

Characterizing the Interactions between a Naturally Primed Immunoglobulin A and Its Conserved *Bacteroides thetaiotaomicron* Species-specific Epitope in Gnotobiotic Mice*

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Background: IgA responses are important for human gut mucosal barrier function.

Results: Gnotobiotic *Rag1*^{-/-} mice harboring a human gut bacterial symbiont and a single, naturally primed IgA revealed species/strain/epitope level specificities and biological effects of the antibody.

Conclusion: *Bacteroides thetaiotaomicron* maintains a highly conserved immunogenic epitope important for its fitness *in vivo*.

Significance: Our approach can yield diagnostic antibodies and new understanding of microbiota-immune system co-evolution.

The adaptive immune response to the human gut microbiota consists of a complex repertoire of antibodies interacting with a broad range of taxa. Fusing intestinal lamina propria lymphocytes from mice monocolonized with *Bacteroides thetaiotaomicron* to a myeloma fusion partner allowed us to recover hybridomas that captured naturally primed, antigen-specific antibody responses representing multiple isotypes, including IgA. One of these hybridomas, 260.8, produced a monoclonal antibody that recognizes an epitope specific for *B. thetaiotaomicron* isolates in a large panel of hospital- and community-acquired *Bacteroides*. Whole genome transposon mutagenesis revealed a 19-gene locus, involved in LPS O-antigen polysaccharide synthesis and conserved among multiple *B. thetaiotaomicron* isolates, that is required for 260.8 epitope expression. Mutants in this locus exhibited marked fitness defects *in vitro* during growth in rich medium and in gnotobiotic mice colonized with defined communities of human gut symbionts. Expression of the 260.8 epitope was sustained during 10 months of daily passage *in vitro* and during 14 months of monocolonization of gnotobiotic wild-

type, *Rag1*^{-/-}, or *Myd88*^{-/-} mice. Comparison of gnotobiotic *Rag1*^{-/-} mice with and without subcutaneous 260.8 hybridomas disclosed that this IgA did not affect *B. thetaiotaomicron* population density or suppress 260.8 epitope production but did affect bacterial gene expression in ways emblematic of a diminished host innate immune response. Our study illustrates an approach for (i) generating diagnostic antibodies, (ii) characterizing IgA responses along a continuum of specificity/degeneracy that defines the IgA repertoire to gut symbionts, and (iii) identifying immunogenic epitopes that affect competitiveness and help maintain host-microbe mutualism.

The human gut microbiota and immune system co-develop following birth with each influencing the properties of the other to maintain a homeostasis that is mutually advantageous (1, 2). The biological barrier that supports this peaceful co-existence is made up of mucus (3, 4), immunoglobulins, and antimicrobial peptides “backed up” by an epithelial monolayer knitted together by tight junction proteins. Studies in animal models have shown that innate signaling pathways in the gut mucosa couple sensing of microbial ligands with secretion of antibacterial peptides that prevent microbes from penetrating the mucus layer and underlying epithelium (5–8). Activation of the adaptive arm of the immune system in the intestinal mucosa induces production of antibodies, notably secretory IgA, directed against gut microbes (9, 10).

IgA responses can develop in a T cell-dependent or -independent manner (11) and may exhibit either broad or limited reactivity (12–15). Although class switching and plasma cell maturation in the gut were classically described in Peyer patches, evidence suggests that these processes may also occur locally in the intestinal lamina propria in response to cues from the microbiota, epithelium, and stromal compartment (16). In this model, a decentralized immune response permits the host to rapidly respond to microbial encroachment by activating the

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Draft genome sequences for *B. thetaiotaomicron* strains BT7330-1 and DH3731 and 16 S rRNA gene sequences have been deposited in the European Nucleotide Archive (ENA study accession number PRJEB8679). GeneChip and RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO accession number GSE65456).

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adaptive immune system at the site of insult. To study the kinetics and plasticity of this response, Hapfelmeier *et al.* (9) used a model in which germ-free mice were reversibly colonized with an auxotrophic strain of *Escherichia coli* that was unable to replicate within the gut, leaving these animals germ-free after a brief period of colonization. Induction of a specific IgA response in these animals required high doses of bacteria ($\sim 10^9$ colony-forming units (cfu)). Subsequent colonization with a stable microbiota lacking *E. coli* decreased titers of anti-*E. coli* antibody, demonstrating the lability of the intestinal IgA response. Together, these observations support the notion that IgA responses develop in both secondary intestinal lymphoid structures and the lamina propria, and where the antibody repertoire is altered continuously in response to local or regional antigenic stimulation (17).

Characterizing the specificity of IgA responses has been a challenge due to the complexity of the response and the microbiota (12, 13, 18–21). Cullender *et al.* (19) reported that Toll-like receptor 5 drives production of IgA and that this response modulates Toll-like receptor 5 ligand (flagellin) expression. More recently, we have used fluorescence-activated cell sorting (FACS) to identify bacterial targets of gut mucosal IgA responses in fecal samples obtained from children with healthy growth phenotypes or with varying degrees of undernutrition, as well as fecal samples harvested from gnotobiotic mice harboring the microbiota and fed the diets of these human gut community donors. FACS-purified viable IgA-targeted bacterial taxa were also transferred to a second round of germ-free animals to examine their functional properties. The results established that IgA responses can be used as biomarkers of disease in undernourished children, that they mediate a diet-dependent enteropathy characterized by small intestinal and colonic epithelial barrier dysfunction, and that certain IgA-targeted microbes purified from healthy donor microbiota can prevent development of this mucosal barrier disruption (22).

To explore the species, strain, and epitope level specificities of the IgA response and its effects on the biological properties and fitness of targeted components of the microbiota, we created a simplified, defined gnotobiotic mouse model (23). *Bacteroides thetaiotaomicron* is a prominent member of the adult human gut microbiota. The genome of the type strain, VPI-5482, encodes a larger complement of carbohydrate-active enzymes (glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases) than the number of these enzymes specified by our human genome. This repertoire of carbohydrate-active enzymes is embedded in 88 polysaccharide utilization loci, composed of 866 genes comprising 18% of its genome, that allow *B. thetaiotaomicron* to sense, acquire, and degrade otherwise indigestible polysaccharides in our diets and to forage on mucus glycans when these polysaccharides are absent from the diet (24, 25). Colonization of adult germ-free C57BL/6J mice with *B. thetaiotaomicron* VPI-5482 leads to increased levels of serum IgA and IgG (particularly IgG3 and IgG2b) within 14 days. Fusing intestinal lamina propria B cells isolated from these monocolonized gnotobiotic animals with a myeloma fusion partner allowed us to recover hybridoma cell lines that “captured” naturally primed, antigen-specific antibody responses representing multiple isotypes, including IgA. Using a

transposon mutant library of *B. thetaiotaomicron*, we identified the cognate epitope for the IgA product of one of the hybridoma cell lines, 225.4, as the product of one of its eight capsular polysaccharide synthesis (CPS)⁴ loci, *CPS4*. This 225.4 hybridoma cell line was then injected subcutaneously into the backs of adult germ-free mice with no T or B cells (C57BL/6J *Rag1*^{−/−}). After a period had elapsed during which time the hybridomas grew to a substantial size, animals were colonized with *B. thetaiotaomicron* VPI-5482. Comparisons of mono-colonized mice with and without the 225.4 hybridoma “backpack” revealed that the presence of the monoclonal antibody (mAb 225.4) decreased 225.4 epitope expression by the bacterium. When an *in vivo* competition was performed using isogenic wild-type and a *CPS4*-null (225.4-negative) mutant strain, we observed that the presence of mAb 225.4 reduced the wild-type to mutant ratio by a factor of 10 (23). Moreover, GeneChip-based whole genome profiling of bacterial and intestinal transcriptional responses disclosed that without the 225.4 IgA *B. thetaiotaomicron* elicited a more robust innate immune response (*e.g.* inducible NOS) and reacted to this response by inducing bacterial genes that metabolize host oxidative products (*e.g.* those involved in nitrate metabolism). Thus, the presence of the IgA reduced intestinal proinflammatory signaling and bacterial epitope expression, thereby balancing suppression of the oxidative burst with the negative impact of the antibody on bacterial fitness.

To determine whether these features were unique to mAb 225.4 or apply more generally to the naturally primed antibody repertoire elicited by colonization, we have now characterized another monoclonal IgA (mAb 260.8) generated from a different lamina propria fusion. Unlike mAb 225.4, which was specific for the VPI-5482 type strain, mAb 260.8 exhibits strong specificity and sensitivity for multiple *B. thetaiotaomicron* isolates from established clinical microbiology laboratory culture collections as well as new culture collections generated from previously frozen human fecal samples. Genetic mapping revealed a 19-gene locus required for expression of the 260.8 epitope. Mutants in this locus, which appears to be involved in LPS O-antigen polysaccharide synthesis and is conserved among multiple *B. thetaiotaomicron* isolates, display a profound fitness defect *in vitro* and *in vivo*. Unlike the capsular polysaccharide-specific 225.4 IgA, studies in gnotobiotic C57BL/6J *Rag1*^{−/−} mice with and without 260.8 hybridoma backpacks indicate that expression of the 260.8 epitope is not down-regulated in the presence of its cognate IgA. Moreover, epitope expression was sustained during 300 days of continuous growth *in vitro* and during 14 months of monocolonization of wild-type C57BL/6J gnotobiotic mice. Together, our results indicate that bacterial surface epitopes may vary widely within a species both in terms of their genetic conservation and expression. Our findings emphasize how a human gut symbiont can maintain highly conserved, species-specific genetic loci that encode immunogenic epitopes that, despite being targeted by host IgA and important for fitness *in vitro* and *in vivo*, are not

⁴ The abbreviations used are: CPS, capsular polysaccharide synthesis; BHI, brain heart infusion; INSeq, insertion sequencing; RNA-Seq, RNA sequencing; NEMC, New England Medical Center.

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transcriptionally suppressed in the presence of a specific IgA or negatively selected against during adaptation to a mammalian gut. Finally, the approach provides a means for creating new clinical diagnostic reagents.

EXPERIMENTAL PROCEDURES

Colonization of Germ-free Mice—C57BL/6J wild-type, *Rag1*^{-/-}, and *Myd88*^{-/-} mice were obtained from The Jackson Laboratory. Mice were rederived as germ-free and maintained in plastic, flexible film gnotobiotic isolators under a strict 12-h light cycle. Animals were fed an autoclaved, polysaccharide-rich, standard rodent chow diet (B&K Universal, East Yorkshire, UK) *ad libitum*. All experiments involving mice were performed using protocols approved by the Washington University Animal Studies Committee.

B. thetaiotaomicron strain VPI-5482 was harvested from stationary phase cultures that had been grown in TYG medium (1% tryptone, 0.5% yeast extract, 0.2% glucose) supplemented with 100 mM potassium phosphate buffer, pH 7.2, 4.1 mM cysteine, 200 mM histidine, 6.8 mM CaCl₂, 140 nM FeSO₄, 81 mM MgSO₄, 4.8 mM NaHCO₃, 1.4 mM NaCl, 1.9 mM hematin, and 5.8 mM vitamin K₃. 10⁸ cfu were gavaged (in 100 μ l of TYG medium) into 6–8-week-old male germ-free recipient animals.

Generating the 260.8 Hybridoma Cell Line from Lamina Propria Fusions—Wild-type C57BL/6J gnotobiotic mice were killed 2 weeks after gavage with just wild-type *B. thetaiotaomicron* VPI-5482. The small intestine from a given mouse was removed immediately and flushed with Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 units/ml) and streptomycin (100 μ g/ml). The excised small intestine was opened along its cephalocaudal axis and minced into small (~2–5-mm) pieces. The pieces were incubated for 15 min at 37 °C in 50 ml of Hanks' buffered salt solution containing 5 mM EDTA to remove epithelial cells and intraepithelial lymphocytes. Tissue fragments were placed in DMEM containing collagenase (100 units/ml; Sigma) and Dispase (0.5 unit/ml; Fisher) and incubated for 2 h at 37 °C with vigorous shaking for 30 s every 30 min over a 2-h period. Released cells were separated from the remaining tissue fragments by sedimentation on the bench top for 1 min, and the resulting cell suspension (supernatant fraction) was passed through a Nytex filter (70- μ m pore diameter; BD Biosciences). Cells in the filtrate were then combined with the myeloma fusion partner (P3X63.Ag8). The combined population was washed three times in serum-free DMEM, and cells were fused by adding PEG1500 (50%, w/v; Roche Applied Science; Refs. (26 and 27)). Cloned IgA-producing lines were identified using procedures detailed in a previous publication (23).

Purification of mAb 260.8 and mAb 225.4—Hybridoma cell lines were grown in bioreactors, and their monoclonal antibodies were subsequently purified from the bioreactor supernatant through a two-step process that used standard ammonium sulfate precipitations followed by hydroxyapatite column chromatography (Leinco Technologies Inc., St. Louis, MO).

