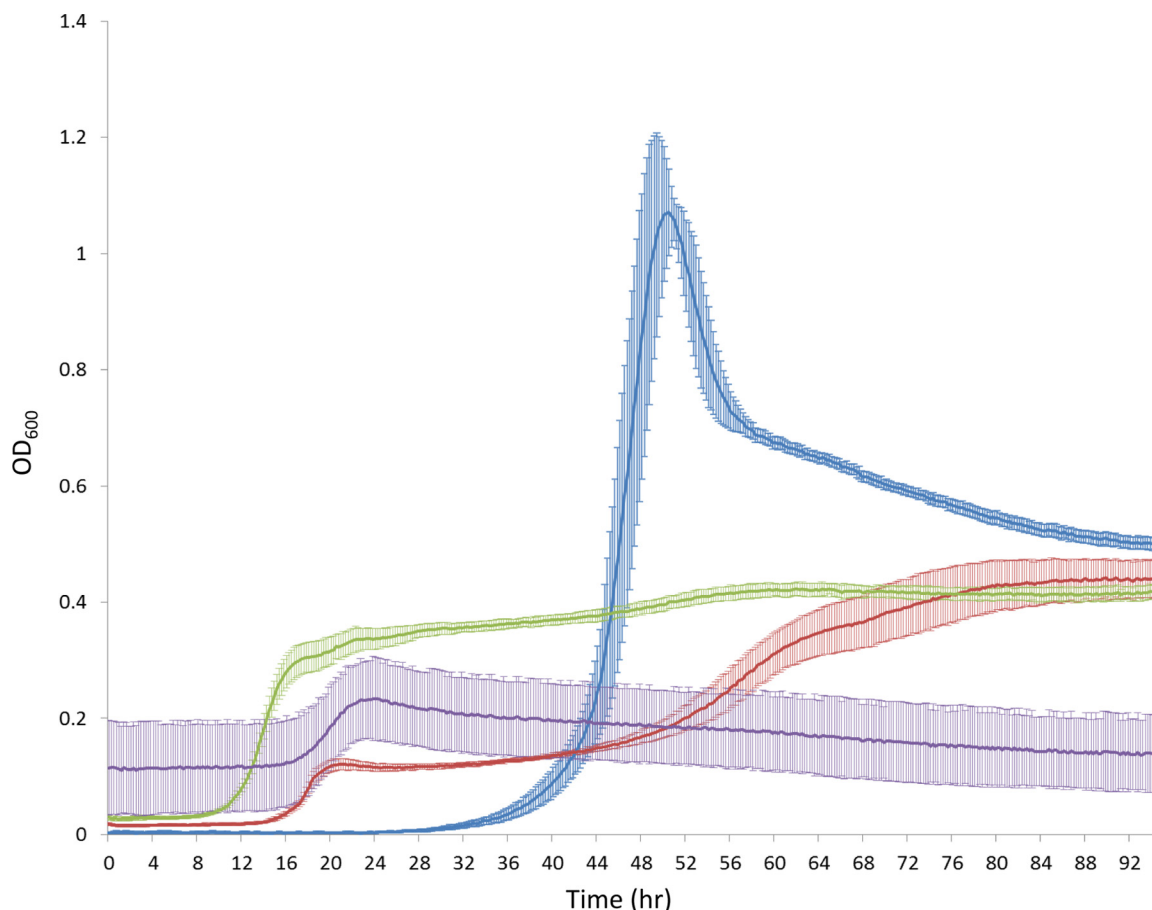
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FIG 3 Growth of *B. thetaiotaomicron* VPI-5482 on individual α-glucan-based EPS components reuteran (red) and IMMP (purple) compared to LrEPS (green) and glucose (blue) controls. The lines indicate averages from six growth experiments, and error bars indicate the standard deviations for these experiments.

