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Functional Genomic and Metabolic Studies of the Adaptations of a Prominent Adult Human Gut Symbiont, *Bacteroides thetaiotaomicron*, to the Suckling Period*[S]

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The adult human gut microbiota is dominated by two divisions of Bacteria, the Bacteroidetes and the Firmicutes. Assembly of this community begins at birth through processes that remain largely undefined. In this report, we examine the adaptations of *Bacteroides thetaiotaomicron*, a prominent member of the adult distal intestinal microbiota, during the suckling and weaning periods. Germ-free NMRI mice were colonized at birth from their gnotobiotic mothers, who harbored this anaerobic Gram-negative saccharolytic bacterium. *B. thetaiotaomicron* was then harvested from the ceca of these hosts during the suckling period (postnatal day 17) and after weaning (postnatal day 30). Whole genome transcriptional profiles were obtained at these two time points using custom *B. thetaiotaomicron* GeneChips. Transcriptome-based *in silico* reconstructions of bacterial metabolism and gas chromatography-mass spectrometry and biochemical assays of carbohydrate utilization *in vivo* indicated that in the suckling gut *B. thetaiotaomicron* prefers host-derived polysaccharides, as well as mono- and oligosaccharides present in mother's milk. After weaning, *B. thetaiotaomicron* expands its metabolism to exploit abundant, plant-derived dietary polysaccharides. The bacterium's responses to postnatal alterations in its nutrient landscape involve expression of gene clusters encoding environmental sensors, outer membrane proteins involved in binding and import of glycans, and glycoside hydrolases. These expression changes are interpreted in light of a phylogenetic analysis that revealed unique expansions of related polysaccharide utilization loci in three human alimentary tract-associated Bacteroidetes, expansions that likely reflect the evolutionary adaptations of these species to different nutrient niches.

Our adult gut is colonized with a community of 10–100 trillion microbes. This microbiota, and its collective genome (microbiome), provide us with important physiological attributes that are not encoded in our own human genome, including the ability to break down otherwise indigestible nutrients that are delivered to the distal gut, such as dietary plant polysaccharides (1, 2). A recent comprehensive 16 S rRNA-based enumeration study of the distal intestinal microbiota of a small number of healthy adult humans demonstrated that >99% of detected phylogenetic types (phylotypes) belong to two of the 70 bacterial divisions (superkingdoms) currently known in nature: the Bacteroidetes and the Firmicutes (3, 4). Within each division there is great diversity at the species and subspecies levels. Moreover, these shallow lineages show considerable variation between individual humans (3, 4).

The mechanisms controlling assembly of our microbiotas remain ill defined. This issue can be framed as follows. What is the effect of the microbial community that is available to colonize a host at the time of birth (the legacy effect)? What is the effect of the gut environment itself on shaping the available community, and is the gut selecting for properties that are common or unique to members of bacterial divisions (the host effect)? Recent reciprocal transplantation experiments have emphasized the importance of host habitat: when the gut microbiota of a conventionally raised mouse is introduced into a germ-free zebrafish recipient, and vice versa, the recipient gut acts as a biological filter to amplify members of the donor community that most closely resemble its normal (native) species. (5).

Our gut-associated Firmicutes and Bacteroidetes have not been identified outside of the intestinal habitat. Thus, they are likely acquired by transmission from our mothers or other family members soon after birth. This notion is supported by several observations. Studies using C57Bl/6J mice revealed that offspring inherit microbiotas similar to those of their mothers (6). The effect of kinship was evident across generations: C57Bl/6J mothers who are sisters have gut microbiotas that are similar to one another and to those of their offspring (6). Culture-based studies of humans indicate that infants, who are germ-free *in utero*, also acquire their initial microbiota from the vaginal and fecal microbiota of their mothers (7, 8). The extent to which host genotype influences the composition of the postnatally acquired microbiota is unclear. 16 S rRNA fingerprinting studies of a small number of adult monozygotic twin pairs indicated their fecal microbiotas were more similar to one another than to their marital partners. However, the same trend was true for dizygotic twins, underscoring the importance of a shared mother (9).

Although many studies have been carried out to decipher the

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composition of the fecal microbiota during the postnatal period, the majority have used culture-based methods and hence are subject to sampling bias and to limited sensitivity (10, 11). To date, there are no reports describing the results of a comprehensive 16 S rRNA sequence-based enumeration of gut microbial communities during the postnatal period, in related or unrelated infants. Therefore, we do not know whether there is an orderly succession of colonizers leading to a climax community, or whether there is a period of open occupation followed by selection of what will become persistent (autochothonous) members of the microbiota.

Culture-based enumeration studies have yielded disparate results that may reflect host and/or legacy effects. Facultative anaerobes are commonly identified among the early colonizers and are succeeded by obligate anaerobes (12–15). However, prominent obligate anaerobes that are members of the adult microbiota have been identified as early as postnatal day 3 in vaginally delivered infants (14, 16), suggesting that adult-associated species establish an early presence in this environment.

In the present study, we address the question of how dominant members of the adult microbiota are able to persist during the suckling period so that they can expand to levels of prominence after the transition from a diet of mother's milk to one rich in plant-derived complex polysaccharides. For this analysis, we selected *Bacteroides thetaiotaomicron*, a Gram-negative anaerobe that comprised 6% of all bacteria and 12% of all Bacteroidetes in the most comprehensive 16 S rRNA sequence-based enumeration of the adult human colonic microbiota published to date (3). One indication of *B. thetaiotaomicron's* adaptation to life in the adult gut is its large collection of genes dedicated to the acquisition and metabolism of plant and other dietary polysaccharides encountered in our postweaning diets (17). The *B. thetaiotaomicron* genome is predicted to encode 226 glycoside hydrolases and 15 polysaccharide lyases, allowing it to break down polysaccharides that we cannot process on our own because our proteome lacks the requisite enzymes (*e.g. B. thetaiotaomicron* contains 64 arabinosidases, xylanases, and pectate lyases, whereas we have none; see Carbohydrate Active Enzymes data base (CAZy)[3] at afmb.cnrs-mrs.fr/CAZY/for a comprehensive annotation). The *B. thetaiotaomicron* genome also contains 209 paralogs of two cell surface proteins that bind starch (107 paralogs of SusC; 102 paralogs of SusD). SusC paralogs are predicted TonB-dependent, β-barrel-type outer membrane proteins; thus, in addition to binding polysaccharides, they likely participate in energy-dependent transport of these carbohydrates into the periplasmic space (18). SusD paralogs are predicted to be secreted proteins with an N-terminal lipid tail that allows them to associate with the outer membrane (19). *B. thetaiotaomicron* also contains an expanded set of environmental sensors and regulators that includes 50 extracytoplasmic function (ECF) σ factors and 26 anti-σ factors, 79 members of classic two-component systems, plus 32 novel "hybrid" two-component systems that incorporate all of the domains encountered in a two-component system into a single polypeptide (17). At least some of the latter proteins are used by *B. thetaiotaomicron* to sense polysaccharides and coordinately regulate genes involved their metabolism (20).

Genes encoding SusC and SusD paralogs are typically positioned adjacent to one another in the *B. thetaiotaomicron* genome (102 of 107 loci containing *susC* paralogs) and are often part of larger gene clusters encoding enzymes involved in carbohydrate metabolism (62 of 107 loci) (17). At least 18 of the 62 clusters that include glycoside hydrolases also contain ECF-type σ factors and adjacent anti-σ factors. This arrangement suggests that the polysaccharide binding, import, and catabolism functions represented in these clusters are co-regulated to allow for efficient, prioritized, and adaptive foraging of glycans based on their availability. Our analysis, described below, of the adaptations of *B. thetaiotaomicron* to the suckling period in a simplified gnotobiotic mouse model supports this view: it emphasizes the importance of host mucosal polysaccharides in supporting *B. thetaiotaomicron* during the suckling period (habitat effect) and links the evolution of these polysaccharide utilization clusters to their coordinate regulation.