Enzyme-linked Immunosorbent Assay (ELISA)—Cultured bacterial strains, bacterial cell lysates or capsular polysaccharides prepared from the bacterial strains, or extracts of cecal contents harvested from gnotobiotic mice colonized with *B.*

thetaiotaomicron VPI-5482 (for details, see below) were assayed by ELISA in 96-well plates (Nunc Maxisorp, Nalge Nunc, Rochester, NY). Samples were diluted in sodium bicarbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, and 3 mM sodium azide, pH 8.5). All assays were performed either at 4 °C overnight or at room temperature for 2 h. mAb 260.8 or 225.4 was added (10 μ g/ml in PBS containing 1% BSA; 50 μ l/well) followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (Southern Biotech; 1:1,000 dilution, 50 μ l/well). All ELISAs were developed using 1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Roche Applied Science) in citrate buffer (100 mM citric acid, 50 mM sodium phosphate, pH 4.2) containing 0.03% H₂O₂. Reactions were read in an ELISA plate reader (ThermoMax, Molecular Devices) at 405 nm.

Bacterial Lysates—Lysates were generated by suspending 10¹⁰ cfu of bacteria in 0.5 ml of sterile PBS and sonicating the mixture in a Misonix XL-2020 sonicator (10 min at a setting of 10). This mixture was then diluted 1:1,000 in bicarbonate coating buffer, and a 50- μ l aliquot was added to each well of an ELISA plate. Because these lysates contained capsular antigens, membrane and cell wall antigens, and intracellular antigens, they were used for initial screening of hybridoma fusions.

Cell Surface Glycans—Surface glycans (capsular polysaccharides and LPS) were isolated from *B. thetaiotaomicron* VPI-5482 cells that had been harvested after they entered stationary phase in TYG medium. Briefly, bacteria were incubated at 65 °C in phenol:water (1:1) with constant stirring. Phases were separated by gravity, and the top phase was dialyzed using 10-kDa-cutoff dialysis tubing for 3 days at 4 °C against deionized water. The dialyzed material was subjected to ultracentrifugation (65,000 \times g for 2 h at 4 °C) and then lyophilized. This material was suspended in sterile PBS at a concentration of 1 mg/ml and used in the ELISA after additional dilution in bicarbonate coating buffer.

Extracts of Cecal Contents from Mice Monocolonized with *B. thetaiotaomicron*—Cecal contents were harvested and frozen at –80 °C immediately after gnotobiotic mice were killed. After thawing, 0.5 ml of a 1:100 dilution of cecal contents (in PBS) was added to 0.5 ml of phenol, and the mixture was incubated at 65 °C for 2 h with vortexing (30 s every 30 min). Following centrifugation at 20,000 \times g for 30 min at 4 °C, the upper phase was recovered, serial dilutions (in bicarbonate buffer) were made, and an ELISA was performed.

To estimate the amount of mAb 260.8-reactive epitope present in cecal contents, ELISA results were standardized to a curve constructed from serial dilutions of stationary phase *B. thetaiotaomicron* (grown in TYG medium) in bicarbonate buffer. This stationary phase culture was arbitrarily designated as having 1 \times 10⁹ epitope units/ml, which is equal to the number of cfu under these conditions; *i.e.* in TYG medium-grown *B. thetaiotaomicron*, 1 260.8 epitope unit is equivalent to 1 cfu.

Isolation of mAb 260.8-reactive Colonies of *B. thetaiotaomicron* from Human Fecal Samples—Fecal samples were collected from four monozygotic and two dizygotic twin pairs and their mothers enrolled in the Missouri Adolescent Female Twin Study (28). Procedures for recruitment of participants and protocols for sample collection and deidentification were approved by the Human Research Protection Office of Washington Uni-

versity. Samples were frozen at -20°C immediately after they were produced and stored at -80°C .

The presence of *B. thetaiotaomicron* was detected by PCR of fecal DNA using primers directed at variable region 2 of bacterial 16 S rRNA genes, and the resulting amplicons were subjected to multiplex 454 pyrosequencing (FLX standard chemistry) (28). An aliquot of each selected frozen fecal sample harboring *B. thetaiotaomicron* 16 S rRNA genes was plated on brain heart infusion (BHI) blood agar supplemented with 200 $\mu\text{g}/\text{ml}$ gentamicin. Plates were incubated at 37°C in an anaerobic chamber (atmosphere, 5% H_2 , 20% CO_2 , 75% N_2 ; Coy Laboratory Products, Grass Lake, MI). Plates were screened for the presence of 260.8-positive colonies using a filter-based assay (29). Single 260.8-positive colonies were restreaked on fresh BHI blood agar supplemented with 200 $\mu\text{g}/\text{ml}$ gentamicin, and four to eight single colonies from each individual donor microbiota were tested by ELISA for 260.8 reactivity. Colonies confirmed to be positive were inoculated into single wells of a sterile 96-well plate with each well containing 200 μl of TYG medium. After an overnight incubation under anaerobic conditions at 37°C , 25 μl of each culture was transferred into 25 μl of coating buffer in fresh 96-well plates (Maxisorp) for ELISA. Colonies positive by ELISA were restreaked, and single colonies were picked and tested once again by an ELISA. The colony with the highest signal was then cultured in TYG broth. DNA was isolated from an aliquot of the culture using the Qiagen DNeasy kit (glycerol stocks were prepared from the remaining culture). The 16 S rRNA gene was amplified using standard PCR primers 8F and 1391R. Amplicons were purified with the Qiagen PCR cleanup kit and sequenced using the dideoxy chain termination method and the following three primers: 8F (5'-AGAGTTTGATCCTGGCTCAG), 1391R (5'-GACGGGCGGTGWGTRCA), and 515F (5'-GTGCCAGCMGCCGCGGTAA) (W = A or T; R = A or G; M = A or C).

XplorSeq (Phyloware) was used to assemble contigs and trim sequence files based on a Phred Q40 quality cutoff. Sequences were aligned using ClustalW and trimmed to the shortest common sequence length. Errors in alignment were manually corrected in MEGA. Phylogenetic trees were produced in MEGA (30) using the nearest neighbor algorithm based on "number of differences."

Whole Genome Transposon Mutagenesis—Mutagenesis of *B. thetaiotaomicron* VPI-5482 was performed using pEP4351 (λ pir-dependent R6K oriV; RP4 oriT; Cm^r Tc^r (Em^r); Tn4351 mutagenesis vector (31, 32)). Mutants were isolated based on their growth on BHI blood agar plates containing erythromycin (10 $\mu\text{g}/\text{ml}$ to select for transposon-containing *B. thetaiotaomicron* strains) and 600 $\mu\text{g}/\text{ml}$ gentamicin (to select against any persistent *E. coli* harboring the transposon mutagenesis vector). Individual colonies were picked into 96-well plates and grown overnight in TYG medium in anaerobic jars ($n = 55$ plates; 4,800 mutants). Plates were replicated with one replicate frozen in 25% glycerol for later studies. Each isolate in each well was then subjected to ELISA screening with mAb 260.8 and mAb 225.4 (the latter mAb served as a positive control for the presence of *B. thetaiotaomicron* VPI-5482; see the Introduction).

Sites of transposon insertion in 225.4-positive, 260.8 antibody-negative colonies were identified using arbitrarily primed

PCR. The arbitrarily primed PCR protocol consisted of a nested PCR with the following primers: Round 1, S3794 (5'-ATCAGTATGCTTTGTGTGTG) and either AR7 (5'-GGCCACGCGTCGACTAGTACN₁₀GTAAT) or AR8 (5'-GGCCACGCGTCGACTAGTACN₁₀GATGC); Round 2, ISF (5'-TCGGTTATATGTTTGTCTCATCTGC) and AR2 (5'-GGCCACGCGTCTCGACTAGTAC). The PCR cycling conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min (a total of 30 cycles). Round 2 used the same conditions but for 40 cycles. The product of the second round of PCR was purified (QIAquick PCR purification kit, Qiagen) and sequenced from the right arm of the transposon into flanking chromosomal DNA using the primer IS4908S (5'-ATCCATTTCAGAGTGAGAGAAAG). The results were compared with the genome sequence of *B. thetaiotaomicron* VPI-5482 (GenBankTM accession number NC_004663) and Tn4351 (GenBank accession number M17124); sequences aligning to both were considered to be positive hits.

A second library of transposon mutants was independently generated with a modified *mariner* transposon delivery vector, pSAM, that contains (i) an antibiotic resistance cassette flanked by MmeI-modified *mariner* inverted repeats, (ii) the *himar1C9* transposase, and (iii) machinery for replication in the donor strain and for conjugative transfer (33). The MmeI sites allow cleavage at chromosomal sites located 16–17 bp from the inserted transposon so that the mutants can be mapped (see below). Approximately 4,200 colonies from this second library were picked and grown within 96-well plates containing TYG medium ($n = 48$ plates; each plate replicated so that frozen glycerol stocks could be archived). The contents of each well were screened by ELISA using both mAb 260.8 and mAb 225.4. DNA from each mutant strain that was mAb 260.8-negative and mAb 225.4-positive was subjected to arbitrary PCR to map the site of insertion of the transposon.

A third library was constructed using the pSAM vector: this library was clonally arrayed into 96-well plates, and the location of each mutant was defined using a bar-coding strategy (33). This mapped, arrayed library was composed of 7,155 members and was used to identify additional mutants in the region defined from libraries 1 and 2 as producing a 260.8-negative/225.4-positive phenotype. The pattern of 260.8 and 225.4 reactivity in these newly identified mutants was subsequently determined using ELISA.

***B. thetaiotaomicron* CPS Locus Mutants**—*B. thetaiotaomicron* VPI-5482 contains eight distinct capsular polysaccharide synthesis loci, each composed of 15–32 genes. A panel of eight isogenic mutants, generated with the suicide vector pGERM to block expression of genes in each locus (23), was used to test the CPS epitope specificity of mAb 260.8.

Comparative Genomic Hybridization Analyses—Genomic DNA was prepared from a number of *B. thetaiotaomicron* isolates with a mAb 225.4-positive and a 260.8-positive (or 260.8-negative) phenotype. DNA was sonicated on ice for 10 min using a Misonix XL-2020 sonicator (10 min at a setting of 10). Fragmentation efficiency was confirmed on a 1% agarose gel (300–400-bp fragments). DNA (5 μg) was labeled using the Enzo BioArray terminal labeling kit. Samples were denatured at 95°C for 5 min and hybridized to custom GeneChips generated

TABLE 1

Type strains and Salyers culture collection

ELISA reactivity and 16 S rRNA sequence comparisons for type strains and *Bacteroides* isolates are shown. ID, nucleotide sequence identity; RDP, Ribosomal Database Project, and (T) indicates type strain.

Isolate name	Percentage of VPI-5482 260.8 ELISA	Percent ID to VPI-5482 16 S rRNA	Best match in RDP database of 16 S rRNA sequences from type strains	Percent ID to best hit in RDP
<i>Bacteroides stercoris</i> 2030	0	93.1	<i>B. stercoris</i> (T); ATCC 43183; X83953	99
<i>Bacteroides caccae</i> 43185T	25	94.5	<i>B. caccae</i> (T); ATCC 43185T; X83951	98
<i>Bacteroides coprocola</i>	0	90.9	<i>B. coprocola</i> (T); M16; AB200224	100
<i>Bacteroides dorei</i>	0	90.7	<i>B. dorei</i> (T); JCM 13471; 175; AB242142	100
<i>Bacteroides finegoldii</i>	1	96.5	<i>B. finegoldii</i> (T); JCM 13345; 199T; AB222699	100
<i>B. fragilis</i> 9343	2	94.5	<i>B. fragilis</i> (T); NCTC 9343; CR626927	100
<i>Bacteroides intestinalis</i>	2	93.9	<i>B. intestinalis</i> (T); JCM 13265; 341; AB214328	100
<i>Bacteroides ovatus</i>	1	96.4	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99
<i>Bacteroides plebeius</i> M35	0	89.6	<i>B. plebeius</i> (T); M12; AB200217	98
<i>Bacteroides uniformis</i>	0	93.9	<i>B. uniformis</i> (T); JCM 5828T; AB050110	98
<i>Bacteroides vulgatus</i>	11	90.8	<i>B. vulgatus</i> ATCC 8482; CP000139	99
BT0633-1	99	99.8	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT3164	90	99.6	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT3443	101	99.2	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT5951	98	99.8	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT7853	113	99.8	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT8669	105	99.5	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT8702	1	97.8	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	97
BT8713	86	99.8	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
BT8736	104	97.6	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98
J19-343	103	99.6	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
DH3730	120	99.4	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
DH3731	136	99.7	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
DH3941	2	96.6	<i>B. ovatus</i> (T); JCM 5824T; AB050108	98
DH4072	3	97.0	<i>Bacteroides xylanisolvens</i> (T); XB1A; AM230650	99
DH4087	105	99.2	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98
DH4108	96	98.8	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98
DH4111	102	99.0	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99
WH2	0	94.9	<i>Bacteroides cellulosilyticus</i> (T); CRE21;	98
WH302	2	96.9	<i>B. xylanisolvens</i> (T); XB1A; AM230650	99
WH305	1	96.8	<i>B. xylanisolvens</i> (T); XB1A; AM230650	99
WH406		90.8	<i>Bacteroides massiliensis</i> (T); B84634; AY126616	99
BT-DOT2	103	99.7	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99

from the finished *B. thetaiotaomicron* VPI-5482 genome sequence (24). Genomic hybridization analysis was performed using the DNA Chip Analyzer software package (dChip).

