IgA Response to Symbiotic Bacteria as a Mediator of Gut Homeostasis

Daniel A. Peterson,1,2 Nathan P. McNulty,2 Janaki L. Guruge,2 and Jeffrey I. Gordon2,*

1Department of Pathology and Immunology
2Center for Genome Sciences
Washington University School of Medicine, St. Louis, MO, 63108 USA
*Correspondence: jgordon@wustl.edu
DOI 10.1016/j.chom.2007.09.013

SUMMARY

Colonization of germ-free mice with a normal gut microbiota elicits bacteria-specific IgA antibody responses. The effects of these responses on microbial and host biology remain poorly defined. Therefore, we developed a gnotobiotic mouse model where the microbiota is reduced to one bacterial species, and the antibody repertoire to a single, monoclonal IgA against the bacterium’s capsular polysaccharide. Bacteroides thetaiotaomicron was introduced into germ-free wild-type, immunodeficient Rag1−/−, or Rag1−/− mice harboring IgA-producing hybridoma cells. Without IgA, B. thetaiotaomicron elicits a more robust innate immune response and reacts to this response by inducing genes that metabolize host oxidative products. IgA reduces intestinal proinflammatory signaling and bacterial epitope expression, thereby balancing suppression of the oxidative burst with the antibody’s negative impact on bacterial fitness. These results underscore the adaptive immune system’s critical role in establishing a sustainable host-microbial relationship. Immunoselection of bacterial epitope expression may contribute to the remarkable strain-level diversity in this ecosystem.

INTRODUCTION

Our adult bodies are home to trillions of microbes, producing a “supraorganism” whose microbial cell population exceeds the number of human cells by an estimated order of magnitude. Our gut contains the vast majority of our bacterial and archaeal partners (Ley et al., 2006a). Over 90% of the bacterial phylogenetic types (phyotypes) belong to just two divisions (superkingdoms)—the Bacteroidetes and the Firmicutes. Each of us appears to harbor a distinct collection of species- and strain-level phyotypes belonging to these two dominant bacterial divisions. The presence of relatively few bacterial and archaeal divisions in this environmental ecosystem indicates that strong selective pressures operate to shape the community (Ley et al., 2006a).

Deciphering how an individual’s immune system and microbiota coevolve should help provide answers to a number of intriguing questions. How is our microbiota selected? How does it manifest compositional diversity and functional stability? How does it adapt to changes in our lifestyles? How do perturbations in microbial ecology contribute to certain pathologic states such as infectious diarrhea, inflammatory bowel diseases, and metabolic abnormalities, for example (Frank et al., 2007; Ley et al., 2006b; Turnbaugh et al., 2006)? How can the representation of components of our microbiota be intentionally manipulated for therapeutic benefit?

Changes in gut microbial ecology have been documented in mice with immune deficiencies (Fagarasan et al., 2002; Suzuki et al., 2004). Colonization of germ-free mice with members of the normal gut microbiota elicits bacteria-specific IgA responses (Shroff et al., 1995). In the few reports where the specificities of these antibodies have been determined, their effects on microbial and host biology have not been described (Macpherson et al., 2000). However, when immunodeficient Rag1−/− or SCID mice, which lack a functional adaptive immune system, are colonized with single or multiple species of bacteria, they display a more robust innate immune response than their immunocompetent wild-type counterparts (Keilbaugh et al., 2005; Cash et al., 2006). This indicates that in normal mice the adaptive immune response plays a critical role in minimizing activation of the innate immune system by the gut microbiota. In models of T cell-mediated colitis produced either by adoptive transfer of CD45RBhi T cells into SCID recipients or by gene knockouts, immunoglobulin (Ig) impacts mucosal inflammation (Gerth et al., 2004; Kanai et al., 2006). Moreover, serum responses to microbial antigens are associated with inflammatory bowel disease in mice and humans (Landers et al., 2002), as are defects in the innate and adaptive immune system (Cobb et al., 2006; Izcue et al., 2006).
immune system is reduced to one naturally primed Ig directed against an identified capsular polysaccharide epitope expressed by the bacterium in vivo.