reuteran, where characteristic high activity was seen with amylopectin (Fig. 6, lane 4), with only limited activity observed on reuteran, producing relatively small amounts of glucose and maltose (Fig. 6, lanes 2 and 3). The SusG product profile was similar to the profile observed from activity of the *B. thetaiotaomicron* culture supernatants from reuteran growth experiments containing SusG enzyme (Fig. 5); therefore, it is likely that SusG contributes to partial growth of *B. thetaiotaomicron* on reuteran. However, this suggests the enzyme itself is not able to sufficiently hydrolyze the polymer. Reuteran resembles starch in its composition and α-1,4- and α-1,6-glucosidic linkages, except that alternating α-1,4 and α-1,6 linkages are much more complex in reuteran than in starch, making it difficult for SusG to access the substrate (Fig. 1). Thus, reduced enzymatic activity of SusG on reuteran would indeed partially contribute to reduced bacterial growth on this substrate.

To further investigate which CAZymes are able to degrade reuteran, several commercially available α-glucan-specific CAZymes were tested for their activity on reuteran. Enzymes with specific activity on α-1,6-linkages, such as pullulanases (endo-α-1,6-glucosidases, GH13 subfamily 14) and isoamylases, were able to degrade reuteran most efficiently (Fig. 7A). *B. thetaiotaomicron* has two pullulanase genes in its genome, BT1663 and BT4689, both predicted to be surface enzymes; however, neither pullulanase is associated with a specific PUL, and

neither enzyme was identified in the proteomics analysis of reuteran culture supernatants. Therefore, attempts were made to induce the expression of these pullulanases by adding equal amounts of isomaltose, maltose, or panose, in addition to reuteran, to the *B. thetaiotaomicron* growth media. The addition of these sugars did not yield any further activity on reuteran by culture supernatants (Fig. 7B), while strong growth was observed only in the cultures containing additional maltose. In the TLC analysis it is apparent that *B. thetaiotaomicron* is not able to transport either isomaltose (6-O-α-D-glucopyranosyl–D-glucose) or panose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucose] glucooligosaccharides, since these sugars still are present in culture supernatants. The addition of pullulan components did not promote further activation of α-glucan-specific CAZymes with activity on reuteran. Although *B. thetaiotaomicron* has pullulanases encoded by its genome, it is not known what induces expression of pullulanases or other non-PUL-associated CAZymes with activity on α-glucans in *B. thetaiotaomicron*. These results demonstrate that reuteran is partially resistant to SusG activity but has the potential to be degraded by *B. thetaiotaomicron* genome-encoded pullulanases; however, these enzymes are not produced by the bacterium in the presence of reuteran or mixed-linkage glucooligosaccharides.

SusD forms an essential component of the SusC/D transport system and is essential for the metabolism of starch by *B.*
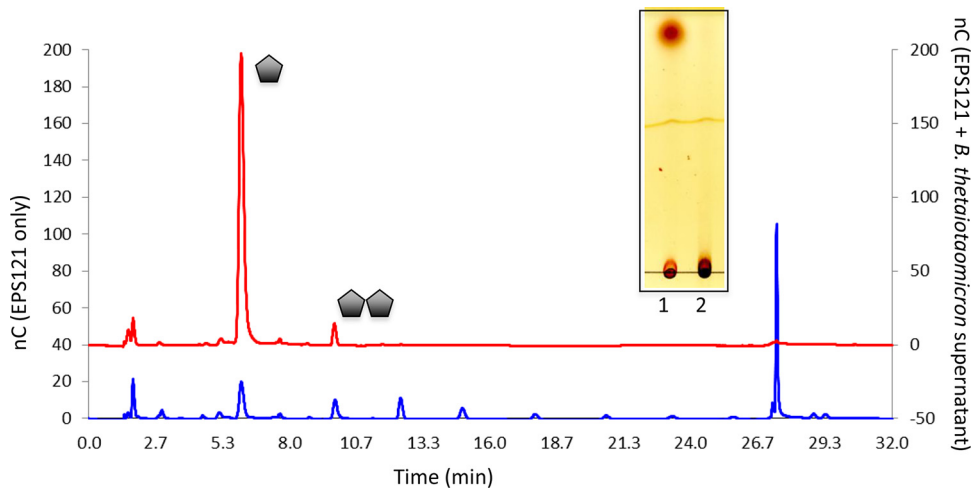
FIG 4 Thin-layer chromatography analysis and HPAEC-PAD profile of *L. reuteri* 121 β-fructan (levan) EPS degradation. The graph shows the levan (blue line) control and products from levan hydrolysis by *B. thetaiotaomicron* culture supernatants after growth on levan (red line). The inset is the corresponding TLC profile from enzymatic activity of CAZymes present in *B. thetaiotaomicron* culture supernatants from growth on wild-type LrEPS activity on LrEPS (lane 1) and reuteran (lane 2). The *y* axes show electric charges (in nanocoulombs [nC]).