Sequencing the Genomes of *B. thetaiotaomicron* Strains DH3731 and BT7330-1—*B. thetaiotaomicron* strains DH3731 and BT7330-1 were isolated from fecal samples obtained from two unrelated normal healthy volunteers. Using an ABI 3730xl capillary sequencer, we obtained $>8\times >Q20$ coverage of each strain's genome. In addition, $>15\times$ coverage was obtained using a 454 pyrosequencer. Hybrid assemblies of 3730xl and 454 reads yielded 205 contigs spanning 7.1 Mb for the BT3731 strain, whereas strain BT7330 was assembled into 478 contigs spanning 6.8 Mb. Predicted ORFs were annotated based on BLASTP queries of the Clusters of Orthologous Groups of proteins (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and NCBI non-redundant (NR) databases (genes assigned to best BLAST hit, e-value $< 10^{-5}$).

Experimental In Vivo and In Vitro Evolution of *B. thetaiotaomicron* VPI-5482—These experiments were designed to determine whether the 260.8 epitope was expressed by *B. thetaiotaomicron* VPI-5482 during periods of prolonged growth in defined medium and over the course of the lifespan of monoclonized gnotobiotic mice. The *in vitro* experimental protocol involved culturing the bacterium anaerobically in minimal medium (100 mM KH_2PO_4 , 15 mM NaCl, 8.5 mM $(\text{NH}_4)_2\text{SO}_4$, 28 mM glucose) at 37 °C in glass culture tubes. A 10- μl aliquot of the culture was transferred every 24 h to fresh medium. This procedure was repeated 300 times in eight replicate experi-

ments. Aliquots of the culture were frozen every 50 transfers and stored in 20% glycerol at -80 °C.

For the *in vivo* experiments, the same starting culture of *B. thetaiotaomicron* VPI-5482 (derived from a single colony) that was used for the *in vitro* experiment was inoculated into 8–10-week-old male germ-free C57BL/6J wild-type, *Rag1* $^{-/-}$, or *Myd88* $^{-/-}$ mice. Gnotobiotic mice were maintained as described above. Animals were individually caged ($n = 4$ wild type, $n = 3$ *Rag1* $^{-/-}$, and $n = 2$ *Myd88* $^{-/-}$). Sterile bedding (Aspen wood shavings, NEPCO) was replaced in each cage every 7 days. Fecal samples were obtained from each animal once a week for the first month of the experiment and every 2 weeks thereafter. Fecal samples were frozen at -80 °C immediately after they had been produced by the animal. The experiment continued throughout the lifespan of each mouse (maximum, ~ 26 months).

Samples from the *in vivo* and *in vitro* experiments were characterized by ELISA. Fecal samples from days 5 and 433 (representing early and late time points in the experiment) were suspended in 500 μl of PBS. Aliquots were taken for ELISA, assay of total DNA (QuantiFluor dsDNA dye, Promega), and serial dilution plating on BHI blood agar. Individual colonies from these fecal samples and from the *in vitro* experiments were tested for 260.8 and 225.4 epitope expression by ELISA following growth to stationary phase in TYG medium (positive control, *B. thetaiotaomicron* VPI-5482; negative control, *B. thetaiotaomicron* strain 8702).

TABLE 2

NEMC culture collection

ELISA reactivity, 16 S rRNA sequence comparisons, and biochemical assignments for *Bacteroides* isolates in the NEMC collection are shown. ID, nucleotide sequence identity; RDP, Ribosomal Database Project, and (T) indicates type strain.

Isolate name	Percent ID to VPI-5482 16 S rRNA	Percentage of VPI-5482 260.8 ELISA	Best match in RDP database 16 S rRNA sequences from type strains	Percent ID to best hit in RDP	Biochemical assignment
22361	93.9	0	<i>B. caccae</i> (T); ATCC 43185T; X83951	98	<i>B. caccae</i>
22547	94.9	0	<i>B. finegoldii</i> (T); JCM 13345; 199T; AB222699	97	<i>B. caccae</i>
22566	93.7	4	<i>B. caccae</i> (T); ATCC 43185T; X83951	97	<i>B. caccae</i>
22599	94.0	0	<i>B. caccae</i> (T); ATCC 43185T; X83951	97	<i>B. caccae</i>
22866	99.6	108	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
22911	99.3	0	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
22913	98.7	93	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
22946	99.3	123	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
22953	98.5	107	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
22959	99.6	92	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
22967	98.9	77	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
22987	99.8	104	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
22998	93.5	8	<i>B. fragilis</i> (T); NCTC 9343; CR626927	98	<i>B. thetaiotaomicron</i>
23041	98.4	83	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23113	99.3	7	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23114	99.4	103	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23149	99.8	112	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23150	98.4	110	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23156	99.7	92	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23161	98.8	103	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23188	99.3	99	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23190	99.4	53	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23205	94.5	3	<i>B. caccae</i> (T); ATCC 43185T; X83951	98	<i>B. thetaiotaomicron</i>
23212	93.8	7	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. thetaiotaomicron</i>
23213	99.8	36	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23218	98.6	90	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23243	99.6	99	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23251	97.8	113	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	97	<i>B. thetaiotaomicron</i>
23257	90.4	0	<i>B. dorei</i> (T); JCM 13471; 175; AB242142	99	<i>B. thetaiotaomicron</i>
23262	92.7	1	<i>B. caccae</i> (T); ATCC 43185T; X83951	96	<i>B. caccae</i>
23269	98.5	3	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23272	99.8	79	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23367	99.4	128	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23368	99.5	110	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23369	96.4	1	<i>Bacteroides salyersiae</i> (T); WAL 10018; AY608696	99	<i>B. thetaiotaomicron</i>
23411	94.5	0	<i>B. caccae</i> (T); ATCC 43185T; X83951	98	<i>B. caccae</i>
23412	94.1	0	<i>B. caccae</i> (T); ATCC 43185T; X83951	98	<i>B. caccae</i>
23418	93.8	0	<i>B. caccae</i> (T); ATCC 43185T; X83951	97	<i>B. caccae</i>
23447	99.8	112	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23500	94.0	1	<i>B. caccae</i> (T); ATCC 43185T; X83951	98	<i>B. caccae</i>
23559	94.0	2	<i>B. uniformis</i> (T); JCM 5828T; AB050110	99	<i>B. uniformis</i>
23575	96.3	1	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99	<i>B. thetaiotaomicron</i>
23576	94.6	2	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. uniformis</i>
23580	99.8	90	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23584	99.8	96	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23596	98.5	107	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23601	93.7	1	<i>B. cellulosilyticus</i> (T); CRE21;	98	<i>B. thetaiotaomicron</i>
23618	94.9	6	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. thetaiotaomicron</i>
23621	96.3	4	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99	<i>B. uniformis</i>
23655	94.5	3	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. thetaiotaomicron</i>
23656	94.9	3	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. ovatus</i>
23657	94.8	3	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. fragilis</i>
23658	94.9	5	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. fragilis</i>
23661	94.8	3	<i>B. fragilis</i> (T); NCTC 9343; CR626927	99	<i>B. fragilis</i>
23673	90.4	3	<i>B. dorei</i> (T); JCM 13471; 175; AB242142	96	<i>B. fragilis</i>
23677	99.0	2	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. fragilis</i>
23680	97.6	3	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. fragilis</i>
23681	95.7	2	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	96	<i>B. vulgatus</i>
23682	90.6	4	<i>B. dorei</i> (T); JCM 13471; 175; AB242142	99	<i>B. vulgatus</i>
23685	99.4	89	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23688	97.6	97	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23689	96.3	2	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99	<i>B. thetaiotaomicron</i>
23692	99.8	98	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. vulgatus</i>
23693	91.0	2	<i>B. dorei</i> (T); JCM 13471; 175; AB242142	97	<i>B. thetaiotaomicron</i>
23698	91.0	3	<i>B. dorei</i> (T); JCM 13471; 175; AB242142	97	<i>B. thetaiotaomicron</i>
23699	99.5	3	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. thetaiotaomicron</i>
23708	96.0	3	<i>B. ovatus</i> (T); JCM 5824T; AB050108	97	<i>B. thetaiotaomicron</i>
23711	94.7	6	<i>B. cellulosilyticus</i> (T); CRE21;	98	<i>B. vulgatus</i>
23722	99.3	107	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	99	<i>B. vulgatus</i>
23723	96.3	2	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99	<i>B. thetaiotaomicron</i>
23724	94.0	3	<i>B. uniformis</i> (T); JCM 5828T; AB050110	99	<i>B. thetaiotaomicron</i>
23728	93.5	3	<i>B. intestinalis</i> (T); JCM 13265; 341; AB214328	99	<i>B. ovatus</i>
23730	98.1	106	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. uniformis</i>
23734	96.3	2	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99	<i>B. thetaiotaomicron</i>
23735	96.0	2	<i>B. ovatus</i> (T); JCM 5824T; AB050108	99	<i>B. ovatus</i>
23742	99.0	92	<i>B. thetaiotaomicron</i> (T); VPI-5482; AE015928	98	<i>B. thetaiotaomicron</i>
23748	96.4	1	<i>B. ovatus</i> (T); JCM 5824T; AB050108	98	<i>B. thetaiotaomicron</i>
23836	92.2	4	<i>B. uniformis</i> (T); JCM 5828T; AB050110	97	<i>B. thetaiotaomicron</i>

IgA Responses and Bacterial Fitness in Gnotobiotic Mice

TABLE 3

Twin isolates

ELISA reactivity and 16 S rRNA sequence comparisons for mAb 260.8-reactive isolates from twin pairs and mothers enrolled in the Missouri Adolescent Female Twin Study are shown. ID, nucleotide sequence identity; RDP, Ribosomal Database Project; MZ, monozygotic; DZ, dizygotic.

Family	Family ID-relationship	Isolate ID	Percent ID to VPI-5482 16 S rRNA	Percentage of VPI-5482 260.8 ELISA	Percentage of VPI-5482 225.4 ELISA	Percent ID to best hit in RDP
1	TS19-MZ twin	APG1.1	98.2	52	0	98
1	TS19-MZ twin	APG1.2	98.0	59	1	98
1	TS19-MZ twin	APG4.10	97.8	78	0	98
1	TS19-MZ twin	APG4.11	97.9	88	0	98
1	TS19-MZ twin	APG4.12	97.5	71	0	97
1	TS19-MZ twin	APG4.13	98.1	91	1	98
1	TS19-MZ twin	APG4.14	97.4	57	3	97
1	TS19-MZ twin	APG4.3	97.8	50	3	98
1	TS19-MZ twin	APG4.5	97.8	64	0	97
1	TS19-MZ twin	APG4.6	98.0	62	1	97
1	TS19-MZ twin	APG4.7	97.8	79	0	97
1	TS19-MZ twin	APG4.9	98.1	79	0	98
1	TS20-MZ twin	APG2.1	99.1	95	0	98
1	TS20-MZ twin	APG2.10	99.1	92	0	99
1	TS20-MZ twin	APG2.11	99.3	91	0	99
1	TS20-MZ twin	APG2.13	97.8	105	0	97
1	TS20-MZ twin	APG2.2	99.8	75	0	99
1	TS20-MZ twin	APG2.3	98.9	91	0	98
1	TS20-MZ twin	APG2.4	99.2	91	0	98
1	TS20-MZ twin	APG2.5	99.8	99	0	99
1	TS20-MZ twin	APG2.6	98.6	99	1	97
1	TS20-MZ twin	APG2.7	99.8	88	0	99
1	TS20-MZ twin	APG2.8	99.7	86	0	99
1	TS20-MZ twin	APG2.9	98.8	93	0	97
1	TS21-mother	APG2.2	98.2	106	0	97
1	TS21-mother	APG3.3	98.6	112	0	98
1	TS21-mother	APG5.6	98.6	110	0	98
1	TS21-mother	APG5.7	98.3	106	0	97
1	TS21-mother	APG5.8	98.3	91	0	97
1	TS21-mother	APG5.9	98.6	106	0	98
1	TS21-mother	APG6.10	96.9	86	0	96
2	TS130-MZ twin	APG1.2	99.6	50	0	99
2	TS130-MZ twin	APG1.3	99.6	67	0	99
2	TS130-MZ twin	APG1.4	99.6	53	0	99
2	TS130-MZ twin	APG1.6	99.6	40	0	99
2	TS131-MZ twin	APG1.1	99.0	81	0	98
2	TS131-MZ twin	APG1.2	98.8	85	0	98
2	TS131-MZ twin	APG1.3	98.9	78	0	98
2	TS131-MZ twin	APG1.4	99.0	34	0	98
2	TS131-MZ twin	APG1.5	99.2	105	0	99
2	TS131-MZ twin	APG1.6	98.9	36	0	98
2	TS131-MZ twin	APG1.7	99.0	38	0	98
2	TS131-MZ twin	APG1.8	99.0	63	0	98
2	TS131-MZ twin	APG1.9	99.1	38	0	99
2	TS132-mother	APG1.1	99.0	103	0	98
2	TS132-mother	APG1.10	99.1	79	0	99
2	TS132-mother	APG1.2	99.1	106	0	98
2	TS132-mother	APG1.3	99.3	117	0	99
2	TS132-mother	APG1.4	99.1	118	0	98
2	TS132-mother	APG1.5	99.1	111	0	99
2	TS132-mother	APG1.6	99.0	93	0	99
2	TS132-mother	APG1.7	99.1	117	1	99
2	TS132-mother	APG1.8	99.2	108	1	99
2	TS132-mother	APG1.9	98.9	114	0	98
3	TS118-DZ twin	APG1.1	99.7	127	0	99
3	TS118-DZ twin	APG1.10	99.5	116	0	99
3	TS118-DZ twin	APG1.11	99.9	111	0	99
3	TS118-DZ twin	APG1.12	99.9	66	0	99
3	TS118-DZ twin	APG1.14	99.7	99	0	99
3	TS118-DZ twin	APG1.15	99.8	91	0	99
3	TS118-DZ twin	APG1.16	99.7	117	1	99
3	TS118-DZ twin	APG1.2	99.7	84	0	99
3	TS118-DZ twin	APG1.3	99.8	80	0	99
3	TS118-DZ twin	APG1.5	99.9	91	0	99
3	TS118-DZ twin	APG1.6	99.7	125	0	99
3	TS118-DZ twin	APG1.7	99.9	99	1	99
3	TS118-DZ twin	APG1.8	99.7	110	0	99
3	TS118-DZ twin	APG1.9	99.5	112	1	99
3	TS119-DZ twin	APG1.1	99.1	71	4	98
3	TS119-DZ twin	APG1.3	99.7	60	0	99
3	TS120-mother	APG1.1	99.4	85	1	99
3	TS120-mother	APG1.2	97.3	88	2	96
4	TS1-MZ twin	APG1.01	99.7	86		99
4	TS1-MZ twin	APG1.02	99.6	83	1	99
4	TS1-MZ twin	APG1.03	99.7	92	0	99
4	TS1-MZ twin	APG1.04	99.8	75	0	99
4	TS1-MZ twin	APG1.05	99.7	89	0	99
4	TS1-MZ twin	APG1.06	99.6	105	0	99
4	TS3-mother	APG1.03	99.8	102	0	99
4	TS3-mother	APG1.05	99.8	16	0	99