RESULTS

We chose \textit{B. thetaiotaomicron} as a model symbiont for our experiments. It possesses a large arsenal of glycoside hydrolases for breaking down dietary polysaccharides that our own human proteome is ill-equipped to process (Sonnenburg et al., 2005) and efficiently colonizes the intestines of adult germ-free C57Bl/6J mice. Twenty-four hours after gavage with 10^8 CFU of the sequenced \textit{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 (Figure S1). ELISA of serum levels of IgG subtypes, IgM and IgA 1 day, 2 days, 4 days, 7 days, 14 days, 28 days, and 6 months after gavage of germ-free recipients disclosed that the IgG2a subtype exhibited the greatest relative increase (75-fold), while IgG1 changed \%1.5-fold (Figure S2), consistent with a CD4 T-helper 1 (Th1) environment (Mazmanian et al., 2005). These observations led us to predict that \textit{B. thetaiotaomicron} reactive B cells would be present within the intestinal wall at the 14 day time point and could be captured by hybridoma fusion, thereby allowing us to immortalize a single naturally primed, bacterial epitope-specific IgA response (see Experimental Procedures for additional details).

\textit{B. thetaiotaomicron}-primed IgA-producing hybridomas were identified (see Experimental Procedures), including one that produced a mAb, named 225.4, specific for the sequenced VPI-5482 type strain. ELISA disclosed that this mAb does not react with bacteria closely related to \textit{B. thetaiotaomicron}, or with any of 1200 bacterial colonies recovered from the ceca of conventionally raised B6 mice and feces of healthy human donors (Figure 1A and data not shown). Using biotinylated 225.4 mAb, we tested fecal pellets for expression of the epitope and found that it was produced by the bacterium in vivo starting from day one of colonization (Figure 1B).

With a finished genome sequence available for \textit{B. thetaiotaomicron} VPI-5482 (Xu et al., 2003), we were able to identify genes required for generation of the 225.4 epitope. A library of 4600 transposon (Tn4351) mutants was generated (see Experimental Procedures for details) and screened by ELISA for colonies that had lost their 225.4 reactivity (Figure 1C). Sixty-five percent of the 225.4-negative mutants contained inserts in the capsular polysaccharide synthesis 4 (CPS4) locus; three of the
To confirm the CPS4 dependence of the 225.4 epitope, we disrupted all eight of the bacterium’s CPS loci using a suicide vector. Each of the resulting isogenic strains harbored a polar insertion in the first gene of each locus. When analyzed by ELISA, only the CPS4 disruption was associated with loss of epitope (Figure 1D). Hot water-

Table 1. Tn4351 Insertion Sites Associated with Markedly Diminished or Absent 225.4 Epitope Expression in B. thetaiotaomicron

<table>
<thead>
<tr>
<th>Insertion Site Gene</th>
<th>Gene Description</th>
<th>Insertion Site Gene</th>
<th>Gene Description</th>
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<tbody>
<tr>
<td>← 100 base pairs upstream of BT1358</td>
<td>BT4576</td>
<td>hypothetical protein</td>
<td>← BT1358 UpxZ homolog</td>
</tr>
<tr>
<td>BT1358 UpxZ homolog</td>
<td>BT4576</td>
<td>hypothetical protein</td>
<td>← BT1357 UpxY homolog</td>
</tr>
<tr>
<td>← BT1356 polysialic acid transport protein kpsD precursor</td>
<td>BT2643</td>
<td>conserved hypothetical protein</td>
<td>← BT1355 polysaccharide biosynthesis protein chain length determinant</td>
</tr>
<tr>
<td>BT1354 flippase</td>
<td>BT2644</td>
<td>DNA topoisomerase I</td>
<td>← BT1353 glycosyltransferase (GT 2)</td>
</tr>
<tr>
<td>BT1351 glucose-1-phosphate cytidylyltransferase</td>
<td>BT2952</td>
<td>SusC homolog</td>
<td>← BT1350 CDP-glucose 4,6-dehydratase</td>
</tr>
<tr>
<td>BT1349 dTDP-4-dehydrorhamnose 3,5-epimerase</td>
<td>BT2950</td>
<td>hypothetical protein</td>
<td>← BT1348 CDP-abequose synthase</td>
</tr>
<tr>
<td>BT1347 glycosyltransferase (GT 2)</td>
<td>BT2948</td>
<td>α-1,2-mannosidase</td>
<td>*← BT1346 capA domain protein</td>
</tr>
<tr>
<td>BT1345 glycosyltransferase</td>
<td>BT3775</td>
<td>mannosyltransferase</td>
<td>*← BT1344 glycosyltransferase (GT 4)</td>
</tr>
<tr>
<td>BT1343 capsule biosynthesis protein</td>
<td>BT1035</td>
<td>β hexaminidase</td>
<td>BT1342 UDP-glucuronic epimerase</td>
</tr>
<tr>
<td>BT1341 UDP-glucose 6-dehydrogenase</td>
<td>BT1033</td>
<td>hypothetical protein</td>
<td>BT1340 lipopolysaccharide biosynthesis glycosyltransferase (GT 2)</td>
</tr>
<tr>
<td>BT1339 undecaprenyl-phosphate acetylglucosaminyltransferase</td>
<td>BT0664</td>
<td>ABC transporter substrate-binding protein</td>
<td>← BT1338 DTDP-4-dehydrorhamnose 3,5-epimerase</td>
</tr>
<tr>
<td></td>
<td>BT0666</td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BT0668</td>
<td>glutathione synthetase</td>
<td></td>
</tr>
</tbody>
</table>