thetaiotaomicron (26). SusD also was found to be present in reuteran culture supernatants; therefore, its production is activated by the presence of reuteran (Table 1). To further characterize SusD interactions with reuteran and IMMP, carbohydrate macroarray analysis was performed with purified and fluorescently labeled SusD on a range of soluble α-glucans. SusD was not able to bind reuteran or IMMP (Fig. 8), which also was confirmed by isothermal titration calorimetry (not shown). However, SusD also did not interact with any soluble glucans tested, including soluble potato starch, amylopectin, and pullulan (Fig. 8), while the appropriate control protein *Tm*CBM41 showed the expected binding pattern (Fig. 8). SusD recognizes amylose helices in starch, and due to its crystallinity, amylose exists as an insoluble polymer. To see whether solubility of the substrate, and not the experimental
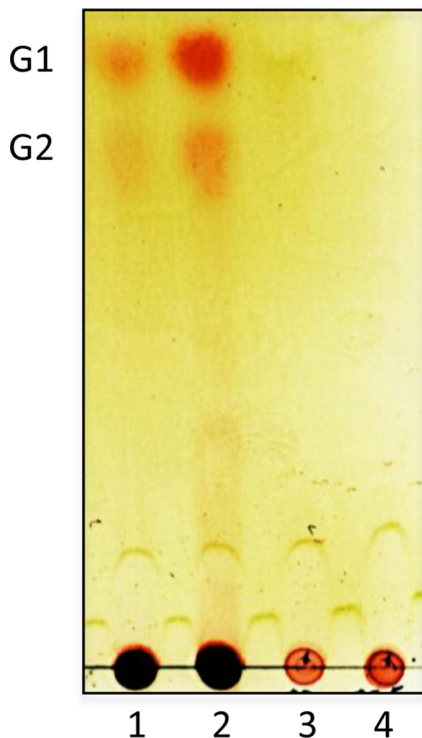