TABLE 3—continued

Family	Family ID-relationship	Isolate ID	Percent ID to VPI-5482 16 S rRNA	Percentage of VPI-5482 260.8 ELISA	Percentage of VPI-5482 225.4 ELISA	Percent ID to best hit in RDP
4	TS3-mother	APG1.06	99.8	96	0	99
4	TS3-mother	APG1.07	99.8	34	0	99
5	TS61-DZ twin	APG1.1	99.6	62	21	99
5	TS61-DZ twin	APG1.10	99.6	123	74	99
5	TS61-DZ twin	APG1.4	99.6	95	14	99
5	TS61-DZ twin	APG1.5	99.6	122	178	99
5	TS61-DZ twin	APG1.6	99.6	98	17	99
5	TS61-DZ twin	APG1.7	99.4	119	125	99
5	TS61-DZ twin	APG1.8	99.6	115	15	99
5	TS61-DZ twin	APG1.9	99.6	99	101	99
5	TS62-mother	APG1.1	99.8	41	0	99
5	TS62-mother	APG1.2	99.8	71	0	99
6	TS4-MZ twin	D6G4	94.5	29	0	99
6	TS6-mother	A7	99.7	88	0	98

Insertion Sequencing (INSeq) Analysis of *B. thetaiotaomicron* VPI-5482 Grown in Vitro and in Gnotobiotic Mice—INSeq is based on the modified *mariner* transposon delivery vector, pSAM, that as noted above contains an antibiotic resistance cassette flanked by MmeI-modified *mariner* inverted repeats, the *himar1C9* transposase, and machinery for replication in the donor strain and for conjugative transfer (33). To identify transposon insertion sites, genomic regions adjacent to the transposons were amplified by linear PCR with a biotinylated primer and subsequently bound to magnetic beads where they were digested with MmeI and linkers were appended to each restriction fragment. After limited PCR amplification, fragments were sequenced with an Illumina GA II instrument (42-nucleotide unidirectional reads), and each read was used to map the location of each transposon mutant in the bacterial genome (33).

The INSeq library (~35,000 mutant strains; 72% of predicted ORFs covered by at least one transposon) was grown in TYG medium in a continuously fed batch fermenter (two independent experiments). Aliquots were subjected to INSeq analysis using an experimental and computational pipeline described in our earlier publication (33). For *in vivo* experiments, the mutant library was introduced alone or in combination with three defined consortia of sequenced members of the normal human gut microbiota. Prior to gavage, community members were assembled in equal proportion under anaerobic conditions (see Ref. 33 for details).

Hybridoma Backpack Experiments in *Rag1*^{-/-} Mice—260.8 hybridoma cells were grown in DMEM supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml) until they were 50–75% confluent and subsequently washed three times in pyrogen-free saline. Cells were introduced into the gnotobiotic isolators in a way that preserved sterility (34) and then injected subcutaneously on the dorsum of germ-free 6–8-week-old male C57BL/6J *Rag1*^{-/-} recipients (2×10^6 cells/mouse). Ten days after implantation, each mouse with a palpable subcutaneous hybridoma was colonized with a single gavage of 10^8 cfu of *B. thetaiotaomicron*. Mice were sacrificed 10 days following colonization, and cecal contents were recovered.

GeneChip-based Transcriptional Profiling of *B. thetaiotaomicron* in Vivo—Cecal contents were flash frozen in liquid nitrogen immediately after their harvest from each mouse in each treatment group ($n = 4$ mice/group with hybridoma backpacks and controls). An aliquot (~200 mg) was thawed in 2–3 volumes of RNAProtect (Qiagen) and centrifuged ($3,000 \times g$ for

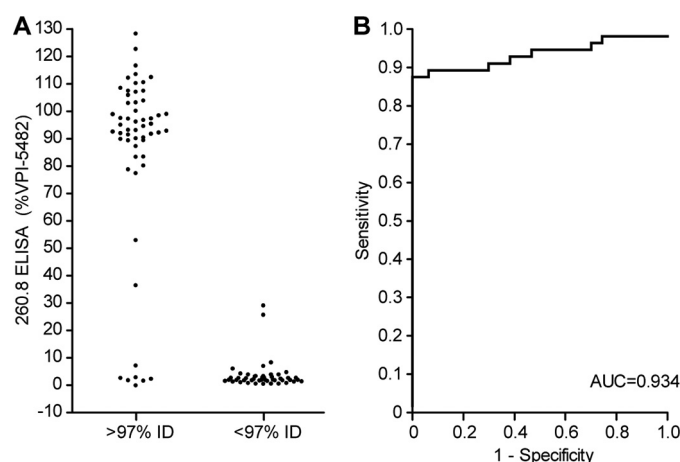


FIGURE 1. Sensitivity and specificity of mAb 260.8 for correct identification of *B. thetaiotaomicron*. A, ELISA reactivity of *B. thetaiotaomicron* isolates. A threshold of $\geq 97\%$ nucleotide sequence identity (% ID) to the 16 S rRNA gene in the type strain VPI-5482 was used to define species membership and to characterize the species specificity of the mAb 260.8 ELISA. B, receiver-operator characteristics of the 260.8 ELISA signal (absorbance measured at 405 nm). AUC, area under the curve.

10 min), and 500 µl of 200 mM NaCl, 20 mM EDTA was added to the resulting pellet together with 200 µl of 20% SDS and 500 µl of phenol:chloroform:isoamyl alcohol, pH 4.5 (125:24:1; Ambion). Acid-washed silica beads (Sigma; 212–300-µm diameter; 250 mg) were added to the mixture, and bacteria were lysed (Mini-Beadbeater, Biospec; “high” setting for 5 min at room temperature). Following centrifugation ($13,000 \times g$ for 3 min at 4 °C), the sample was re-extracted in phenol:chloroform:isoamyl alcohol, precipitated with 60 µl of 3 M sodium acetate, pH 5.2 plus 600 µl of cold isopropanol. RNA was purified (RNeasy kit, Qiagen). Residual genomic DNA was subsequently removed by treatment with DNAfree (Ambion), and the relative proportion of bacterial *versus* host RNA in each preparation was defined by quantitative RT-PCR using primers directed at *B. thetaiotaomicron* 16 S rRNA (forward, 5'-GGT-AGTCCACACAGTAAACGATGAA; reverse, 5'-CCCGTCA-ATTCCTTTGAGTTTC) and mouse 18 S rRNA (forward, 5'-CATTCGAACGTCTGCCCTATC; reverse, 5'-CCTGTGCC-TTCCTTGGA). The results disclosed that $\geq 90\%$ of the recovered rRNA in each RNA preparation was bacterial.

GeneChip targets were generated as described (24). The cDNA product was isolated (QiaQuick spin columns, Qiagen), fragmented by treatment with DNase I (Amersham Biosci-



FIGURE 2. 16S rRNA-based phylogenetic tree of human gut *Bacteroides* isolates and their reactivity with mAb 260.8 as measured by ELISA. Icons accompanying the isolate name are color-coded according to the source (i.e. type strain, culture collection, or the human family with twins from which the isolate was derived). Branch and text color reflect the ELISA reactivity.

ences), and biotinylated (BioArray terminal labeling kit). Standard Affymetrix protocols were used for hybridization of the cDNA targets to each *B. thetaiotaomicron* GeneChip (23, 24). After normalization of the data, model-based expression values were generated (perfect match-mismatch model). Signals from spiked-in control transcripts and oligo-B2 (Affymetrix) were used to assess the quality of target preparation and target hybridization, respectively. Comparisons were performed on specified GeneChip data sets to identify genes up- or down-regulated in the experimental group (*E*) relative to the baseline group (*B*). The following criteria were used to identify significantly up-regulated genes: (i) $E/B > 1.5$; (ii) $E - B \geq 100$; (iii) $E = B$, $p < 0.05$; and (iv) called "Present" in $\geq 75\%$ of "*E*" GeneChips.

Assaying the Effect of the mAbs on Growth and Gene Expression in Vitro—*B. thetaiotaomicron* VPI-5482 was cultured anaerobically in TYG medium to stationary phase, and an aliquot was diluted to an A_{600} of 0.01 in 5 ml of fresh TYG

medium. Optical density was measured over time in three replicate monocultures using a Spectronic 20D+ spectrophotometer (Thermo).

When monocultures grown as described in the preceding paragraph had reached an A_{600} of ~ 0.1 , 50 μ l of either PBS, mAb 225.4 (1 mg/ml in PBS), mAb 260.8 (1 mg/ml in PBS), or an equal mixture of mAb 225.4 plus mAb 260.8 (each at 1 mg/ml) were added, and tubes were resealed. An additional set of cultures was grown anaerobically in the presence of an isotype monoclonal IgA (anti-CD36; 1 mg/ml; clone JC63.1, Abcam). Prior to its use, this antibody was dialyzed at 4 °C against three washes of PBS using a 10-kDa-cutoff membrane (Slide-A-Lyzer 10K Dialysis Cassettes, Thermo) to remove glycerol and traces of azide.

Replicate cultures were harvested into 10 ml of RNAProtect (Qiagen) after 10.5 h of growth when all cultures had reached an A_{600} of 0.5–0.7 (note that no differences in growth kinetics were observed between cultures inoculated with antibody com-

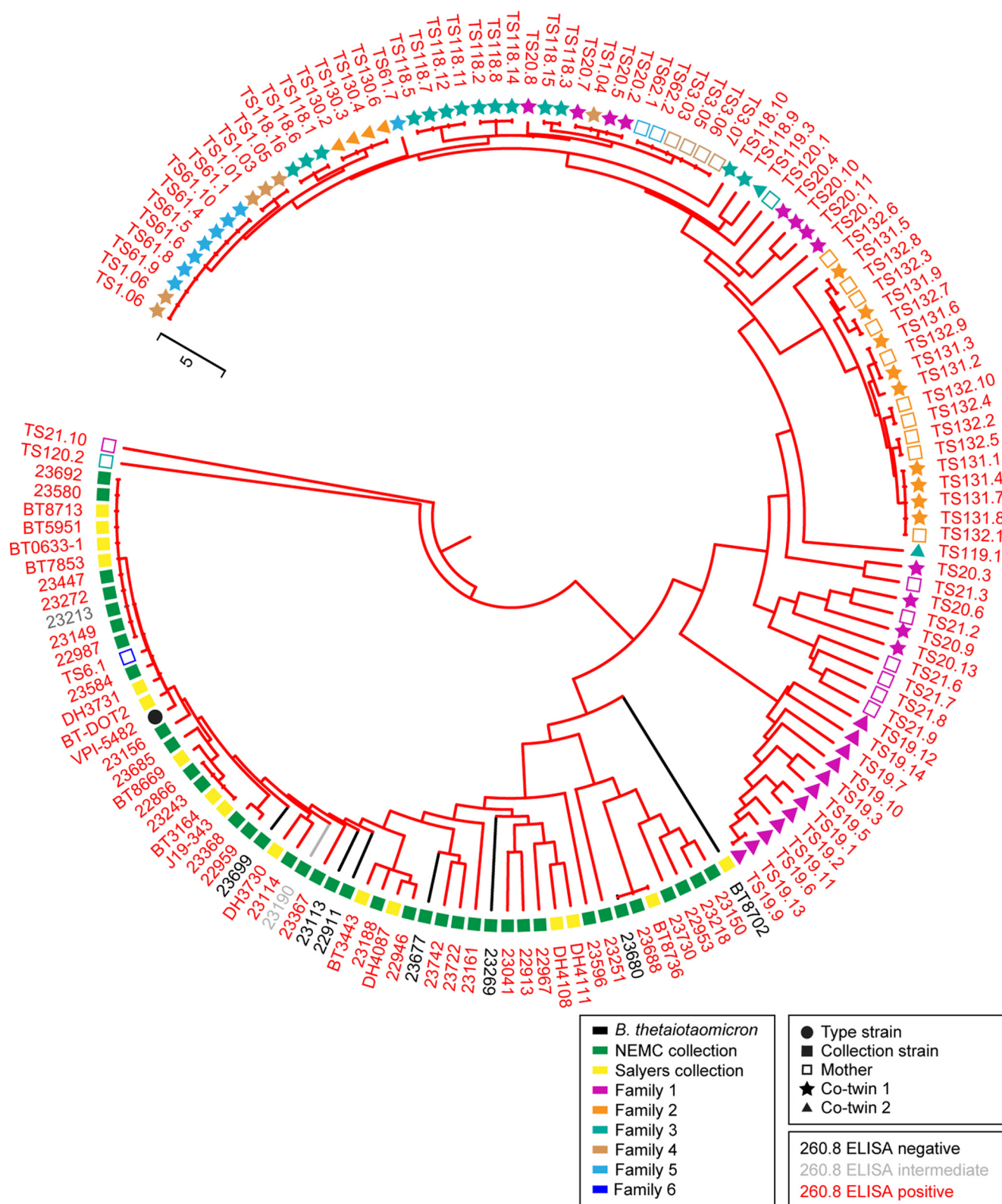


FIGURE 3. **16 S rRNA-based phylogenetic tree of human gut *B. thetaiotaomicron* isolates and their reactivity with mAb 260.8 as measured by ELISA.** Shown is an expansion of the *B. thetaiotaomicron* subgroup depicted in Fig. 2. Icons accompanying the isolate name are color-coded according to the source (i.e. type strain, culture collection, or the human family with twins from which the isolate was derived). Branch and isolate name color reflect the ELISA reactivity.

pared with control cultures inoculated with PBS). After a 5-min incubation at 24 °C, cells were centrifuged at 3,000 × g for 10 min. The supernatant was removed, and cell pellets were frozen at −80 °C until further use.