Arrows indicate the gene that contains the transposon insertion. Downstream genes in known or predicted operons are also listed (Westover et al., 2005). * Indicates two unique mutants were identified in this gene. Glycosyltransferases (GT) are classified based on their assignment to families in the Carbohydrate-Active Enzymes (CAZy) database (http://www.cazy.org/).

other six loci are known or predicted to be related to various aspects of carbohydrate metabolism (Table 1).

To confirm the CPS4 dependence of the 225.4 epitope, we disrupted all eight of the bacterium’s CPS loci using a suicide vector. Each of the resulting isogenic strains harbored a polar insertion in the first gene of each locus. When analyzed by ELISA, only the CPS4 disruption was associated with loss of epitope (Figure 1D). Hot water-

phenol extraction of wild-type bacteria recovered after overnight growth in rich TYG medium revealed that 225.4 reactivity was retained in the water phase of the extract (Figure 1E). In addition, the epitope was resistant to digestion with DNase, RNase, and proteases, but susceptible to acid hydrolysis (data not shown). Thus, a combination of biochemical and genetic data are consistent with the notion that 225.4 recognizes a surface capsular
carbohydrate epitope whose production is directed by the CPS4 locus and influenced by additional genes distributed throughout the genome. The exquisite strain specificity of the antibody is reminiscent of strain-specific responses to encapsulated pathogens such as S. pneumoniae (Coughlin et al., 1998). While it is unclear how well the 225.4 specificity is represented in the normal anti-B. thetaiotaomicron repertoire, our results indicate that it is clearly a member of a naturally primed immune response. The next question we addressed was the impact of this specific antisymbiont IgA in vivo. Therefore, germ-free Rag1\(^{-/-}\) mice, which lack mature T and B cells, were injected with 225.4-producing hybridoma cells under their dorsal skin, creating a hybridoma “backpack” mouse where the only Ig in the serum and gut lumen was 225.4 (Michetti et al., 1992). We waited 10 days before colonizing these backpack animals with wild-type B. thetaiotaomicron: this decision was based on an analysis of the time course of rise of serum 225.4 mAb levels in Rag1\(^{-/-}\) backpack controls (Figure S3A). Variations in levels of 225.4 IgA in the sera of different animals at this time point were linked to differences in the growth of their backpack hybridomas and correlated with levels of antibody excreted into their intestinal lumen (\(r^2 = 0.94\) based on ELISA of fecal pellets; \(n = 12\) mice; Figure S3B). This feature allowed us to conduct a dose-response study of the effects of the capsular epitope-specific IgA on the host-bacterial relationship. The first in vivo effect of the 225.4 antibody response that we measured was on expression of its own epitope. Ten days after colonization of germ-free Rag1\(^{-/-}\) mice with implanted backpacks, and Rag1\(^{-/-}\) controls with no backpacks (\(n = 4–16\) /group, 2 replicate experiments), animals were sacrificed, and hot water-phenol extracts of their cecal contents were prepared. ELISA revealed that (1) levels of the 225.4 antigen were significantly decreased in the ceca of Rag1\(^{-/-}\) backpack mice even though there were no significant differences in bacterial density between the two groups of animals (\(p < 0.01\); Figures 2A and 2B) and (2) there was an inverse relationship between antibody levels and luminal levels of its epitope: i.e., lowest levels of epitope were linked to differences in the growth of their backpack animals with wild-type B. thetaiotaomicron (the representation of the wild-type strain decreased a 1000-fold lower than wild-type; Figures 3A–3C). The ratio of wild-type to isogenic \(\Delta\)CPS4 mutant cells was shifted by an order of magnitude in favor of the mutant in mice with high levels of cecal 225.4 antibody, reflecting a reduction in the dominance of the wild-type bacterium (the representation of the wild-type strain decreased from 99.9% to 99% of the population; Figure 3C). In mice with <5 \(\mu\)g (0.4–2.3 \(\mu\)g) of 225.4 antibody per ml of cecal contents, B. thetaiotaomicron 225.4 epitope expression was significantly reduced as judged by the ratio of wild-type to \(\Delta\)CPS4 mutant CFUs in their ceca (Figure 3C). We next examined the in vivo effect of the 225.4 antibody on global bacterial gene expression. Whole-genome transcriptional profiling with custom B. thetaiotaomicron GeneChips containing probe sets that cover 98.6% of the bacterium’s 4779 protein-coding genes (Sonnenburg et al., 2005) was used to define the impact of this engineered antisymbiont immune response on bacterial physiology in vivo. We compared wild-type B. thetaiotaomicron...
higher expression of (1) an operon (BT1414-1418) that encodes nitrate reductase, (2) another gene (BT0687) involved in nitric oxide metabolism (Rodionov et al., 2005), and (3) an operon encoding subunits of cytochrome D ubiquinol oxidase (implicated in aerotolerance) (Figure 4A).