FIG 5 Thin-layer chromatography analysis of the products of degradation on reuteran and IMMP incubated with culture supernatants (CS) of *B. thetaiotaomicron* grown in the presence of either substrate. Lane 1, reuteran and reuteran CS; lane 2, IMMP and IMMP CS; lane 3, reuteran and glucose CS; lane 4, IMMP and glucose CS. Culture supernatants were incubated with EPS substrate for 1 h before being spotted on a TLC plate.
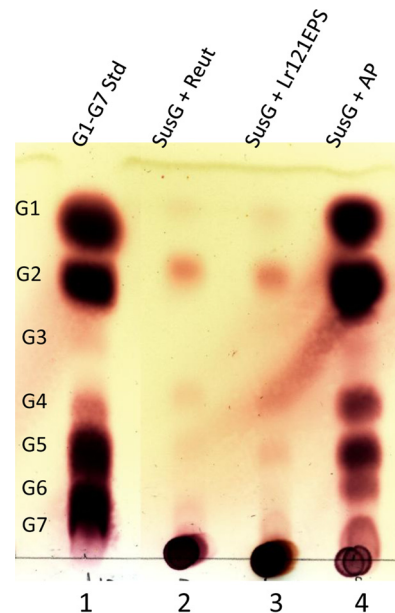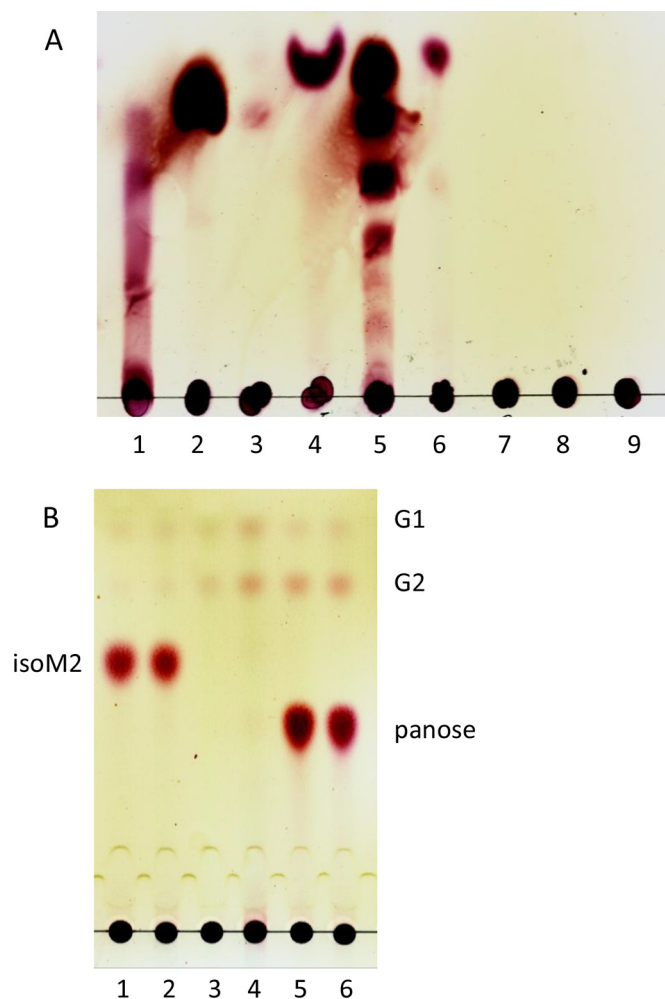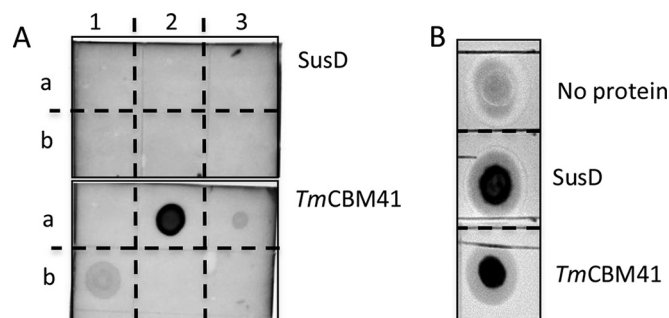

FIG 6 Thin-layer chromatography of reuteran hydrolysis by SusG. All reaction mixtures contain 10 mg/ml polysaccharide and 22 μg SusG in 20 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM CaCl$_2$. Reaction products were sampled after 4 h of incubation at 37°C. Lane 1, G1 to G7 maltooligosaccharide standards (Std); lane 2, reuteran (Reut); lane 3, Lr121EPS; lane 4, amylopectin (AP).