RNA was isolated as above with the exception that following isopropanol precipitation pellets were washed with 750 μl of ice-cold 70% ethanol and resuspended in 100 μl of nuclease-free water (Ambion). RNA was then cleaned up twice using

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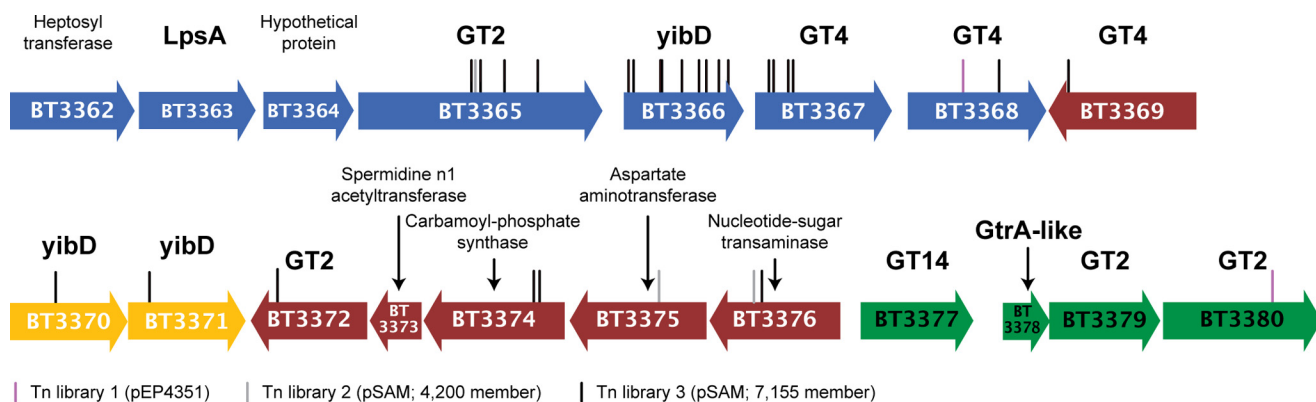


FIGURE 4. The 260.8 epitope is produced by a locus spanning BT3362–BT3380 of the *B. thetaiotaomicron* VPI-5482 genome. Annotation and predicted functions of genes in the locus are shown. Sites of insertion of transposons (*Tn*) from the three mutant libraries that are associated with loss of reactivity to mAb 260.8 in the ELISA are indicated by color-coded vertical lines.

MEGAclear (Life Technologies) and DNase-treated with TURBO DNase (Life Technologies) and Baseline-ZERO DNase (Epicentre). RNA integrity was verified by gel electrophoresis, and genomic DNA depletion was confirmed using a PCR assay directed at the 16 S rRNA gene (primers 8F and 1391R). rRNA was depleted using Ribo-Zero (Epicentre) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript II kit (Life Technologies) and then sheared for 10 min using a bath sonicator (Bioruptor, Diagenode) with alternating cycles of 30 s of sonication and a 30-s pause (sonication was carried out at 4 °C). Sonicated DNA was concentrated using MinElute kits (Qiagen). Blunt end synthesis and 5' nucleotide phosphorylation was performed using T4 polynucleotide kinase and T4 polymerase (New England Biolabs; incubation at 25 °C for 30 min). A-tails were generated using *Taq* polymerase (Invitrogen; incubation at 75 °C for 20 min). Illumina adapters were ligated to the DNA with T4 DNA ligase (New England Biolabs), and cDNAs were size-selected by 2% agarose gel electrophoresis. PCR of the adapter-ligated product was performed using the following cycling conditions: 98 °C for 30 s, 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 45 s (a total of 18 cycles). Samples were pooled in equal proportion and sequenced using an Illumina HiSeq 2500 instrument (unidirectional 50-nucleotide reads). The resulting microbial RNA-Seq data set was analyzed as described previously (35) with the following exceptions. (i) After splitting barcodes, reads were mapped to the *B. thetaiotaomicron* VPI-5482 reference genome using Bowtie (36), and (ii) analysis of differential expression was performed using the DESeq2 package in R (37). Functional annotation of genes with significantly different expression after false discovery rate correction was performed using the NCBI online genome browser.

RESULTS

Phylogenetic Distribution of 260.8 Epitope—In a previous study, we showed that 24 h after gavage of adult germ-free C57BL/6J mice with 10^8 cfu of the sequenced *B. thetaiotaomicron* type strain VPI-5482 bacteria achieve a density in the small intestine and cecum that does not change significantly over the ensuing 6 months and that within 2 weeks of colonization *B. thetaiotaomicron*-reactive B cells are well represented in the intestine and can be captured by hybridoma fusion (23). This

allowed us to immortalize a single naturally primed, bacterial epitope-specific IgA response (see the Introduction).

One of the cloned IgA-producing hybridoma cell lines, designated 260.8, produces a mAb that recognized polysaccharides extracted with hot water/phenol from wild-type *B. thetaiotaomicron* VPI-5482 grown in rich TYG medium and from the same strain isolated from the ceca of gnotobiotic mice 180 days after monoclonization (data not shown). To define the phylogenetic distribution of the epitope recognized by the 260.8 mAb, we surveyed two existing collections of *Bacteroides* isolates. The “Salysers collection” was provided by Abigail Salysers (University of Illinois, Urbana/Champaign) and contained 22 isolates recovered by the microbiology laboratories of the Virginia Polytechnic Institute (Blacksburg, VA), the Wadsworth Veterans Affairs Hospital (Los Angeles, CA), the Loyola Veterans Affairs Hospital (Maywood, IL), the Carle Hospital (Urbana-Champaign, IL), and the Marine Biology Laboratory (Woods Hole, MA) (Table 1). The “NEMC Collection” from the Special Studies Laboratory at New England Medical Center contained 78 strains isolated by clinical microbiology laboratories within the United States in 2006 (38) (Table 2). These isolates had been characterized by using a commonly used diagnostic method (API RapID ANA II System) and/or by other standard biochemical techniques. Using 16 S rRNA gene sequencing, we found that these methods had misidentified 41% of the isolates, a rate higher than reported by others (39). In addition to these pre-existing strain panels, we tested the ability of mAb 260.8 to identify *B. thetaiotaomicron* from individual human fecal microbiota samples. Aliquots of frozen fecal samples obtained from six healthy adult female twin pairs and their mothers were cultured on BHI blood agar plates under anaerobic conditions, and the resulting colonies were blotted onto nitrocellulose filters. These filters were subsequently incubated with mAb 260.8. Using this approach, we identified mAb 260.8-reactive isolates in 15 of the 18 donors surveyed (Table 3). Single colonies that reacted with the mAb were then cultured in TYG medium and characterized by ELISA using both 225.4 and 260.8 mAbs (see “Experimental Procedures”). DNA was extracted from these isolates; near full-length amplicons were subsequently generated from their 16 S rRNA genes by PCR and sequenced using the dideoxy chain termination method.

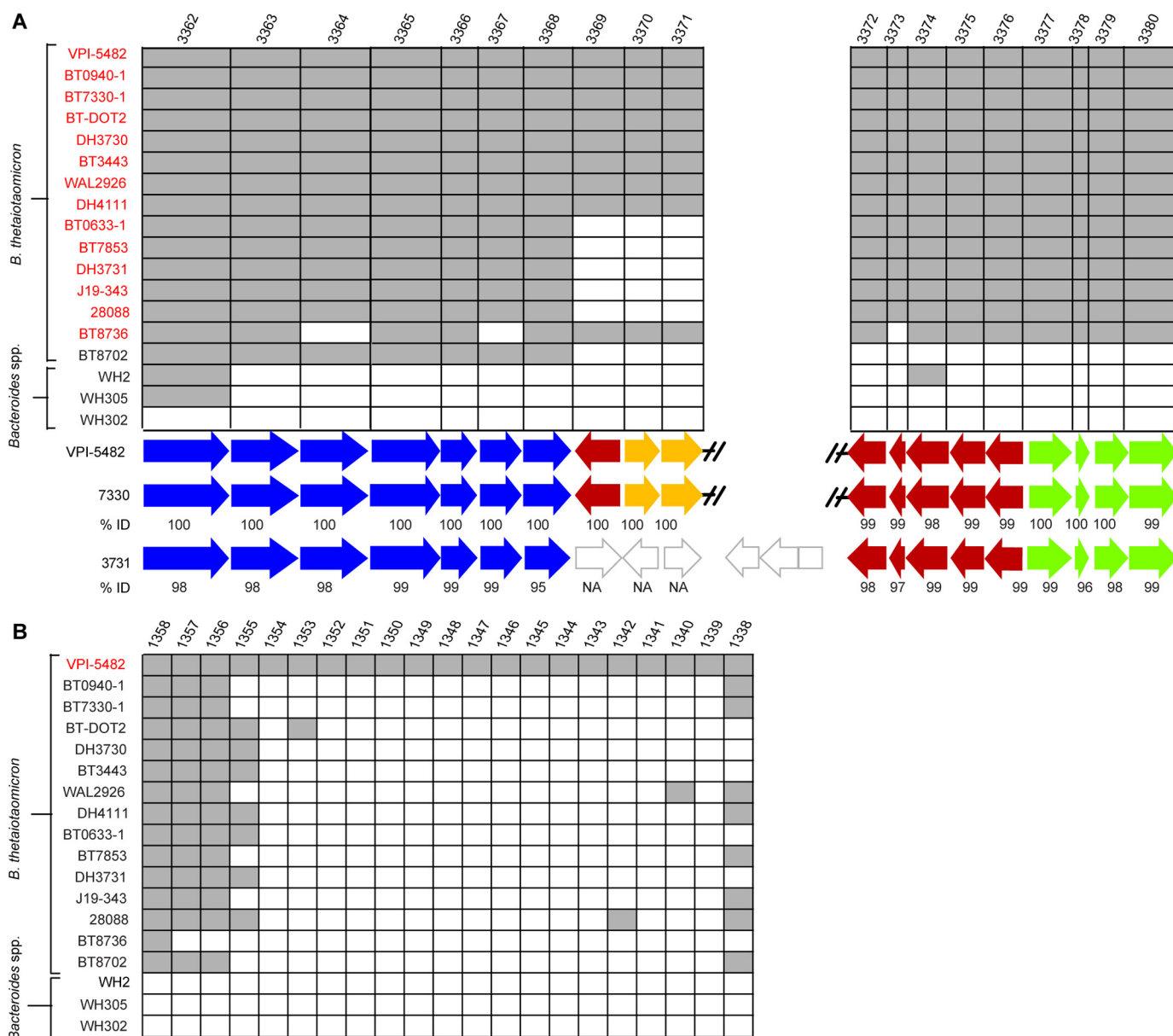


FIGURE 5. Comparative genomic hybridization and analysis of draft genome assemblies of *B. thetaiotaomicron* isolates reveal that the BT3362–BT3380 locus is well conserved. Genomic DNA from the indicated isolates was hybridized to *B. thetaiotaomicron* GeneChips. *A*, isolate strain names colored red are 260.8-positive in the ELISA, whereas those colored black are 260.8-negative. Genes called “present” are shown in gray, whereas those called “absent” are white. The locus required for production of the 260.8 epitope is compared between the VPI-5482 type strain and two 260.8-positive *B. thetaiotaomicron* isolates (DH3731 and BT7330-1). Percent nucleotide sequence identities (% ID) for ORFs in the draft genomes are expressed relative to the type strain. Data obtained from other *Bacteroides* species are shown for reference. *B*, comparable comparative genomic hybridization data for the CPS4 locus that is the source of the epitope recognized by mAb 225.4.