EXPERIMENTAL PROCEDURES

Mother-to-Pup Transmission of B. thetaiotaomicron—All of the experimental manipulations involving mice used protocols approved by the Washington University Animal Studies Committee. Mice belonging to the NMRI-KI inbred line were reared in gnotobiotic isolators (21) under a strict 12-h light cycle (lights on at 6:00 a.m.) and given unlimited access to autoclaved water and an autoclaved standard polysaccharide-rich chow diet (B&K Universal, Grimston, UK).

In a given experiment, one male and two pregnant female NMRI mice were gavaged with 100 μl of an overnight culture of *B. thetaiotaomicron* strain VPI-5482 (ATCC 29148) containing 10^8 colony-forming units in TYG medium (20). Littermates were sacrificed at postnatal day 17 (P17) and P30. The stomachs of all P17 animals were surveyed to verify that there were no chow particles present. The density of bacterial colonization of the cecum and colon was determined based on colony-forming units and quantitative real time PCR assays (20, 22).

Whole Genome Transcriptional Profiling of B. thetaiotaomicron in Vivo—Ceca from P17 mice were snap frozen in liquid nitrogen. After thawing in RNAprotect solution (1 ml/sample; Qiagen), each cecum was cut open and vortexed for 10 s at 25 °C to separate luminal contents from the mucosa, and the mixture was centrifuged (1,200 $\times$ *g* for 10 s) to remove tissue fragments. The resulting supernatants were subjected to a bacterial cell lysis protocol described below. The contents were removed from P30 ceca by manual extrusion from their distal ends and snap frozen in liquid nitrogen. The samples were later thawed in 1 ml of RNAprotect prior to lysis.

Cecal samples from P17 mice were lysed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 1 mg/ml of lysozyme (specific activity 50,000 units/mg; Sigma; 10 min of incubation at room temperature). Cecal samples from P30 mice were combined with 500 μl of 200 mM NaCl/20 mM EDTA, and the cells were lysed by bead beating (6). Total RNA was isolated and

[3] The abbreviations used are: CAZy, Carbohydrate Active Enzymes; ECF, extracytoplasmic function; P*n*, postnatal day *n*; COG, clusters of orthologous groups; GH, glycoside hydrolase; PL, polysaccharide lyase; *CPS*, capsular polysaccharide synthesis.

prepared for hybridization to custom *B. thetaiotaomicron* GeneChips according to Sonnenburg *et al.* (1).[4]

Microarray Suite 5 software (Affymetrix) was used for initial data analysis. Selection criteria for identifying genes that were differentially expressed between P17 and P30 ceca were 100% present calls for the condition where the average signal for a given probe set was higher, and a false discovery rate cut-off of 1% based on significance analysis of microarrays (v2.20) analysis of the data set (23). Genes satisfying these criteria were categorized using COGs (clusters of orthologous groups) (23, 24). Hypergeometric distribution was used to identify significantly overrepresented COGs. Bacterial genes whose expression changed at P17 compared with P30 or vice versa were also searched against (i) the CAZy data base to identify enzymatic activities related to polysaccharide metabolism and (ii) MetaCyc (25) to place genes onto known metabolic and signaling pathways.

GeneChip Profiling of B. thetaiotaomicron during Growth in Vitro—An overnight 10-ml culture of *B. thetaiotaomicron*, grown in an aqueous solution of 10% tryptone (Becton Dickinson Co.) and 1.9 μM hematin (Sigma), was introduced into the 1.2-liter reaction vessel of a Bioflo 110 batch culture fermentor (New Brunswick Scientific) containing 800 ml of the same 10% tryptone medium. The bacteria were then incubated at 37 °C with agitation at 100 rpm under an atmosphere of 20% CO_2, 80% N_2 (sparged at 0.1 liter/min). During mid-log phase ($A_{600} = 0.3$), lactose, glucose, or galactose was added to a final concentration of 0.2%. Aliquots of the culture (10 ml) were removed immediately before and 60 min after carbohydrate addition and placed in RNAprotect. Bacterial cells were collected by centrifugation (3,000 $\times$ *g* for 15 min at 4 °C), and the samples were prepared for hybridization to *B. thetaiotaomicron* GeneChips as above (1).

Biochemical Assays of Cecal Contents—Gas chromatography-mass spectrometry analysis of cecal contents was carried out as detailed in Ref. 1. Other biochemical assays were conducted on cecal contents that had been freeze-dried and resuspended in 400 μl of ice-cold buffer containing 0.2 M NaOH and 1 mM EDTA. Aliquots (80 μl) of this mixture were removed, and an alkaline extract was prepared by incubation at 80 °C for 20 min, followed by placement on ice and neutralization (80 μl of a solution containing 0.25 M HCl and 100 mM Tris base). An acid extract was prepared by taking another 60-μl aliquot, adding 20 μl of 0.7 M HCl, incubating the material for 20 min at 80 °C, placing the solution back on ice, and adding 40 μl of Tris Base. All of the extracts were stored at −80 °C. The levels of ATP and NADH were measured in the alkaline extracts, whereas glucose, galactose, lactose, and NAD^+ were assayed in acid extracts using well established pyridine nucleotide-based enzyme cycling methods (26). The data are expressed relative to the protein content of the cecal sample (Bradford assay; Bio-Rad).

ClustalW Analysis of SusC/SusD Paralogs—Pairs of genes encoding SusC and SusD paralogs were identified in the genome sequences of *B. thetaiotaomicron* VPI-5482 (17), *Bacteroides fragilis* NCTC 9343 (27), *B. fragilis* YCH46 (28), and *Porphyromonas gingivalis* (29) by performing individual BLAST searches against each genome using amino acid sequences of previously annotated SusC and SusD paralogs as queries (30). The low scoring hits from each search (*E* values between 10^{-4} and 10^{-10}) were themselves used as BLAST queries to reveal more divergent putative paralogs in each genome; this process was repeated until no new paralogs were identified. Lists of putative SusC and SusD paralogs were compared for each species. Paralogs were included in a subsequent clustalW analysis based on the requirement that each had a separately predicted, adjacent partner (this process was instrumental in excluding related TonB-dependent hemin, vitamin B_{12}, and iron-siderophore receptors from the list of putative SusC paralogs). The resulting data set included 240 paralog pairs: 102 pairs in *B. thetaiotaomicron*, 69 pairs in *B. fragilis* NCTC 9343, 65 pairs in *B. fragilis* YCH46, and four pairs in *P. gingivalis*.

Multiple sequence alignments were generated for SusC and for SusD paralogs using clustalW (31). Tree determination (neighbor-joining method) and bootstrapping were performed using Paup 4.0b10 (32), based on the clustalW alignment. Cladogram trees were drawn from the consensus of 100 trees that were generated for each alignment, with *P. gingivalis* RagA/RagB (homologs of SusC/SusD) proteins selected as arbitrary roots. Branches with bootstrap values ≥70% were retained.