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FIG 7 (A) Thin-layer chromatography analysis of the products of reuteran digestion by various α-glucan-degrading enzymes. Lane 1, *Pseudomonas* species isoamylase (EC 3.2.1.68); lane 2, *B. licheniformis* α-amylase (EC 3.2.1.1) (the dark spot is sucrose from enzyme storage solution); lane 3, *Microbacterium aurum* amylase A1 (EC3.2.1.1); lane 4, *Aspergillus niger* amyloglucosidase (EC 3.2.1.3); lane 5, *Bacillus acidopullulyticus* pullulanase (EC 3.2.1.41); lane 6, *L. reuteri* GTFA (EC 2.4.1.5); lane 7, GTF180; lane 8, *Microbacterium aurum* amylase B; lane 9, untreated reuteran control. (B) Activity of culture supernatants from *B. thetaiotaomicron* grown on reuteran with the addition of dextran- or pullulan-derived oligosaccharides isomaltose, maltose, and panose. Lane 1, reuteran plus isomaltose supernatant only; lane 2, reuteran plus isomaltose supernatant with reuteran; lane 3, reuteran plus maltose supernatant only; lane 4, reuteran plus maltose supernatant with reuteran; lane 5, reuteran plus panose culture supernatant only; lane 6, reuteran plus panose culture supernatant with reuteran. The addition of small oligosaccharides did not increase the ability of *B. thetaiotaomicron* to degrade reuteran. Results also indicate that isomaltose and panose are not transported by *B. thetaiotaomicron*, since they remain in the culture supernatant.

FIG 8 (A) Carbohydrate macroarray analysis of SusD binding to soluble α-glucans. A1, 1% pullulan; A2, 1% soluble potato starch; A3, 1% amylopectin; B1, 1% reuteran; B2, 1% IMMP; B3, 1% dextran. (B) Pulldown assay of SusD with insoluble wheat starch. *Tm*CBM41 is a control for α-glucan binding activity and displays expected binding patterns (29). FITC-labeled SusD and *Tm*CBM41 binding were carried out in 20 mM Tris, 150 mM NaCl, 5 mM CaCl₂, and 0.05% Tween 20 at room temperature for 1 h. Binding was visualized at 520 nm.

targeted degradation by *B. thetaiotaomicron*, it is likely that the lack of SusD binding with reuteran and IMMP prevents utilization of this polymer. The results of SusG activity and SusD binding experiments suggest it is a combined effect of poor SusD interaction and poor SusG enzymatic activity on IMMP and reuteran substrates that makes reuteran plus IMMP a metabolically poorer growth substrate for *B. thetaiotaomicron* than β-fructan EPS.

DISCUSSION

This study demonstrates that *B. thetaiotaomicron*, a human gut commensal organism, is able to actively target and metabolize microbially synthesized carbohydrates, such as EPS produced by probiotic LAB strains, and achieves this by activating dedicated PUL systems to target EPS molecules. β-Fructan EPS metabolism was most prominent in the experiments where levan EPS activated the fructan-associated PUL, and cell surface levanase enzymes were able to fully metabolize levan to fructose monosaccharide, following previously established data showing that *B. thetaiotaomicron* degrades levan (β-2,6-fructan) but cannot degrade inulin (β-2,1-fructan) (22). Reuteran and IMMP trigger the expression of *sus* and dextran-associated PUL components, respectively (Table 1), as observed in proteomic analysis of culture supernatants. Reuteran and IMMP partial degradation may be linked to two factors. First, reuteran and IMMP are unable to be recognized by the transport component SusD, which is shown to be essential for growth on starch (Fig. 8A) (24, 26). Koropatkin et al. have shown that mutants of *B. thetaiotaomicron* lacking SusD are not able to grow on starch, showing that SusD recognition is essential for substrate utilization by *B. thetaiotaomicron* (26). SusD is closely associated with the transmembrane transporter SusC and facilitates transport of larger (molecules greater than 5 glucose units [>DP5]) maltooligosaccharides into the cell. Without an interaction between SusD and reuteran or IMMP, full metabolism of glucan EPS substrates would not be achieved. Second, the *sus* PUL-encoded CAZyme SusG encounters difficulties in degrading reuteran. Reuteran has a much more complex structure than starch, as it contains a much larger proportion of alternating α-1,4- and α-1,6-glycosidic linkages (Fig. 1A). Although reuteran and IMMP activate the production of starch- and dextran-degrading enzymes, it is most likely the unique architecture of LAB

setup, was the problem, pulldown assays were performed using insoluble starch as a substrate for SusD binding. In this case it was observed that SusD efficiently bound to insoluble wheat starch, suggesting that SusD is able to interact only with crystalline amylose. Thus, it appears that SusD is limited to interacting with insoluble α-1,4-linked glucose polymers, such as amylose, and is not able to interact with soluble α-glucans, including soluble starch molecules, reuteran, and IMMP. Since the interaction of SusD with its cognate PUL substrate is an important step in substrate-