Combining the results of the ELISA and 16 S rRNA gene sequencing revealed that the 260.8 mAb is an excellent diagnostic reagent with strong discriminatory power for *B. thetaiotaomicron*. Receiver operating characteristic curves generated by plotting the fraction of true positives among all positives (true positive rate) versus the fraction of false positives of the negatives (false positive rate) demonstrated an area under the curve of 0.934 for distinguishing isolates whose 16 S rRNA genes had >97% nucleotide sequence identity with the 16 S rRNA gene in the finished genome of the VPI-5482 type strain from isolates whose 16 S rRNA genes had <97% nucleotide sequence identity (Fig. 1, *A* and *B*).

Isolates from the readily cultured component of fecal microbiota samples that were initially identified as positive in the 260.8-based dot blot assay and confirmed by ELISA had a similar distribution of 16 S rRNA sequence identity to strain VPI-5482 as isolates in the previously established Salyers and NEMC collections (see Tables 1 and 2, and Figs. 2 and 3 for 16 S rRNA-based phylogenetic trees of the isolates). Each of the isolates identified by dot blot assay as being positive (260.8-reactive) after three rounds of streaking for clonality was antigen-positive in the ELISA, and 93 of 94 were confirmed to be *B. thetaiotaomicron* by 16 S rRNA sequencing (Table 3). We subsequently screened this collection of 260.8-positive isolates for

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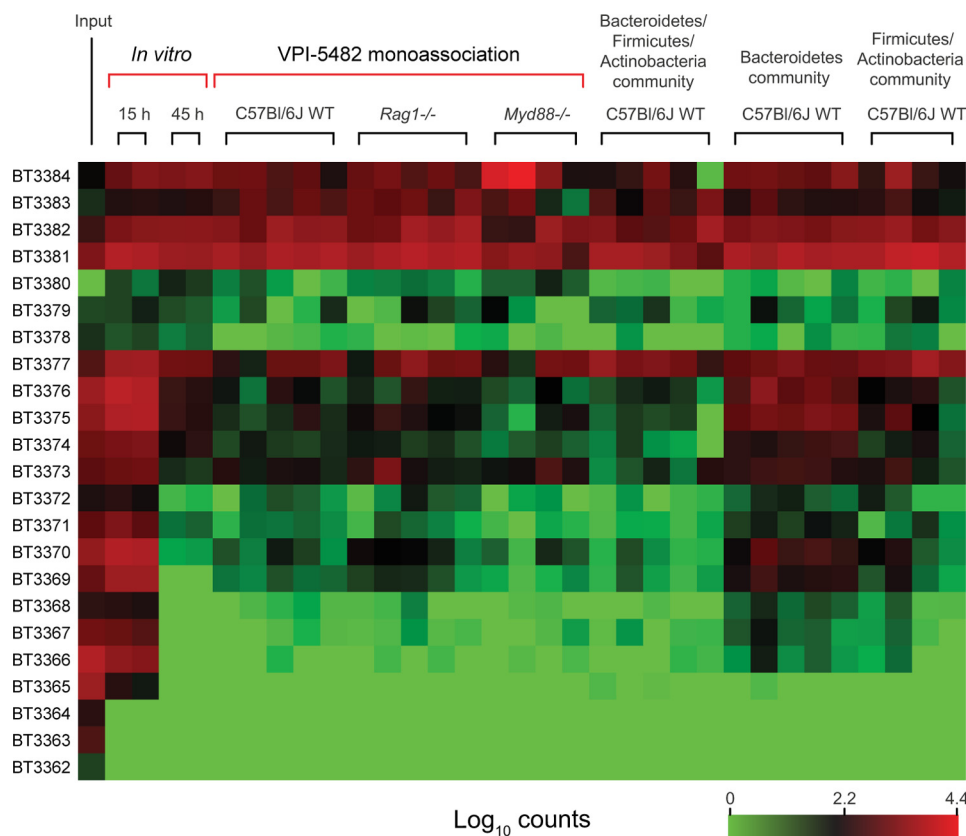


FIGURE 6. INSeq analysis of the importance of genes spanning *BT3362–BT3380* to the fitness of *B. thetaiotaomicron* VPI-5482 under different environmental conditions. The frequency (relative abundances) of mutants in the input INSeq library (left column) is compared with their frequency in output communities recovered (i) after 15 or 45 h of growth in TYG medium in a continuously fed batch fermenter; (ii) 10 days after monocolonization of C57BL/6J wild-type, *Rag1*^{-/-}, or *Myd88*^{-/-} mice; or (iii) 10 days after colonization of mice with a six-member community of human gut Bacteroidetes, an eight-member community of human gut Firmicutes and Actinobacteria, or a 14-member community composed of the Bacteroidetes, Firmicutes, and Actinobacteria. Note that data for *BT3381–BT3384*, which are not part of the locus required for 260.8 epitope production, are included as reference controls.

mAb 225.4 reactivity and found that all strains from all but one of the donors (TS61) were 225.4-negative (Table 3).

Identification of a Conserved Locus in the *B. thetaiotaomicron* Genome Required for Production of the 260.8 Epitope—To determine whether the 260.8 mAb, like the previously characterized 225.4 mAb, recognizes a capsular polysaccharide epitope, cultures of wild-type *B. thetaiotaomicron* VPI-5482 and eight isogenic mutant strains, each harboring a polar insertion of a suicide vector in the first gene of each of the organism's eight capsular polysaccharide synthesis loci, were extracted with hot phenol/water (which retains capsular polysaccharides and LPS), and the extracts were examined by ELISA. Levels of this epitope were not significantly different in the wild-type strain grown to stationary phase in TYG medium compared with any member of the panel of isogenic mutant strains (each strain assayed in triplicate; data not shown).

After eliminating these *CPS* loci as a source of the 260.8 epitope, we screened three libraries of isogenic transposon mutants of *B. thetaiotaomicron* VPI-5482 to identify genes required for its expression (see “Experimental Procedures” for details of how these libraries were generated and characterized). The results allowed us to attribute 260.8 epitope production to a 19-gene region spanning *BT3362–BT3380* (Fig. 4). The genes in this locus are predicted to encode glycosyltransferases belonging to carbohydrate-active enzyme glycosyltransferase families 2, 4, and 14. Mutants in *BT3362–BT3364* were

not observed in any of the three transposon mutant libraries, suggesting that these genes may be essential for *in vitro* growth and survival under the conditions used to create the transposon library. We have provisionally included them in the 260.8 locus on the basis of RNA-Seq data indicating that *BT3362–BT3364* are co-transcribed with the downstream genes that we observed were required for epitope expression (35). *BT3362* and *BT3363* are homologs of *WaaC/YibC* (heptosyltransferase) and *LpsA* involved in 3-deoxy-D-manno-octulosonic acid-lipid A modification, suggesting that at least some of the downstream glycosyltransferases are involved in LPS O-antigen polysaccharide synthesis and modification in *B. thetaiotaomicron*. These findings are consistent with the observation that mAb 260.8 reacts with hot water/phenol-extracted material that contains LPS. None of the transposon mutants had rough colony morphology, indicating that there is likely redundancy in the capacity for LPS O-antigen biosynthesis in the *B. thetaiotaomicron* genome.

To further define which genes in the *BT3362–BT3380* locus are conserved and required for producing the epitope recognized by mAb 260.8, we characterized 17 *B. thetaiotaomicron* strains using a combination of comparative genomic hybridization with custom GeneChips that contained probe sets for >98% of the 4,779 known or predicted genes in the type strain and by generating deep draft assemblies of the genomes of two 260.8-positive human gut-derived isolates from the Salyers col-

lection (strains DH3731 and BT7330-1; N50 contig lengths = 97,716 and 217,107 bp, respectively (Fig. 5A)). Comparison with ELISA data from the same isolates allowed us to determine that several genes in the locus are not required for expression of the 260.8 epitope. For example, several strains that lacked *BT3369–BT3371*, a region that encodes three putative glycosyltransferases, retained ELISA reactivity. Comparative genomic hybridization also established that the one 260.8-negative strain (8702) was missing all but seven of the conserved genes from the *BT3362–BT3380* locus. In contrast, the *CPS4* locus responsible for production of the 225.4 epitope was only conserved in the type strain, consistent with the observed pattern of 225.4 ELISA reactivity (Fig. 5B).

Role of the 260.8 locus in bacterial fitness—Examining transposon mutants under different growth conditions allowed us to define the impact of the genes that are essential for 260.8 epitope formation on fitness *in vitro* and *in vivo*. A library of 34,554 mutants of *B. thetaiotaomicron* generated with a modified mariner transposon that contains MmeI sites at its flanking inverted repeat sites had allowed us to use a method known as INSeq to quantify the proportional representation of mutants in the library before and after a series of selections were applied (33). These selections included (i) growth during early and late log phase in batch fermenters containing TYG medium; (ii) introduction into wild-type, *Rag1*^{−/−}, or *Myd88*^{−/−} C57BL/6J mice without other gut bacterial species for 14 days ($n = 4–5$ mice/group); and (iii) introduction into wild-type mice together with one of three types of defined communities of sequenced members of the human gut microbiota. These communities were assembled from strains representing three bacterial phyla that numerically dominate the human distal gut microbiota and included a community of six other Bacteroidetes, a community composed of eight members of the Firmicutes and Actinobacteria, and a third community consisting of all 14 of these representatives (see Table S7 of Ref. 33 for the species composition of these communities; note that all communities also contained the *B. thetaiotaomicron* INSeq mutant library).

Analysis of these previously generated INSeq data sets (33) now enabled us to identify *BT3362–BT3368* as well as *BT3378–BT3380* in the *BT3362–BT3380* 260.8 locus as required for fitness during growth in TYG medium in a continuously fed batch fermenter and under all *in vivo* conditions tested. Mutants in *BT3373–BT3376* (encoding a putative spermidine *N*¹-acetyltransferase, carbamoyl-phosphate synthase, aspartate aminotransferase, and a nucleotide-sugar transaminase; Fig. 4) transcribed from the opposite strand as the genes required for 260.8 expression exhibited fitness defects in gnotobiotic mice that were robust to wild-type, *Rag1*^{−/−}, and *Myd88*^{−/−} host genotypes but sensitive to microbial community context in wild-type C57BL/6J hosts (mutants were proportionally underrepresented in the Firmicutes/Actinobacteria and full communities compared with the Bacteroidetes-only community) (Fig. 6).

To validate our findings from the INSeq data set and to further assess the impact of the 260.8 epitope (genes) on fitness *in vivo*, we performed a competition experiment in wild-type C57BL/6J mice. A 1:1 mixture of wild-type VPI-5482 and an

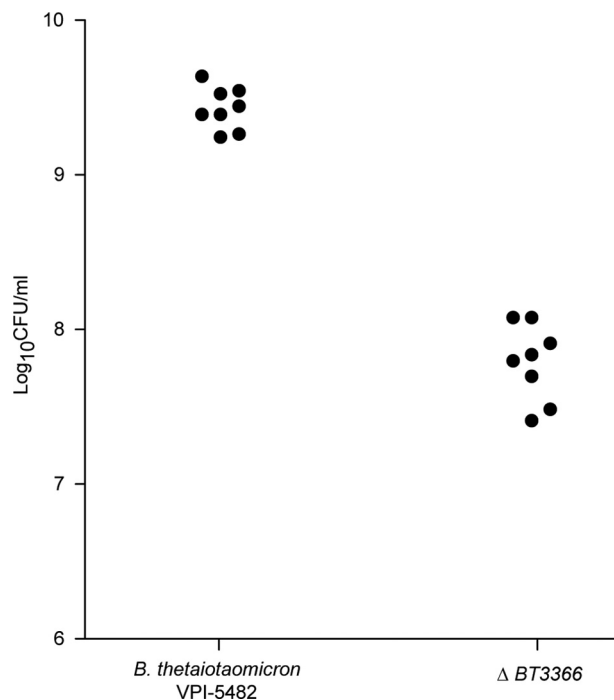


FIGURE 7. **In vivo competition assay between wild-type *B. thetaiotaomicron* and an isogenic strain lacking *BT3366*.** Wild-type germ-free adult male C57BL/6J mice ($n = 8$) received a 1:1 mixture of wild-type VPI-5482 and an isogenic mutant strain containing a transposon disruption of *BT3366*. Animals were sacrificed 10 days later. Wild-type and mutant strains were quantified by replicate plating on selective medium (BHI blood agar with or without erythromycin). Each filled circle represents the results from a given animal.

isogenic mutant strain (3.1.G12) containing a transposon disruption of *BT3366* (*yibD* glycosyltransferase) were introduced into germ-free mice by gavage. After 10 days, we observed a 40-fold lower level of the mutant strain in the cecal contents of these animals ($p < 0.0001$, unpaired *t* test; $n = 8$ mice per group; Fig. 7).

Expression of the 260.8 and 225.4 Epitopes during Growth in Vivo and in Vitro—To determine whether expression of the 260.8 and 225.4 epitopes was stable during prolonged periods of growth *in vitro* and *in vivo*, two experiments were performed. In the first experiment, wild-type *B. thetaiotaomicron* VPI-5482 was inoculated into wild-type, *Myd88*^{−/−}, and *Rag1*^{−/−} mice. Animals were caged individually ($n = 2$ animals/genotype/isolator), and fecal samples were collected regularly throughout the duration of their natural lives with no further intervention. In the second experiment, a separate aliquot of the gavage sample was used to inoculate eight individual broth cultures that were subsequently passaged daily over the course of 300 days of *in vitro* growth.