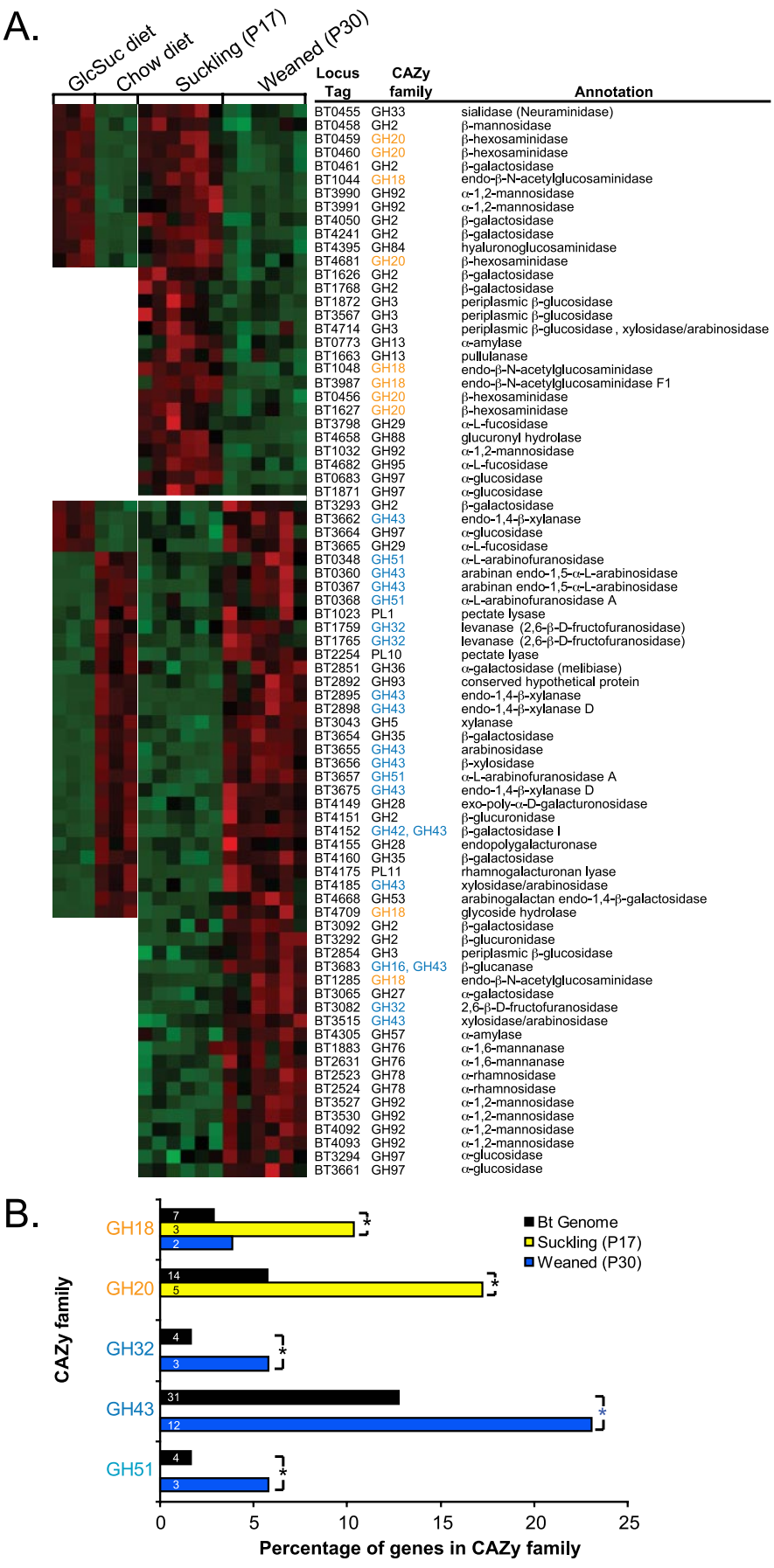
Comparison of the SusC and the SusD cladograms suggested that these two protein families resolve into nearly identical branches (supplemental Table S1), supporting the notion that they diverged in parallel with one another during their expansion in these genomes: 141/240 SusC paralogs (58.8%) were resolved into 24 different clades exhibiting 100% bootstrap values; 139 of these (98.6%) had SusD partners that were resolved into similar (2 of 24) or identical (22 of 24) clades in a SusD cladogram (data not shown). Because of this observation and to increase the resolving power of our cladogram analysis, the predicted amino acid sequences of each protein encoded by a given *susC/susD* pair were joined *in silico* to generate a single amino acid sequence for each pair and then entered into clustalW for neighbor-joining alignment as described above.

RESULTS AND DISCUSSION

B. thetaiotaomicron Transfer from Gnotobiotic Mothers to Their Offspring—Pregnant 10–14-week-old germ-free mice belonging to the NMRI inbred strain were inoculated with *B. thetaiotaomicron* on the 10th day of gestation. After they gave birth, a subset of suckling pups in each litter was sacrificed at P17. Another subset was killed at P30, after weaning to a standard autoclaved polysaccharide-rich chow diet (initiated at P20). Levels of colonization were quantified in the cecum, an anatomically distinct structure interposed between the distal small intestine and colon that allows for ready and reliable harvest of luminal contents, and in the colon.

B. thetaiotaomicron colonized all of the pups surveyed. P17 pups contained $7.03 \pm 1.49 \times 10^{11}$ and $1.39 \pm 0.38 \times 10^{12}$ colony-forming units/ml of cecal and colonic contents, respectively. The corresponding values for P30 mice were $1.42 \pm 0.17 \times 10^{12}$ and $1.78 \pm 0.43 \times 10^{12}$ colony-forming units/ml

[4] All of the GeneChip data used in this study are available from the Gene Expression Omnibus data base (www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE279.



(means ± S.E., $n = 11$ litters, 2–10 mice/litter; $p < 0.01$ for P17 *versus* P30 cecum; no significant difference ($p > 0.05$) for colon)). Thus, in the absence of competitors, *B. thetaiotaomicron* can reliably colonize the suckling mouse intestine at densities that are only slightly lower in the cecum compared with the colon. The levels of colonization in these regions of the distal gut are similar to what we have observed in adult mice who were gavaged once with the same size inoculum at 6–10 weeks of age and then sacrificed 2 or 4 weeks later (1).

In Vivo Transcriptional Profiling of B. thetaiotaomicron at P17 and P30—To gain insight into how *B. thetaiotaomicron* adapts to the cecal habitats of suckling and weaned mice, we performed whole genome transcriptional profiling of the bacterium using GeneChips containing probe sets representing 98.6% of its 4,779 predicted protein-coding genes (1). RNA was isolated from cecal contents and then pooled from three to four P17 pups from each litter (pooling was necessary to obtain sufficient material) or from cecal contents harvested from individual littermates that had been allowed to live until P30. Individual RNA samples from six P30 animals, representing three different litters, and six pools of RNA from twenty-three P17 animals, representing five litters, were used as templates for synthesis of cDNA targets; each of the 12 targets was hybridized to a separate *B. thetaiotaomicron* GeneChip. Analysis of the resulting GeneChip data sets using significance analysis of microarrays software (23) and the selection criteria described under "Experimental Procedures" identified 1,266 genes whose expression was defined as significantly different between the two ages; 583 were higher in *B. thetaiotaomicron* colonizing the ceca of P17 compared with P30 mice, whereas 683 were expressed at higher levels at P30 (for a list, see supplemental Table S2). Differentially expressed genes were then grouped according to their COG assignments (supplemental Fig. S1). By comparing the percentage representation of the groups of regulated genes in a given COG, with the percentage representation all *B. thetaiotaomicron* genes in that COG, we determined that four COGs were significantly "overrepresented" among genes that were differentially expressed in either condition ($p < 0.05$). "Translation" (COG J) was the most overrepresented COG at the suckling stage, whereas "Carbohydrate transport and metabolism" (COG G) and "Cell wall/membrane biogenesis" (COG M) were most overrepresented at the weaned state. COG C ("Energy Production and Conversion") was overrepresented in both states.