Inducible nitric oxide synthase (iNOS) is a prototypic member of the host’s oxidative response pathway, and nitric oxide is an evolutionarily conserved component of the innate immune response (Davidson et al., 2004). qRT-PCR assays of RNA prepared from the distal small intestines of mice belonging to the three different groups established that iNOS expression was, on average, 5-fold higher in Rag1/-/- mice without backpacks compared to Rag1/-/- mice with backpacks, and 23-fold higher compared to wild-type B6 animals (p < 0.01; Figure 4B). Moreover, in Rag1/-/- mice with and without backpack tumors there was a direct and significant correlation between levels of expression of BT1417 (nitrite reductase locus member) and iNOS (r² = 0.75; Figures 4C and 4D).

These results indicate that, in the absence of a secreted IgA, B. thetaiotaomicron elicits a more robust oxidative response in the host and adapts by inducing bacterial genes involved in the metabolism of products of the host response. They provide evidence that a quiescent relationship between B. thetaiotaomicron and its host is predicated on the IgA response and are consistent with a model where an iterative set of adaptations involving symbiont and host results in a coevolved homeostasis. In both Rag1/-/- and wild-type gnotobiotic mice, possessing CPS4 gives the bacteria a competitive advantage over isogenic strains that lack it. There appears to be an “optimal” level of anti-CPS response: at high levels of 225.4 IgA, CPS4-associated epitope expression is decreased, but not extinguished (Figures 2A–2C), and innate cell activation is diminished; however, if epitope levels decrease too much under immune pressure B. thetaiotaomicron may elicit a more robust innate immune response (as suggested by Figure 4E).

The final effect of the antisymbiont antibody response examined was its impact on intestinal gene expression. Transcriptional profiling of distal small intestinal RNA prepared from the same mice as those described above revealed that, in addition to iNOS, wild-type B. thetaiotaomicron-colonized Rag1/-/- animals without backpacks exhibited a marked upregulation of other genes involved in innate inflammatory responses when compared to colonized Rag1/-/- mice with backpacks or wild-type B6 animals (n = 3–5/group; see Figure S5 and Table S2 for gene lists). Ingenuity Pathway Analysis (IPA) software was subsequently used to organize these regulated genes, as well as genes that are expressed but not regulated, into known interaction networks. A large network composed of complement components, phospholipase A2 group 2A (PLA2G2A), regenerating islet-derived 1-β (Reg1-β), interleukin-1 receptor-antagonist (IL1-RN), and iNOS (with concomitant decreases in expression of arginase 2, a competitive iNOS inhibitor) is shown in Figure 5 and Tables S3 and S4. Downstream signaling pathways are also upregulated in Rag1/-/- mice without backpacks.
compared to controls, including signal transducer and activator of transcription-3 (STAT3) and STAT6, and interferon regulatory factor-8. In addition, other known markers of innate immune cell activation not in this network are affected, such as chemokine CX3CL1 (Fracktalkine), nuclear factor kB (NFkB), and Reg3-g. Since Rag1−/−/C0−/C0 mice lack B and T lymphocytes, these transcriptional profiles reflect activation of genes in intestinal epithelial cells (including members of the Paneth lineage), natural killer cells, and/or macrophages/dendritic cells (DC) that are present in these animals.