α-glucan EPS polymers that causes inaccessibility issues for α-amylase SusG and also for SusD to bind to the substrate. The complexity of the substrate also may be why there is an apparent lack of growth on IMMP, a dextran-like molecule containing large stretches of α-1,6-linked glucose residues. As yet there are no studies on dextran metabolism by *B. thetaiotaomicron* arising from dextran-associated PUL activation, making it difficult to speculate why IMMP was not degraded by *B. thetaiotaomicron*. Other studies on LAB α-glucan-based EPS molecules have shown that large α-glucan polymers synthesized by purified glucansucrases from lactic acid bacteria are fermented in fecal inocula (34, 36). Thus, even though *B. thetaiotaomicron* cannot fully metabolize these polymers, there still may be cross-feeding that occurs between bacterial taxa in the gut environment.

EPS degradation by *B. thetaiotaomicron* may confer two main advantages to the bacterium. First, in addition to dietary or mammalian carbohydrate sources, microbial sources also would provide an abundant and continuous nutritional source for *B. thetaiotaomicron* which would further add to its success as an established human gut symbiont. Second, by digesting EPS from competing gut microbiota in the intestinal tract, *B. thetaiotaomicron* would leave the competition vulnerable to clearance from the gastrointestinal tract, as the competition lacking EPS would lose its ability to efficiently adhere to the intestinal cell wall, while removal of the protective outer EPS layer would expose competing bacteria to cells of the innate immune system that reside in the intestinal mucosal layer.

Overall, our results describe a novel role for PUL activation in *B. thetaiotaomicron* directed at metabolism of LAB-derived EPS molecules. Activation of PULs in response to microbially derived carbohydrates has, to our knowledge, not been described before. EPS molecules are differentially metabolized, with levan being completely consumed, reuteran being partially consumed in the absence of levan, and very little IMMP consumed. If we consider the observations by McNulty et al. (20), who demonstrated that genes for levan and starch carbohydrate metabolism are upregulated in commensal bacteria in the presence of probiotic species, the results of this study using the model glycolytic bacterium *B. thetaiotaomicron* provide evidence for the first time that increased activation of genes for carbohydrate metabolism in commensal species is connected to the metabolism of EPS molecules produced by probiotic LAB strains. Other studies indicate EPS from *Bifidobacterium* as fermentable substrates for intestinal bacteria (37, 38), suggesting a more widespread mechanism of EPS metabolism by human gut commensal bacteria occurring in the gastrointestinal tract.

Conclusion. *B. thetaiotaomicron* activates distinct polysaccharide utilization loci to degrade microbially synthesized polysaccharides, describing a novel role for PULs within *Bacteroides*. *L. reuteri* EPS molecules were differentially metabolized in the order of levan, reuteran, and IMMP. This phenomenon provides an explanation as to why genes for carbohydrate metabolism, specifically levanases and enzymes for starch metabolism, are significantly upregulated by commensal bacteria during probiotic supplementation (20). Bacterial EPS is likely an important alternative energy source for commensal bacterial species residing in the gut and may play an important role in how microbial communities are shaped in our gastrointestinal tract. Future research is aimed at further studying how EPS, and other microbially derived carbohydrates, are metabolized by *B. thetaiotaomicron* via the action of genome-based PULs and assessing the importance of microbially derived carbohydrates associated with transient and persistent microbial colonizers of the gut.

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