B. thetaiotaomicron Exhibits Increased Growth Rate in the Ceca of Suckling Mice—Sixty-four percent (50 of 78) of the genes in the translation COG that were significantly up-regulated during the suckling period encode proteins associated with either 30 or 50 S ribosomal subunits or were tRNA synthetases; together, they represent 80% (16 of 20) of all 30 S ribosomal genes and 76% (25 of 33) of all 50 S ribosomal genes in the *B. thetaiotaomicron* genome (supplemental Table S2). In addition, two operons, one encoding a polyamine (spermidine/putrescine) transport system (*BT1268–BT1291*) and the other encoding a Na^+/sulfate symporter and acquisition system (*BT0412–BT0415*), were significantly up-regulated at P17 compared with P30 (supplemental Fig. S2*A*). Increased polyamine synthesis is an established biomarker of increased translation rate in bacteria (33), whereas sulfate can be targeted to L-cysteine and L-methionine biosynthetic pathways to sustain increased translation.

Biochemical analysis of cecal contents from *B. thetaiotaomicron* mono-associated mice confirmed that free glucose and lactose levels are significantly higher at P17 compared with P30 ($n = 3–4$ animals/group; $p < 0.05$; supplemental Fig. S2*B*). Two glucose/galactose transporters (*BT4311* and *BT4653*) are expressed at higher levels in the ceca of P17 animals (supplemental Table S2), consistent with the notion that *B. thetaiotaomicron* utilizes the abundant pools of glucose and lactose present. We also noted that predicted operons encoding (i) two different Na^+ translocating NADH·ubiquinone oxidoreductase complexes (*BT0617–BT0621* and *BT1155–BT1160*); (ii) components of an electron transfer flavoprotein (*BT1804–BT1805*), which likely assists in passing electrons from NADH dehydrogenase to ubiquinone (34); and (iii) a V-type ATPase (ATP synthase) involved in Na^+/H^+ transport (*BT1296–BT1299*), are all expressed at higher levels during the suckling period, as are a Na^+/solute symporter (*BT1082*) and a H^+/peptide symporter (*BT0580*) (supplemental Table S2), suggesting that these functions aid in maintaining Na^+ and H^+ gradients for importing sugars and metabolites (35, 36).

When cultures of *B. thetaiotaomicron* were grown to mid-log phase in a batch culture fermentor containing 10% tryptone (tryptic digest of the prominent milk protein casein), and glucose, galactose, or lactose was added ($n = 2–4$ biological replicates/condition), a rapid increase in growth rate ensued (supplemental Fig. S2*C*). Moreover, GeneChip profiling prior to and 60 min after the addition of these sugars revealed a dominant increase in expression of genes in the translation COG (supplemental Fig. S2*D*), including 95% (19 of 20) of the 30 S ribosomal genes of the organism, and 100% (33 of 33) of its 50 S ribosomal genes (supplemental Fig. S2*A*). This positive correlation between *B. thetaiotaomicron* growth rate *in vitro* and increased expression of translation functions supports the notion that *B. thetaiotaomicron* divides more rapidly in the ceca of P17 compared with the P30 mice, at least in part because of the increased amounts of free lactose, glucose, and galactose in

FIGURE 1. **Regulation of GH and PL of *B. thetaiotaomicron*.** *A*, heat map of bacterial GH and PL genes with significantly changed expression in suckling (P17) *versus* weaned (P30) ceca and in adult mice fed a glucose-sucrose (*GlcSuc*) *versus* a polysaccharide-rich chow diet. *Red* indicates higher levels and *green* indicates lower levels of gene expression compared with the mean value for all conditions. Of the 241 known or predicted *B. thetaiotaomicron* GH and PL genes, 29 show significantly higher expression during the suckling period, and 50 show significantly higher expression during the weaned period. Note the overlap of bacterial genes up-regulated in the ceca of adult gnotobiotic mice fed a glucose-sucrose diet and in P17 mice. The absence of *red* or *green boxes* in the columns marked *GlcSuc* indicates that the level of expression of these genes was not significantly changed compared with a standard chow diet. *B*, disproportionate representation of genes belonging to five families of GH and PLs annotated in the CAZy data base, relative to their representation in the *B. thetaiotaomicron* genome. "Percentage of genes" refers to the number of genes within a given CAZy family (indicated by the *number* within the *bars*) that belong to a given group (suckling or weaned or the genome) divided by the total number of *B. thetaiotaomicron* genes belonging to all CAZy GH and PL families. *, $p < 0.05$.

mother's milk that it can access under noncompetitive conditions.

B. thetaiotaomicron Uses Host-derived Glycans during the Suckling Period—To further investigate the *B. thetaiotaomicron* metabolic response to the cecal nutrient milieu present in the suckling *versus* weaning period, we examined expression of genes encoding glycoside hydrolases (GH) and polysaccharide lyases (PL). Of the 79 GH and PL genes exhibiting significantly changed expression during the two periods, 29 were expressed at higher levels during the suckling period (Fig. 1*A*). Based on their genome annotations and the functional classification scheme described in the CAZy data base (afmb.cnrs-mrs.fr/CAZY/), these 29 enzymes exhibit significantly enriched representation in two families (GH18 and GH20; Fig. 1*B*). They are predicted to cleave a range of glycosidic linkages, including some that are commonly associated with host *O*- and *N*-linked glycans and glycosaminoglycans that are present in mucus and the underlying gut epithelium (five β-hexosaminidases, three β-*N*-acetylglucosaminidases, a sialidase, a hyaluronoglucosaminidase, and a glucuronyl hydrolase). We also observed that five β-galactosidases, three α-1,2-mannosidases, two α-fucosidases, and two α-glucosidases were also induced at P17; these activities are predicted to target monosaccharide linkages present in *both* host-derived and dietary glycans.

Our previous studies of germ-free adult NMRI mice that had been colonized for 10 days with *B. thetaiotaomicron* alone and fed a polysaccharide-rich chow diet or a polysaccharide-deficient glucose-sucrose diet revealed that in the absence of dietary polysaccharides, bacteria turn to host glycans as a nutrient source (1). Twelve of the bacterial GH genes induced in the ceca of adult mice fed a glucose-sucrose diet are also induced in the ceca of suckling P17 animals, including activities that likely facilitate utilization of sugars contained primarily in host polysaccharides, *e.g.* hexosamines and sialic acids (Fig. 1*A*).

In humans, milk oligosaccharides contain core structures composed of lactose and *N*-acetylglucosamine with terminal fucose and sialic acid residues (37), some of which are resistant to hydrolysis by host glycoside hydrolases (38). *In silico* reconstructions of *B. thetaiotaomicron* metabolic pathways, obtained by placing the enzyme products of genes identified from our GeneChip data sets as being up-regulated in P17 mice onto the MetaCyc maps (25), revealed that components of pathways involved in catabolism of host- or milk-derived monosaccharides are up-regulated in concert with the GH and PL activities that liberate them. Examples include an operon involved in the metabolism of fucose, as well as genes involved in metabolism of *N*-acetylglucosamine (supplemental Fig. S3*A*). Taken together, these results led us to conclude that within the ceca of P17 mice *B. thetaiotaomicron* degrades milk- and host-derived glycans and assimilates these nutrients as energy sources.