Two observations emphasize that the impact of the engineered 225.4 IgA response on the host-microbial relationship is the result of recognition of a bacterial surface epitope, rather than a unique property of the CPS4 epitope per se. Colonization of germ-free Rag1−/− mice without backpacks (no antibody response) and wild-type B6 mice with the ΔCPS4 strain produced the same effects on iNOS expression as the wild-type strain (Figure 4 and Figure S6). Moreover, in the wild-type B6 mice, where 225.4 specificity is only one component of the adaptive polyclonal IgA response to colonization, iNOS but not 225.4 expression was suppressed, and there was no significant impact the ratio of wild-type to ΔCPS4 mutant cells in the cecum (Figure S6).

We found that the antisymbiont IgA response not only suppresses CPS4 expression; it also induces expression of another capsular locus (CPS5; Figure S6), which, in turn, could initiate another round of host immune responses. Studies of another gut Bacteroides, B. fragilis (Krinos et al., 2001), have provided important insights about the complex genetic regulation of CPS locus expression through inversion of upstream, locus-associated promoters. We did not find any evidence of inversion in the proximal promoter of CPS4 but cannot rule out that control of CPS4 expression could be dependent on invertible elements positioned elsewhere in the B. thetaiotaomicron genome. Our findings provide an additional view of this regulation, one where anti-capsular antibodies modulate expression of loci producing their cognate epitopes. Gut symbionts exhibit an enormous capacity for surface variation, not only in response to immune pressure, but also as a result of other forces including nutrient availability (Sonnenburg et al., 2005; Bjursell et al., 2006). The genomes of prominent human gut Bacteroides species including B. thetaiotaomicron, B. fragilis, B. vulgatus, and B. distasonis have revealed that CPS loci are prominent sites of variation, in part due to lateral gene transfer (Xu et al., 2007). Moreover, comparisons of the B. thetaiotaomicron type strain VPI-5482 and two additional human...
DISCUSSION

Our study supports the notion that the role for antibody in the gut is to mediate tolerance. Tolerance in the gut may be viewed as a quiescent homeostasis predicated on immune recognition of members of its microbiota. Bacteria that elicit excessive levels of IgA may suffer a greater competitive disadvantage than those that elicit low to moderate amounts. This requires that long-term residents modulate their immunodominant determinants continually, likely providing one explanation for the extraordinary level of strain-level diversity observed in the gut ecosystem. Although relatively few human gut-associated Bacteroides genomes have been sequenced, those that have reveal a large representation of genes involved in the generation of surface carbohydrates (Xu et al., 2003, 2007; Kuwahara et al., 2004; Sonnenberg et al., 2005; Cerdeno-Tarraga et al., 2005; Krinos et al., 2001; Fletcher et al., 2007).

Our observations are consistent with the adaptive immune system being a driver of diversification of these surface structures, with the beneficial outcome being promotion of a noninflammatory relationship between gut symbiont and host.

The innate immune response to bacteria, including the generation of NO, is highly conserved in invertebrates and vertebrates (Davidson et al., 2004). A key evolved role of the adaptive immune system in vertebrates may be to accommodate more complex microbial communities, even at the added risk of susceptibility to colonization with pathogens, and/or autoimmunity (Hedrick, 2004; McFall-Ngai, 2007). Our results support this concept and provide evidence that a major role for the adaptive immune system is to maintain ‘‘connection’’ with the gut microbiota by selectively generating immune responses to bacteria that stimulate the innate system. This arrangement allows the host to detect new bacterial phylotypes, and to ignore the presence of those that it has previously encountered (memory). The result would be support of greater diversity without sacrificing the essential protective role of the innate immune system in maintaining the mucosal barrier.