ELISA of the intact fecal bacterial communities sampled at early and late time points from both experiments revealed no change in overall levels of 225.4 or 260.8 epitope expression (data not shown). Because phase variable expression of the capsular polysaccharide has been demonstrated in a related *Bacteroides* spp. (*Bacteroides fragilis*; Ref. 40) and hypothesized for several others (41), we examined the extent to which expression of these epitopes varied among members of the population during prolonged growth *in vivo* and *in vitro*. Thus, for each sample, 10 or more colonies were isolated from early and late time

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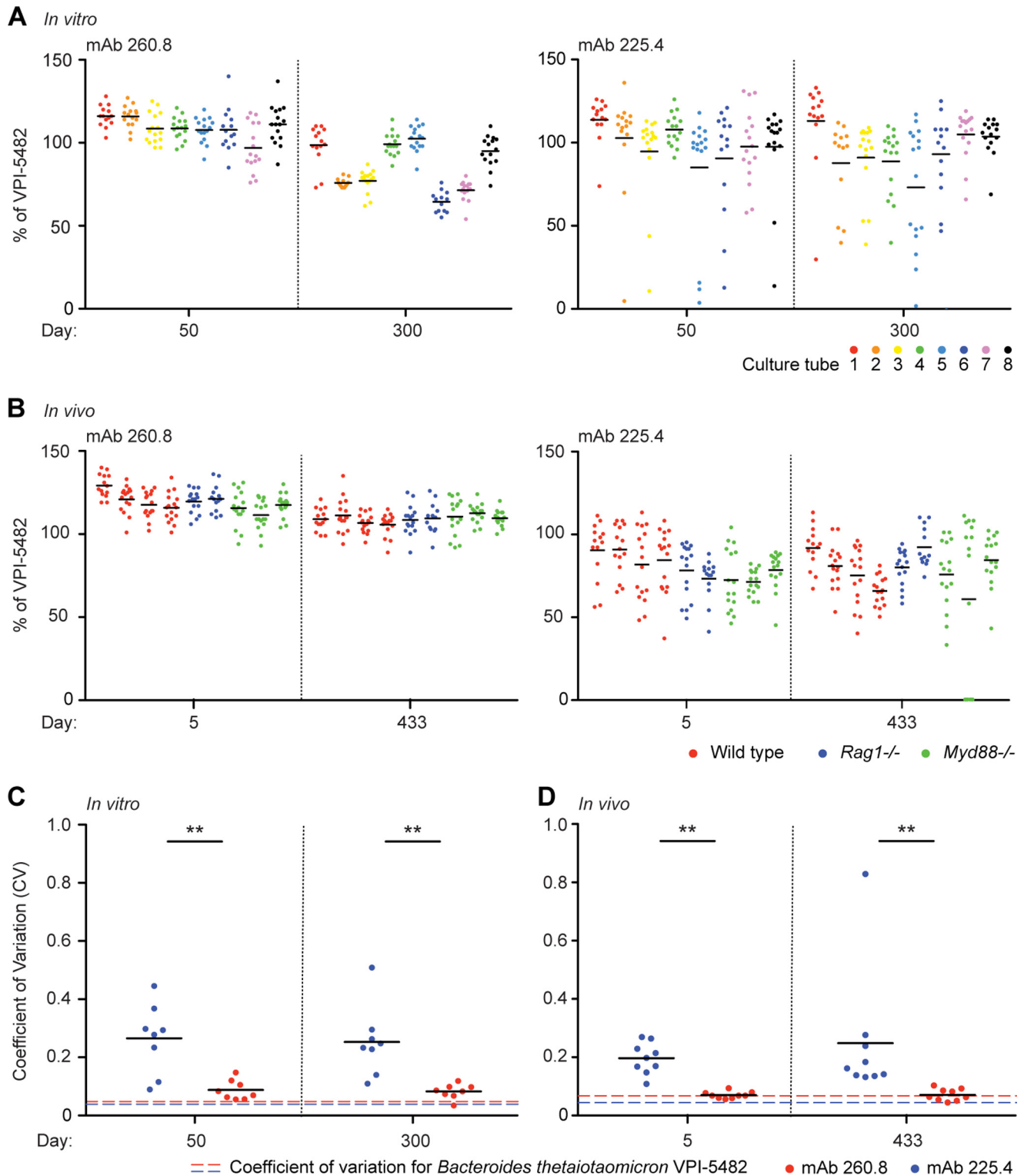


FIGURE 8. Expression of the 260.8 and 225.4 epitopes in monoclonalized gnotobiotic mice and continuously passaged cultures. *A*, samples taken at the indicated time points from *B. thetaiotaomicron* VPI-5482 monocultures that were passaged daily for 300 days in eight separate culture tubes containing minimal medium. *B*, fecal samples collected from wild-type, *Rag1*^{-/-}, or *Myd88*^{-/-} gnotobiotic mice 5 and 433 days after monocolonization with *B. thetaiotaomicron* were streaked onto TYG agar. Individual colonies were then picked into TYG broth, grown to late stationary phase, and analyzed by ELISA. Epitope expression was normalized to a late stationary phase culture of the type strain. Each colored circle represents results from a single colony. Horizontal lines represent means for each sample type at the time point surveyed. *C* and *D*, coefficients of variation (CV) for the group of samples obtained at the indicated time points during the *in vitro* and *in vivo* experiments. **, $p < 0.01$ for the comparison between mAb 225.4 and mAb 260.8 (Wilcoxon rank sum test).

point samples (*in vivo*, days 5 and 433; *in vitro*, days 50 and 300). Colonies were picked into TYG medium, grown to stationary phase, and evaluated by ELISA. These experiments allowed us

to conclude that the 260.8 epitope is expressed at a relatively uniform level between isolates from a given sample both *in vitro* and *in vivo* (Fig. 8). In contrast, individual isolates varied widely

with respect to expression of the 225.4 epitope at all time points for nearly all *in vitro* and *in vivo* samples (Fig. 8). These patterns of ELISA reactivity support a model in which the expression of the 260.8 epitope is relatively stable across isolates in a given sample, whereas expression of the 225.4 epitope is under phase variable control. Consistent with this model, homologs of *UpxY* and *UpxZ*, which help mediate phase variation of capsular polysaccharides in *B. fragilis* (42), are present in the 225.4 locus but not in the 260.8 locus.

The Effects of the 260.8 IgA on *B. thetaiotaomicron* in Vivo—To examine the impact of the 260.8 IgA on *B. thetaiotaomicron* *in vivo*, adult male germ-free *Rag1*^{-/-} mice were injected subcutaneously with 260.8 hybridoma cells (*n* = 11 animals). Germ-free *Rag1*^{-/-} and wild-type C57BL/6J controls (*n* = 5/group) did not receive hybridoma cells. After 10 days, all groups of animals were colonized with a single gavage of wild-type *B. thetaiotaomicron* VPI-5482 and then sacrificed 10 days later. At the time of sacrifice, IgA was detected in the serum and cecal contents of animals with the hybridoma backpack and their wild-type counterparts (cecal concentrations, 124 ± 18 and 372 ± 65 μg/ml, respectively; Fig. 9A) but not in *Rag1*^{-/-} (no backpack) controls. No statistically significant differences in bacterial density were observed among the treatment groups as measured by quantifying cfu obtained after culturing cecal contents on BHI blood agar plates (data not shown). ELISA of hot phenol/water-extracted cecal contents indicated no significant change in 260.8 epitope levels (or the control 225.4 epitope in the absence of a circulating IgA that recognizes it) in *Rag1*^{-/-} (mAb 260.8) backpack mice compared with *Rag1*^{-/-} (no backpack) or wild-type controls (Fig. 9B).

Whole genome transcriptional profiling using *B. thetaiotaomicron* GeneChips and RNA prepared from cecal contents harvested at the time of sacrifice revealed a total of 63 bacterial genes whose levels of expression satisfied our criteria (see “Experimental Procedures”) as being significantly different in *Rag1*^{-/-} (mAb 260.8) backpack and wild-type C57BL/6J mice versus *Rag1*^{-/-} (no backpack) controls (Fig. 10). Unlike the 225.4 epitope associated with *CPS4* (BT1338–BT1358), there was not a significant difference in expression of genes in the BT3362–BT3380 locus that specifies the 260.8 epitope. Like previously reported *Rag1*^{-/-} mice with 225.4 hybridoma backpacks, we observed significantly lower levels of expression of bacterial oxidative stress genes (Fig. 10) as well as genes encoding nitrite reductase (BT1415 and BT1416). In this earlier study, we showed that bacterial nitrite reductase expression directly correlates with host inducible NOS expression (23). Although we did not directly measure inducible NOS levels in the current study, our results are consistent with the notion that the 260.8 mAb-bacterium interaction leads to a more muted innate immune response. Finally, unsupervised clustering of *B. thetaiotaomicron* GeneChip data sets showed that the monoclonized *Rag1*^{-/-} (260.8) backpack mice cluster with monoclonized wild-type animals, indicating that the effects of the monospecific IgA immune system on the bacterium shares similarities with the effects of a far less restricted polyclonal immune response to colonization in wild-type gnotobiotic animals.

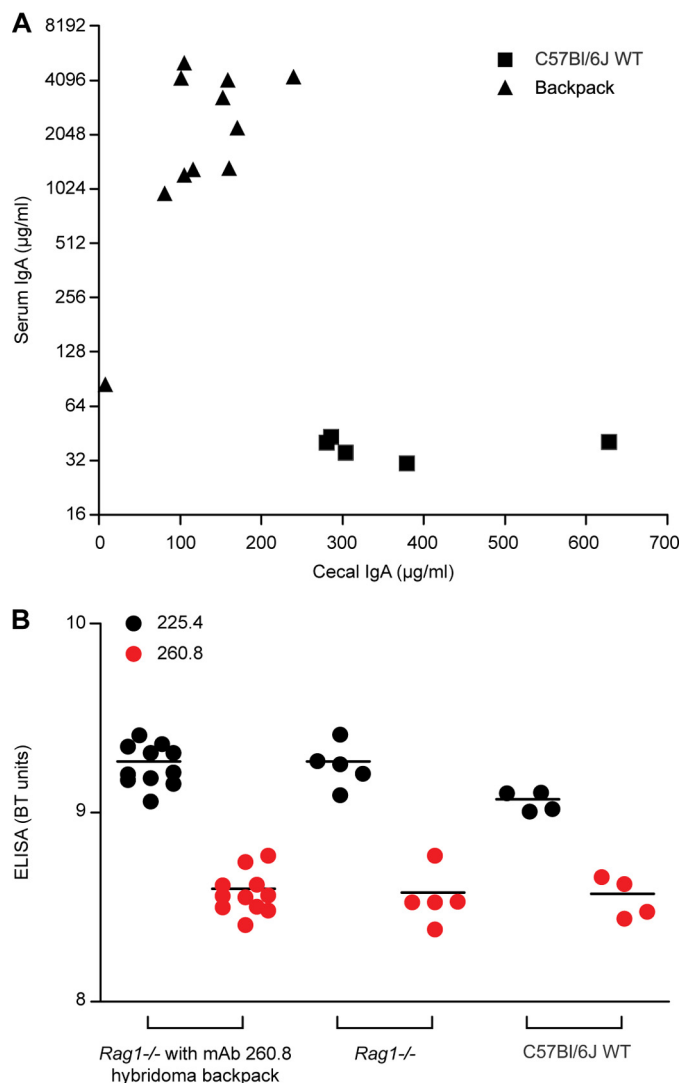


FIGURE 9. *In vivo* analysis of the effects of mAb260.8 production on expression of its epitope in gnotobiotic mice monoclonized with *B. thetaiotaomicron* VPI-5482. A, *Rag1*^{-/-} mice were injected with 260.8 hybridoma cells. Ten days later they were gavaged with *B. thetaiotaomicron*. At the time of sacrifice, 10 days after gavage, IgA was measured in serum and in cecal contents. B, 260.8 epitope expression in cecal contents of monoclonized *Rag1*^{-/-} mice injected subcutaneously with 260.8 hybridoma cells (hybridoma backpack) and wild-type and *Rag1*^{-/-} C57BL/6 mice without hybridoma cells. Cecal contents were extracted with hot phenol/water, and epitope levels were measured by ELISA using the 260.8 IgA or 225.4 IgA mAb. BT, *B. thetaiotaomicron*.