B. thetaiotaomicron Forages on Plant-derived Dietary Polysaccharides in the Fully Weaned Intestine—The *B. thetaiotaomicron* populations in P30 mice experience a different gut environment relative to that in P17 mice, resulting from the removal of mother's milk and introduction of a chow diet rich in plant-derived polysaccharides (plant cell walls and starch). Plants cell walls are composed of three principal types of polysaccharides: cellulose (β1–4-linked glucose polymer),

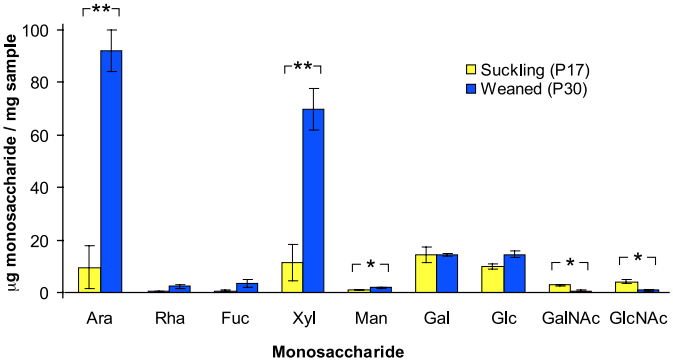
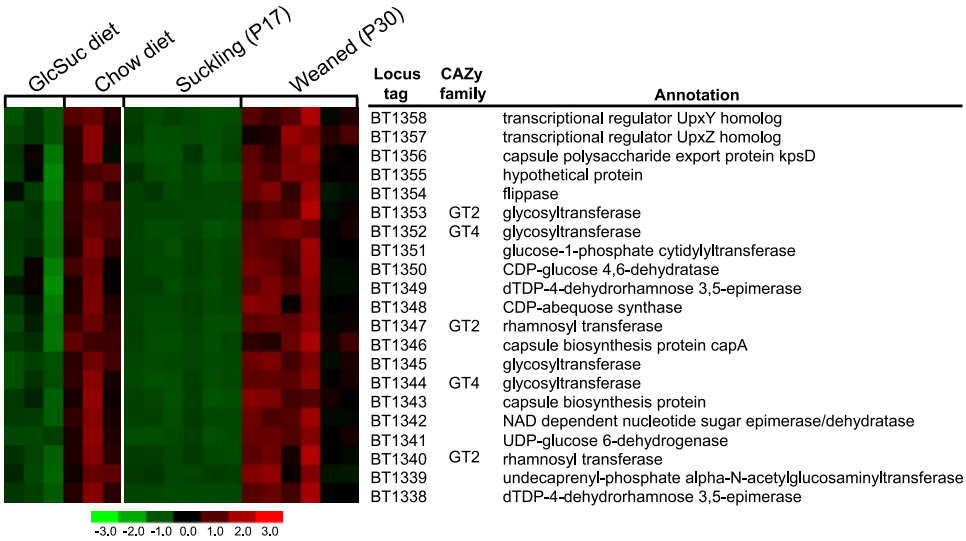


FIGURE 2. **Gas chromatography-mass spectrometry analysis of carbohydrates present in the ceca of *B. thetaiotaomicron*-colonized suckling and weaned gnotobiotic mice.** Cecal contents were subjected to acid hydrolysis to liberate monosaccharides from oligo- and polysaccharides ($n = 3$ animals independently assayed/time point). *, $p < 0.05$; **, $p < 0.01$.

hemicellulose (contains primarily glucose, xylose, and arabinose), and pectin (largely composed of galacturonic acid, galactose, and rhamnose) (39). Consistent with the monosaccharide composition of plant cell walls and starch, the predominant neutral sugars in the chow consumed by our weaned mice are glucose, arabinose, xylose, and galactose (10:8:5:1 ratio) (1). Acid hydrolysis and subsequent gas chromatography-mass spectrometry analysis of the cecal contents of P17 *versus* P30 mice revealed that arabinose and xylose are significantly more abundant in P30 compared with P17 cecal contents (Fig. 2). Fifty of the 79 GH and PL genes identified as differentially regulated in our GeneChip analysis exhibited increased expression in the ceca of P30 mice (Fig. 1 and supplemental Fig. S4). This group is enriched for activities predicted to participate in liberating sugars from plant polysaccharides (arabinosidases, xylanases/xylosidases, polygalacturonases, levanases, rhamnosidases, and pectate lyases); this is reflected by the overrepresentation of CAZy families 32, 43, and 51 (Fig. 1). Transcriptome-directed *in silico* reconstruction of *B. thetaiotaomicron* metabolism revealed that genes in pathways for xylose, arabinose, and rhamnose utilization are expressed at significantly higher levels in the ceca of fully weaned compared with suckling animals (supplemental Fig. S3*B*), supporting the conclusion that liberated plant monosaccharides are utilized by *B. thetaiotaomicron*.

We also noted that in the P30 cecum *B. thetaiotaomicron* selectively increases expression of one of its eight capsular polysaccharide synthesis (*CPS*) loci, *CPS4* (Fig. 3). The *CPS4* locus contains two predicted rhamnosyl-transferases and two predicted D-TDP-4-dehydrorhamnose 3,5,-epimerases, indicating that rhamnose and/or rhamnose derivatives are substrates for *CPS4* biosynthetic enzymes. Moreover at P30, *B. thetaiotaomicron* exhibits increased expression of two α-rhamnosidases (*BT2523* and *BT2524*) contained in a predicted operon, as well as an operon required to import and metabolize rhamnose so that it can enter the glycolytic pathway (Fig. 1*A* and supplemental Fig. S3*B*). These findings add to an evolving set of observations that *Bacteroides* species modulate expression of their *CPS* loci in response to changes in glycan availability (1, 20, 40).



Locus tag	CAZy family	Annotation
BT1358		transcriptional regulator UpxY homolog
BT1357		transcriptional regulator UpxZ homolog
BT1356		capsule polysaccharide export protein kpsD
BT1355		hypothetical protein
BT1354		flippase
BT1353	GT2	glycosyltransferase
BT1352	GT4	glycosyltransferase
BT1351		glucose-1-phosphate cytidylyltransferase
BT1350		CDP-glucose 4,6-dehydratase
BT1349		dTDP-4-dehydrorhamnose 3,5-epimerase
BT1348		CDP-abequose synthase
BT1347	GT2	rhamnosyl transferase
BT1346		capsule biosynthesis protein capA
BT1345		glycosyltransferase
BT1344	GT4	glycosyltransferase
BT1343		capsule biosynthesis protein
BT1342		NAD dependent nucleotide sugar epimerase/dehydratase
BT1341		UDP-glucose 6-dehydrogenase
BT1340	GT2	rhamnosyl transferase
BT1339		undecaprenyl-phosphate alpha-N-acetylglucosaminyltransferase
BT1338		dTDP-4-dehydrorhamnose 3,5-epimerase

FIGURE 3. **Up-regulation of *B. thetaiotaomicron CPS* locus 4 in weaned mice.** Genes in this locus are up-regulated on average 11.9-fold after the suckling-weaning transition. Four of the genes in the cluster encode enzymes involved in rhamnose metabolism (two epimerases and two rhamnosyl transferases). *CPS4* is the only *B. thetaiotaomicron CPS* locus that exhibits consistent changes in its expression between the suckling (P17) and weaned (P30) state.