There is an emerging model of mucosal immunity where differentiation of B cells to IgA-producing plasma cells is locally induced in the lamina propria of the small intestine by local factors (Fagarasan et al., 2001); e.g., it appears that T cell-independent class switching in the gut is induced by expression of costimulatory molecules such as CD40 ligand, APRIL, BlyS, and TGF-β by epithelial and DCs (Litinskiy et al., 2002; Macpherson and Uhr, 2004; He et al., 2007). The result of locally controlled IgA production is to create a simple feedback loop that bypasses the systemic immune system: i.e., where DCs, and epithelial cells drive IgA production independent of T cells until sufficient levels of IgA block microbial stimulation. This model could be tested in the future by creating a quasi-monoclonal (gnotobiotic) mouse with a T cell-deficient genetic background that expresses the 225.4 antibody as a B cell receptor knocked into the immunoglobulin gene locus (Cascalho et al., 1996): colonization of this mouse with B. thetaiotaomicron would reveal how efficiently IgA class switching occurs in a highly defined system, and the degree to which IgA production is antigen specific and T cell dependent.
One extrapolation from our observations is that inflammatory bowel disease (IBD) could be viewed, at least in part, as a failure to generate an effective adaptive immune response to the resident gut microbiota: such a response would normally prevent presentation of microbial antigens to T cells specific for members of the community. Consistent with this model, the antibody response of TCRα/β mice to gut microbes changes from polyclonal to oligoclonal as they develop colitis. Moreover, passive transfer of Ig into this model ameliorates disease (Mizoguchi et al., 1996, 1997).

Thus, instead of just searching for microbial targets that are enriched in the IBD-associated antibody repertoire, identifying where “holes” exist in the repertoire may be informative and could suggest new therapeutic strategies based on active or passive immunization. The identification of such holes should be facilitated by the upcoming human microbiome project, which will sequence (1) the genomes of a large number of cultured representatives of the phylogenetic types present in the gut microbiota, and (2) samples of gut microbial communities obtained from healthy individuals as well as those with diseases such as IBD. Moreover, these concepts likely apply to other human body habitats that harbor a microbiota and can be further explored using elaborations of the type of gnotobiotic mouse model described in this report.

EXPERIMENTAL PROCEDURES

Colonization of Germ-Free Mice

C57Bl/6J (B6) mice were obtained from the Jackson Laboratory. Germ-free B6 wild-type and Rag1−/− mice were housed in gnotobiotic isolators (Hooper et al., 2002) under a strict 12 hr light cycle and fed an autoclaved, polysaccharide-rich, standard rodent chow diet (B&K Universal, East Yorkshire, UK). All experiments were performed using protocols approved by the Washington University Animal Studies Committee.

Six- to eight-week-old males were colonized with a single gavage of 108 CFU of wild-type B. thetaiotaomicron strain VPI-5482, and/or an isogenic mutant strain with a disrupted CPS4 locus (see below). Bacteria were harvested from stationary phase cultures 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.

Hybridomas Obtained from Lamina Propria Fusions

Gnotobiotic mice were killed 2 weeks after gavage with wild-type B. thetaiotaomicron. The small intestine from a given mouse was immediately removed and flushed with Dulbecco’s modified Eagle’s medium (DMEM) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). The gut was then opened along its cephalocaudal axis and minced into small (≤2–5 mm) pieces. Following a 15 min incubation at 37 °C in 50 ml Hank’s buffered salt solution (HBSS) containing 5 mM EDTA, tissue fragments were placed in DMEM containing collagenase (100 units/ml; Sigma, St. Louis, MO) and dispase (0.5 units/ml; Fisher) and incubated for 2 hr at 37 °C, with vigorous shaking for 30 s every 30 min. 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 filtered through a Nytex filter (70 μm diameter pore; BD Biosciences, Bedford, MA). 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 medium, and the cells were fused by adding PEG1500 (50% w/v; Roche, Mannheim, Germany; Kohler and Milstein, 1975). The ratio of IgA-, IgM-, and IgG2b-producing hybridomas was 7:11:14 (n = 61 colonies scored representing three animals, note that control experiments using intestinal lamina propria lymphocytes recovered from age-matched, conventionally raised wild-type B6 animals produced hybridomas with a similar isotype distribution).

**B. thetaiotaomicron ELISAs**

All ELISAs were performed using standard protocols (Velazquez et al., 2001) in 96-well plates (Nunc-Maxisorb, Naique-Nunc, Rochester, NY). Bacterial strains suspended in PBS, lysates prepared from strains, capsular polysaccharides purified from strains, or extracts of cecal contents harvested from colonized gnotobiotic mice were assayed by ELISA (see following paragraphs for descriptions of how samples were prepared).