The Effects of the 260.8 and 225.4 IgA on *B. thetaiotaomicron* in Vitro—To test whether the changes in gene expression that we observed *in vivo* were directly mediated by antibody binding or involved host-dependent pathways, we cultured *B. thetaiotaomicron* VPI-5482 in the presence of (i) mAb 260.8 alone, (ii) mAb 225.4 alone, (iii) an equal mixture of the two antibodies, or (iv) a mouse isotype control IgA that does not bind *B. thetaiotaomicron*. There were no differences in the growth characteristics of *B. thetaiotaomicron* in any of the four treatment conditions compared with control cultures inoculated with PBS alone (Fig. 11, A and B). We subsequently performed microbial RNA-Seq of midlog phase cultures. The results disclosed that the changes in gene expression we observed *in vivo* were not recapitulated by antibody binding *in*

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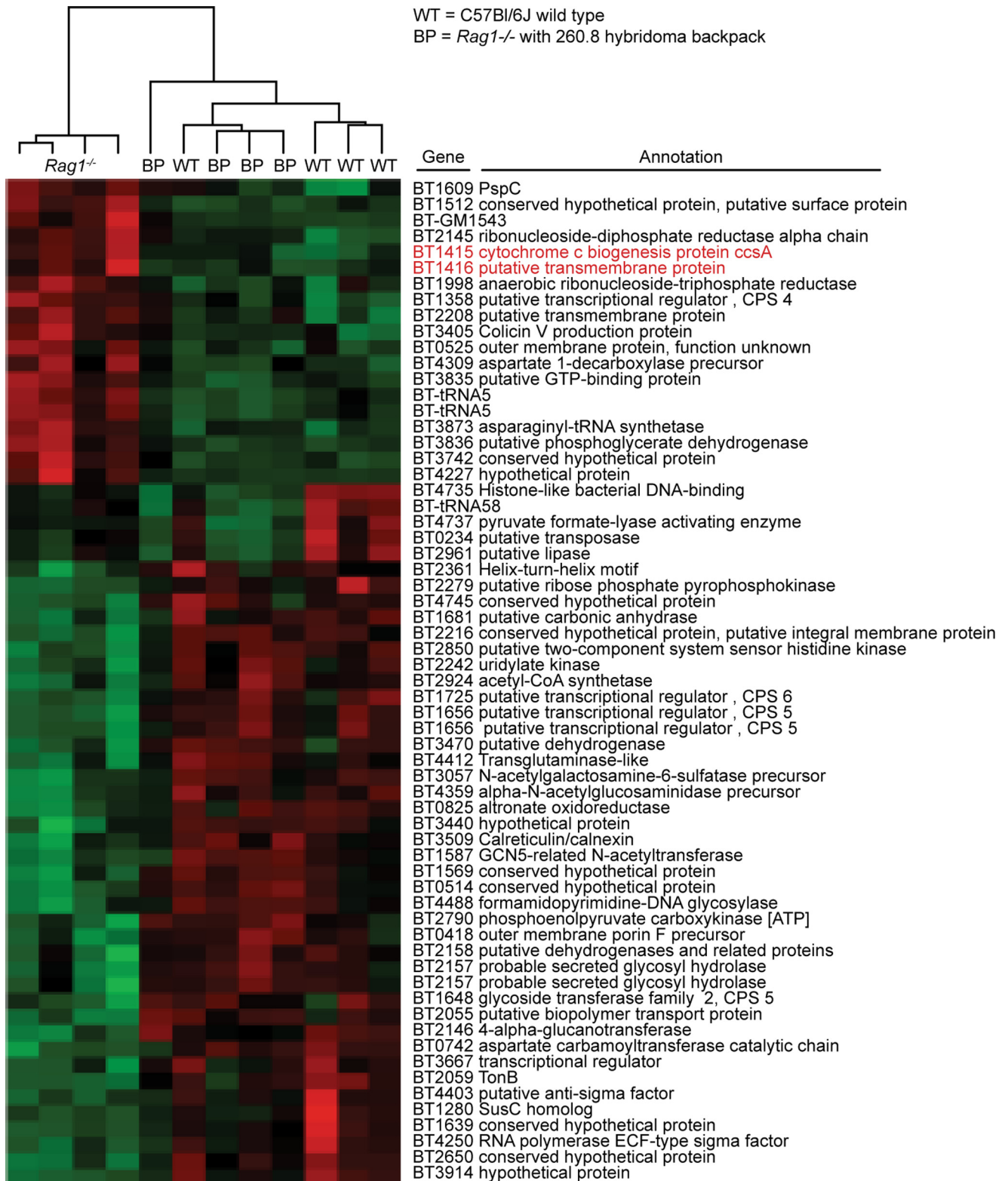


FIGURE 10. **GeneChip profiling of the effects of mAb 260.8 on *B. thetaiotaomicron* gene expression.** Hierarchical clustering of GeneChip data sets generated from *B. thetaiotaomicron* VPI-5482 harvested from the ceca of *Rag1*^{-/-} mice with and without 260.8 hybridoma backpacks (BP) and from wild-type C57BL/6J controls is shown. Genes whose expression was defined as significantly different in either direction (increased *versus* decreased; see "Experimental Procedures") between *Rag1*^{-/-} (mAb 260.8) backpack and *Rag1*^{-/-} (no backpack) and between *Rag1*^{-/-} and wild-type C57BL/6J mice are shown. Duplicated gene names (*i.e.* Bt-tRNA5 and BT2157) reflect the presence of these probe sets at multiple sites on the GeneChip where they served as internal controls. Genes involved in nitrite reduction are highlighted in red.

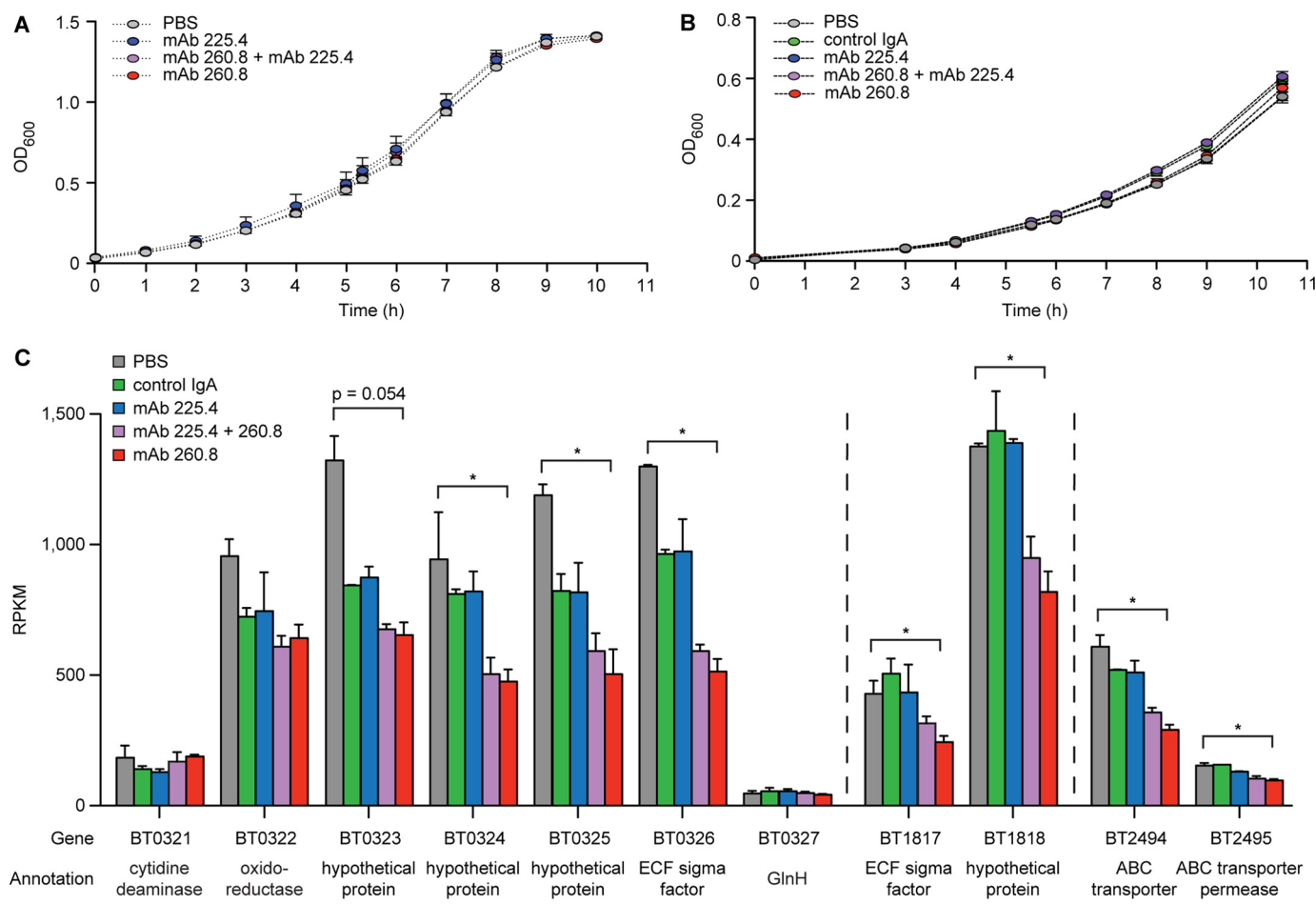


FIGURE 11. Assaying the effects of mAb 260.8 and mAb 225.4 on *in vitro* growth of *B. thetaiotaomicron* and its pattern of gene expression. *A*, *B. thetaiotaomicron* VPI-5482 was grown in TYG medium in the presence of mAb 225.4, mAb 260.8, an equal amount of both mAbs, or the antibody buffer (PBS) alone. *B*, cultures grown under the same conditions as those used in *A* that were used for microbial RNA-Seq (note that an additional isotype control has been included). Mean values \pm S.D. (error bars) are shown in *A* and *B*. *C*, microbial RNA-Seq results. Results are expressed as reads per kilobase per million (RPKM) for three loci that were differentially expressed in the presence of mAb 260.8 (mean values \pm S.E. (error bars) are plotted). *, $p < 0.05$ (Wald test). ABC, ATP-binding cassette; ECF, extracytoplasmic function.

in vitro. *In vitro* binding of the 260.8 or the 225.4 mAb produced no change in expression of the loci responsible for production of their epitopes. No genes were up-regulated by the addition of IgA. Modest down-regulation in expression of three loci (BT0323–BT0326, BT1817–BT1818, and BT2494–BT2495) was observed in cultures grown in the presence of mAb 260.8. BT0323–BT0326 and BT1817–BT1818 each specify an extracytoplasmic function-type σ factor, a group of transcription factors involved in transduction of signals from the extracellular environment (43). INSeq analysis revealed that genes encoding these two extracytoplasmic function σ factors (BT1817 and BT0326) and a hypothetical protein (BT0323) were not present above the limit of detection used to identify membership in our input INSeq library (indicating that they are presumably essential for growth under the *in vitro* conditions used to generate the library), whereas two hypothetical proteins (BT0324 and BT1818) were significant fitness determinants in hosts with or without deficient innate or adaptive immune systems.

DISCUSSION

The adaptive immune response to the gut microbiota consists of a complex repertoire of potential antibody specificities

interacting with a broad range of microbial species and strains. We have developed an approach that examines the specificity of individual antibodies from a naturally primed immune response to a prominent human gut mutualist. The 260.8 mAb, like our previously characterized 225.4 mAb, is specific to *B. thetaiotaomicron*; mAb 225.4, which recognizes a product of one of the organism's capsular polysaccharide synthesis loci, exhibits strain specificity. The 260.8 mAb, which recognizes the product of a conserved *B. thetaiotaomicron* locus apparently involved in LPS O-antigen polysaccharide synthesis, has species specificity that encompasses the vast majority of strains we tested from many different human hosts. As such, the process by which we produced and immortalized the naturally primed 260.8 antibody illustrates a method for generating diagnostic antibodies of value and perhaps antibodies that may have therapeutic utility.

Undoubtedly, this approach will yield antibodies along a continuum of specificity and degeneracy that define the aggregate repertoire. It remains to be seen precisely how this repertoire shapes the microbiota. We can conceptualize the immune response as a malleable tool that can be forged to “fit” any microbial community, responding to both common and unique

epitopes. We show that whereas the presence of the *CPS4*-specific 225.4 IgA leads to down-regulation of epitope expression *in vivo*, we did not see that adaptability in the case of the 260.8 epitope. It is interesting that the epitope that was more responsive in expression (225.4) is specified by a non-conserved locus in the *B. thetaiotaomicron* pan-genome, whereas expression of the conserved 260.8-associated locus was not changed significantly by the presence of the antibody *in vivo*. We speculate that it is beneficial for a bacterial species/strain to have immunogenic epitopes that impact its competitiveness. If it were impervious to IgA effector functions, then it could expand or penetrate the functional barrier created by IgA and in this sense behave as a pathogen. A successful evolutionary strategy may be balanced immunogenicity as well as susceptibility to the immune system to preserve a place for an organism in the gut community while preventing overgrowth of that species.

Both 260.8 and 225.4 IgAs could represent the innate/natural antibody/B1 B cell repertoire as they both recognize bacterial polysaccharides and were developed early in the immune response to colonization (*i.e.* within 2 weeks). However, the level of specificity that we have observed in both hybridomas does not fit that concept. Nonetheless, recent studies support the concept of bacterium-specific natural antibodies and memory formation independent of T cells (44, 45). The antibodies we have characterized thus far are not responsive to a universal bacterial epitope such as phosphocholine but rather to structures that are likely restricted to *B. thetaiotaomicron*. Because these epitopes are polysaccharides and repetitive, we postulate that they can be both low affinity and high specificity. As such, it is questionable how important T cell help is in the generation of this B cell response. As future experiments examine the immune response to the gut microbiota, there is a need to address further this general question of the specificity and degeneracy of antibody responses directed against individual strains within a (defined) microbial community. Additionally, further experiments will be needed to delineate how binding of intestinal IgA affects the fitness and behavior of microbes along the length of the gut and whether IgA binding leads to changes in the localization of these organisms within the mucus layer or their spatial distribution between the mucosa and luminal contents.

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