Polysaccharide Utilization Functions Cluster into Coordinately Regulated, Genomically Linked Units—Differential expression of *B. thetaiotaomicron* polysaccharide utilization genes in the ceca of P17 and P30 mice highlights the concept that the bacterium "tunes" its transcriptome to coincide with available polysaccharide resources. As noted in the Introduction, our previous analysis of the *B. thetaiotaomicron* genome revealed that genes encoding GH and PL activities frequently co-localize adjacent to SusC and SusD paralogs (17). Furthermore, these clusters often contain paired genes encoding an ECF-σ factor and an anti-σ factor. These observations led us to hypothesize that these linked genes comprise "polysaccharide utilization loci" that encode the functions necessary for sensing, binding, and degrading specific polysaccharide substrates (17). If this hypothesis is correct, genes linked together in the same utilization locus should exhibit simultaneous expression when their targeted polysaccharide species become available. These loci could also provide insights into the genomic foundations for and the nature of the evolved niche (profession) of *B. thetaiotaomicron* in a given gut habitat (address).

To explore this issue, we examined our data sets of *B. thetaiotaomicron* genes exhibiting increased expression in the ceca of either P17 or P30 mice for the presence of expressed GH or PL functions encoded by genes that were physically linked to expressed SusC/SusD paralogs and/or to transcription factors. We identified six such loci (Fig. 4*A*); four contain ECF-σ/anti-σ factors. Three of these four loci are up-regulated in the P17 cecum. Two other loci contain genes encoding hybrid two-component proteins and are induced at P30.

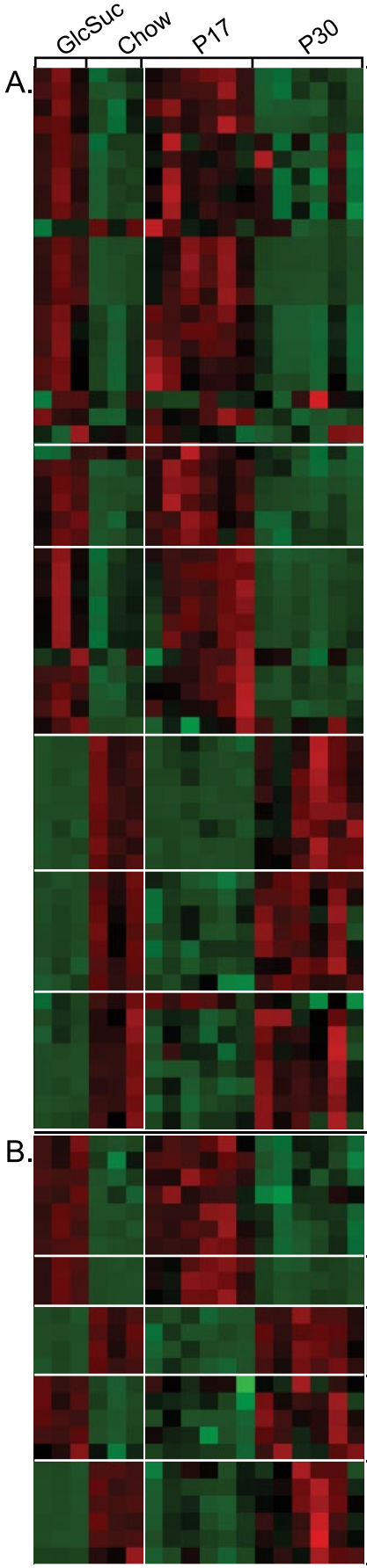
Each of the four loci harboring ECF-σ/anti-σ pairs contains a specialized *susC* encoding an outer membrane protein with an extra N-terminal domain (41). This domain shares amino acid similarity, determined using a hidden Markov model (41), to the N-terminal domain of the *Escherichia coli* FecA iron-dicitrate receptor protein. This domain allows FecA to interact directly with an anti-σ factor (FecR) and control gene expression through modulation of an associated ECF-σ factor (FecI) (42). Based on their similarity with *E. coli* FecA, these four *B. thetaiotaomicron* SusC paralogs contained within the four loci likely have polysaccharide binding/import as well as regulatory functions, *i.e.* the genomic linkage and coordinate expression of these specialized SusC paralogs and anti-σ factors reinforce the idea that they act together to utilize their targeted polysaccharide species (Fig. 4*A*).

We also identified five other coordinately regulated loci containing some, but not all, of these functions (Fig. 4*B*); two loci (*BT0455–BT0461* and *BT3654–BT3657*) only contain GH activities, two loci (*BT0865–BT0867* and *BT4667–BT4672*) contain glycoside hydrolase genes linked to *susC/susD* paralogs, and one locus (*BT3663–BT3667*) contains GH activities linked to a predicted helix-turn-helix regulator. These observations suggest that *B. thetaiotaomicron* possesses "incomplete" polysaccharide utilization loci that may either be regulated by unlinked environmental sensors/regulators or act in concert with unlinked loci containing SusC/SusD paralogs.

Evolutionary Relationships of SusC/SusD Homologs and Their Patterns of Regulation—The host- and plant-derived polysaccharides that *B. thetaiotaomicron* has evolved to utilize contain different monosaccharide species with myriad arrangements and glycosidic linkages. Based on previous studies demonstrating that SusC and SusD are required for the ability of *B. thetaiotaomicron* to bind starch, it is logical to conclude that at least some of the remaining 101 paralogous SusC/SusD pairs in the genome of this organism are fundamental to its ability to bind and utilize other polysaccharides.

The frequent inclusion of *susC/susD* gene pairs in polysaccharide utilization loci with mechanistically diverse regulatory mechanisms and enzymatic activities (9 of 13 loci in Fig. 4) represents the most common feature through which these loci may be compared. To visualize the evolutionary expansion of SusC/SusD homolog pairs in members of the Bacteroidetes, we generated a cladogram depicting the relationship of all homolog pairs encoded by the genomes of three species associated with the human alimentary tract: 102 pairs from *B. thetaiotaomicron*; 69 pairs from *B. fragilis* strain NCTC 9343 plus 65 pairs from *B. fragilis* strain YCH46; and four pairs from *P. gingivalis* (Fig. 5). The two *Bacteroides* species are normal members of the human colonic microbiota. *P. gingivalis* is a member of the oral microbiota and thus has evolved mechanisms to survive in a distinct habitat.

The results of our analysis suggest that many similar SusC/SusD pairs are "shared" between *B. thetaiotaomicron* and *B. fragilis* (Fig. 5). One example is the prototypic *B. thetaiotaomicron* SusC/SusD pair present in the starch utilization system



Locus tag	CAZy family	Annotation
BT1032	GH92	α-1,2-mannosidase
BT1033		conserved hypothetical protein
BT1034		putative signal transducer
BT1035*		conserved hypothetical protein
BT1036*		hypothetical protein
BT1037*		conserved hypothetical protein
BT1038*		hypothetical protein
BT1039*		SusD homolog
BT1040*		SusC homolog
BT1041*		integrase
BT1042		SusC homolog
BT1043		SusD homolog
BT1044	GH18	endo-β-N-acetylglucosaminidase
BT1045		patatin-like protein
BT1046		SusC homolog
BT1047		SusD homolog
BT1048	GH18	endo-β-N-acetylglucosaminidase
BT1049		patatin-like protein
BT1050		hypothetical protein
BT1051*	GH20	β-hexosaminidase
BT1052		Anti-α factor
BT1053*		ECF-α factor
BT1617		RNA polymerase ECF-type sigma factor
BT1618		putative anti-sigma factor
BT1619		SusC homolog
BT1620		SusD homolog
BT1621*	GH20	beta-hexosaminidase
BT1622*		N-acetylgalactosamine-6-sulfatase
BT3983		SusC homolog
BT3984		SusD homolog
BT3985		hypothetical protein
BT3986		patatin-like protein
BT3987	GH18	endo-β-N-acetylglucosaminidase
BT3988		peptidoglycan bound protein
BT3989*		hypothetical protein
BT3990	GH92	α-1,2-mannosidase
BT3991	GH92	α-1,2-mannosidase
BT3992		Anti-α factor
BT3993*		ECF-α factor
BT2892	GH93	conserved hypothetical protein
BT2893		SusD homolog
BT2894		SusC homolog
BT2895	GH43	endo-1,4-β-xylanase
BT2896		NHL-repeat protein
BT2897		hybrid two-component system
BT2898	GH43	endo-1,4-β-xylanase D
BT2899		hypothetical protein
BT3043	GH5	xylanase
BT3044		hypothetical protein
BT3045		SusD homolog
BT3046		SusC homolog
BT3047		NHL-repeat protein
BT3048		hypothetical protein
BT3049		hybrid two-component system
BT4705*		RNA polymerase ECF-type sigma factor
BT4706		anti-sigma factor
BT4707		SusC homolog
BT4708*		SusD homolog
BT4709	GH18	glycosyl hydrolase
BT4710		conserved hypothetical protein
BT4711		conserved hypothetical protein
BT4712*		hypothetical protein
BT0455	GH33	sialidase
BT0456	GH20	β-hexosaminidase
BT0457		sialic acid-specific 9-O-acetylesterase
BT0458	GH2	β-mannosidase
BT0459	GH20	β-hexosaminidase
BT0460	GH20	β-hexosaminidase
BT0461	GH2	β-galactosidase
BT0865		chitobiase
BT0866		SusD homolog
BT0867		SusC homolog
BT3654	GH35	β-galactosidase
BT3655	GH43	arabinosidase
BT3656	GH43	β-xylosidase
BT3657	GH51	α-L-arabinofuranosidase A
BT3663*	GH43	β-xylosidase
BT3664	GH97	α-glucosidase
BT3665	GH29	α-L-fucosidase
BT3666		conserved hypothetical protein
BT3667		AraC type HTH transcriptional regulator
BT4667*	GH2	β-galactosidase
BT4668	GH53	arabinogalactan endo-1,4-β-galactosidase
BT4669		hypothetical protein
BT4670		SusD homolog
BT4671		SusC homolog
BT4672		hypothetical protein

(Sus) operon (*BT3702–BT3701*); this SusC/SusD pair is most similar to paralog pairs from *B. fragilis* (*BF3146–BF3145* in strain NCTC 9343; *BF3307–BF3306* in strain YCH46). Of note, these *B. fragilis* pairs are part of loci also encoding predicted amylases (*BF3143* in NCTC 9343, and *BF3304* in YCH46), suggesting that they target similar substrates as the related *B. thetaiotaomicron* Sus locus (amylose and amylopectin) (30). Extrapolating, it is tempting to postulate that the occurrence of numerous related SusC/SusD pairs between these two *Bacteroides* species indicates that their respective polysaccharide utilization loci target identical or similar substrates in the distal gut and therefore denote potential areas of niche overlap.

However, in addition to the numerous related SusC/SusD pairs shared between *B. thetaiotaomicron* and *B. fragilis*, the cladogram reveals that each bacterial species also has substantial expansions of SusC/SusD pairs that do not have closely related partners in the other species (Fig. 5). Evidence of this comes from one large branch containing 43 related SusC/SusD pairs, of which 39 (90.7%) belong to *B. thetaiotaomicron* (*group A* in Fig. 5). Two smaller branches reveal similar unique expansions of SusC/SusD paralogs in *B. fragilis*; in these branches 25 of 32 (78.1%) and 7 of 7 (100%) paralogs are exclusive to this species (*groups B* and *C* in Fig. 5). These observations suggest that each *Bacteroides* species has evolved and retained its own specialized sets of polysaccharide-binding SusC/SusD homologs that may assist it in using nutrient resources that may not be directly accessible to other members of the microbiota.

We identified a total of 41 *B. thetaiotaomicron* SusC and SusD paralogs belonging to genomically linked paralog pairs, which exhibit significant expression changes between the suckling and weaned states; 30 belong to pairs in which both partners are up-regulated. Twenty-one of the 41 paralogs had higher expression at P17 compared with P30, whereas 20 had increased expression at P30 (supplemental Table S1 and supplemental Fig. S5). By mapping these changes in expression onto the cladogram (Fig. 5), we found that two closely related pairs of SusC/SusD paralogs were responsive to different *in vivo* conditions. One set was induced at P17 (indicated by *arrows* and *yellow tick marks* in Fig. 5), whereas another set was induced at P30 (*arrows* and *blue tick marks*). Thus, these two closely related proteins may have evolved unique substrate specificities and are expressed in response to the presence of different glycans. Alternatively, they may have retained the ability to bind to similar substrates but have been duplicated during genome evolution so that they may be expressed under the control of different environmental sensors/regulators in response to different nutrient environments.

Our analysis of gene expression in tryptone medium revealed that none of the SusC/SusD paralogs that were up-regulated *in vivo* at P17 responded to the addition of lactose by significantly changing their levels of expression. Moreover, of the 29 glycoside hydrolases up-regulated in the P17 cecum, only four (including two β-galactosidases, *BT1626* and *BT4050*) were also up-regulated *in vitro* after exposure to this disaccharide (supplemental Fig. S6). Thus, *B. thetaiotaomicron* loci encoding SusC/SusD paralogs, GHs and PLs that show altered expression during the suckling period appear to be reacting to the presence of more complex carbohydrate structures represented in milk and host glycans.

Prospectus—In this study, we have used gnotobiotic mice to describe the transcriptional and metabolic adaptations of *B. thetaiotaomicron* to the suckling and weaned distal gut. Although their simplified gut ecosystem does not recapitulate all of the features present with a complete microbiota, our findings provide a foundation for future experiments examining the behavior of more complex consortia composed of sequenced gut microbes.

We show that during the suckling period, *B. thetaiotaomicron* relies heavily on the host for nutrients, in addition to carbohydrates from mother's milk. The ability of *B. thetaiotaomicron* to access host-derived polysaccharides (*e.g.* those in mucus) highlights the importance of habitat effects in shaping the population structure of the microbiota. The ability of the bacterium to access relatively stable and coveted mucosal glycans in the neonatal gut should help it establish a foothold in this ecosystem so that it can later become a dominant member of the adult microbial community as dietary polysaccharides are introduced.

A noteworthy feature of the responses of *B. thetaiotaomicron* to changing nutrient landscapes in the gut is co-regulation of chromosomally linked genes encoding functions involved in polysaccharide utilization. Despite the fact that *B. thetaiotaomicron* VPI-5482 is a type strain originally isolated from humans, it effectively controls expression of its polysaccharide utilization genes in response to host mucus- and diet-derived nutrients. However, because only a fraction of the polysaccharide utilization apparatus of *B. thetaiotaomicron* shows differential regulation in our gnotobiotic model of postnatal gut development, it is likely that signals specific to other host species, microbial colonization states, or diets control expression of other components of its versatile glycobiome.

The organization of the polysaccharide utilization loci depicted in Fig. 4*A* is similar to the prototypic starch-utilization system locus (*susRABCDEFG*), which contains genes involved in starch binding (*susC*, *susD*, *susE*, and *susF* (30)) and catabolism (*susA*, *susB*, and *susG* (43)). Regulation of this locus by SusR is based on sensing of maltose disaccharides liberated during starch catabolism (44). SusR is predicted to be a helix-turn-helix transcription factor and therefore is structurally dissimilar to both the ECF-σ/anti-σ and hybrid two-component system proteins that are part of the six loci we identified. This observation suggests that *B. thetaiotaomicron* has adapted at least three different sensory mechanisms to control polysaccharide utilization functions.

FIGURE 4. **Expression of genes in polysaccharide utilization loci.** *A*, "complete" polysaccharide utilization loci encoding genomically linked transcriptional regulators, *susC/susD* paralogs and GH/PL genes. *B*, "incomplete" loci that lack regulatory and/or *susC/susD* genes. These genes may be regulated *in trans* and/or might process substrates imported by SusC/SusD proteins encoded by genes located elsewhere in the genome. *Gray boxes* and *arrows* indicate the predicted *B. thetaiotaomicron* operons (45) and the direction of transcription. An *asterisk* indicates that the genes are not significantly changed between the suckling and weaned states.

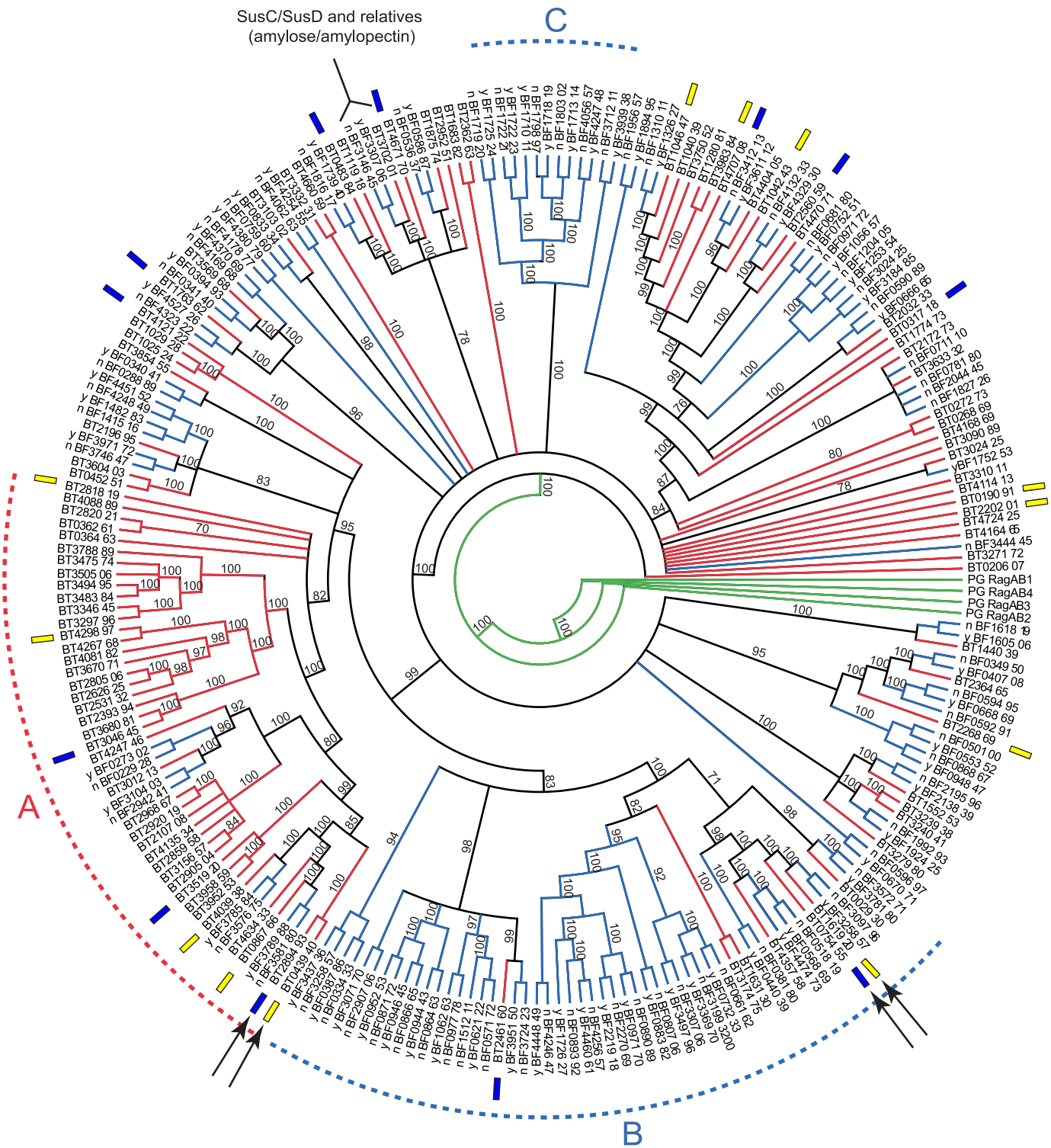


FIGURE 5. **Cladogram analysis of SusC/SusD pairs.** Branches containing SusC/SusD pairs that are unique to a single species are color coded as follows: *B. thetaiotaomicron* (*red*), *B. fragilis* (*blue*), and *P. gingivalis* (*green*). Taxon names for each *B. thetaiotaomicron* and *B. fragilis* SusC/SusD pair are provided according to the locus tag identifier in the annotation of each respective genome and are listed with the SusC paralog first. Names for *B. fragilis* SusC/SusD pairs are preceded by either *n* or *y* to indicate their origin in the genome sequences of strain NCTC 9346 or strain YCH46, respectively. *P. gingivalis* SusC/SusD homologs are denoted by their previously assigned RagA/RagB designations. The *B. thetaiotaomicron* amylose/amylopectin-binding SusC and SusD proteins encoded by members of the Sus operon are indicated along with their nearest homologs in *B. fragilis*. SusC/SusD pairs exhibiting increased expression in either suckling (*yellow*) or weaned (*blue*) mice are indicated by bars next to appropriate taxon names. Two pairs of closely related SusC/SusD paralogs that exhibit differential expression between the suckling and weaned states are denoted by *arrows*. Species-specific SusC/SusD expansions are indicated by *dashed lines* around the cladogram edge and are colored according to the species in which expansion has occurred. *Expansion A* is enriched for *B. thetaiotaomicron* homologs (39 of 43), whereas *expansions B* and *C* are enriched for *B. fragilis* homologs (25 of 32 and 7 of 7, respectively; note that pairs of presumed identical relatives from each *B. fragilis* genome were only counted once). The bootstrap values (70–100%) are indicated for each branch point, except for branches that occur between nearest relatives from each *B. fragilis* genome.

Based on the similar organization of polysaccharide utilization loci encoding transcriptional regulators and SusC/SusD paralog pairs, the coordinate expression of these loci in certain environments and, the nature of their encoded enzymatic functions, predictions can be made about some of the biochemical characteristics of the polysaccharide species that are targeted by these loci, as well as how they are regulated. For example, the P17-induced locus *BT1617–BT1622* encodes an ECF-σ/anti-σ regulatory pair (*BT1617–BT1618*) and a linked SusC paralog (*BT1619*) with an N-terminal extension predicted to interact directly with an anti-σ factor (likely *BT1618*). This locus also encodes a SusD paralog (*BT1620*) and two enzymatic activities, a β-hexosaminidase (*BT1621*), and a sulfatase (*BT1622*). Thus, this locus may be expressed in response to, and target, sulfated glycans containing hexosamines (*e.g.* sulfated mucosal *O*-glycans or glycosaminoglycans like chondroitin sulfate), a notion consistent with the observation that it is expressed in both P17 suckling animals and adult mice fed a glucose-sucrose diet (Fig. 4*A*).

In summary, the regulated polysaccharide utilization loci identified in this report can serve as useful starting points and models for future genetic and biochemical studies designed to investigate the general question of how a successful gut mutualist adapts to alterations in nutrient availability prompted by changes in host development and diet.

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