Samples to be assayed were diluted in sodium bicarbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, and 3 mM sodium azide [pH 8.5]). All steps were performed either at 4°C overnight, or room temperature for 2 hr. Following addition of supernatants from hybridomas (~1–5 μg IgA/ml), goat horseradish peroxidase (HRP)-conjugated anti-mouse IgA (Southern Biotech; 1:1000 dilution, 50 μL/well) was added. All ELISAs were developed using ABTS [2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] (1 mM; Roche) 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 OD 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 1:1000 in bicarbonate buffer, and a 50 μl aliquot was added to each well of an ELISA plate. Lysates contained capsular antigens, membrane and cell wall antigens, plus intracellular antigens and were therefore used for initial screening of hybridomas.

**Capsular Polysaccharides**

Capsular polysaccharides were isolated from *B. thetaiotaomicron* VPI-5482 cells, harvested at the stationary phase of growth in TYG medium, using the hot water-phenol method (Jann et al., 1965). Briefly, bacteria were extracted for 2 hr in phenol:water (1:1) at 65°C with constant stirring. Phases were separated by gravity, and the top phase was dialyzed for 3 days at 4°C against tap water. The dialyzed material was subsequently lyophilized and suspended in sterile PBS (1 mg/ml). LPS was removed by ultracentrifugation (65,000 x g for 2 hr at 4°C).

**Cecal Contents**

Extractions were carried at 65°C in 1.5 ml eppendorf tubes containing 0.5 ml of a 1:100 dilution of cecal contents in PBS, and 0.5 ml phenol. Samples were vortexed for 30 s, every 30 min over a 2 hr period. Following centrifugation at 20,000 x g for 5 min at 4°C, the upper phase was recovered, serial dilutions (in bicarbonate buffer) were made, and ELISA was performed. To estimate the amount of 225.4 epitope present in cecal contents, ELISA reactivity was standardized to a curve constructed from serial dilutions of *B. thetaiotaomicron* (grown in TYG to stationary phase) into bicarbonate buffer. This stationary culture was arbitrarily designated as having 1 x 108 units per ml (the equivalent of CFU under these conditions). Therefore, in TYG-grown *B. thetaiotaomicron*, one 225.4 epitope unit is the equivalent to one colony-forming unit.

**Isotype ELISA**

ELISA plates were coated overnight at 4°C with goat anti-mouse IgG (Southern Biotech; reacts with heavy and light chains), then washed and blocked with 1% BSA-PBS (30 min at room temperature). Serial dilutions of serum samples, obtained from *B. thetaiotaomicron*-colonized mice at the time of their sacrifice, were added to the 96-well plates (n = 3 biological replicates/per time point), followed by HRP-conjugated, isotype-specific, secondary antibodies (1:1000 dilution in PBS-BSA; Southern Biotech). Fold change at each time point following colonization was calculated as the isotype level of each mouse relative to the mean of the germ-free control group.

**Transposon Mutagenesis**

Mutagenesis was performed using the donor plasmid pEP4351 and *B. thetaiotaomicron* VPI-5482 (Salyers et al., 2000; Shoemaker et al., 1986). Mutants were isolated based on their growth on Brain Heart Infusion-sheep red blood cell agar plates containing erythromycin (10 μg/ml; to select for transposon-containing *B. thetaiotaomicron*) and 800 μg/ml gentamicin (to select against any persistent E. coli). Individual colonies were picked into 96-well plates and grown overnight in TYG medium in anaerobic jars. Plates were split: one replicate was frozen in 25% glycerol for later studies; the other plate was frozen for subsequent ELISA screening with the 225.4 mAb.

Sites of transposon insertion in 225.4 antibody-negative colonies were identified using an arbitrarily primed polymerase chain reaction (AP-PCR). The AP-PCR protocol consists of a nested PCR with the following primers: Round 1, S3794 (5′-ATCAGTATGCTTTGTGTGTG) and either AR7 [5′-GGGCAACGCCTGCTGACTACGTACGTCTGTAAT] or AR8 [5′-GGGCAACGCCTGCTGACTACGTCTGTCGATTAGTTGTTGTGGCTGTAAT] and AR2 (5′-GGGCAACGCCTGCTGACTACGTCTGTCGATTAGTTGTTGTGGCTGTAAT). The product of the second round of PCR was purified (Qiagen PCR Purification Kit; QIAEN) and then sequenced from the right arm of the transposon into the chromosomal DNA using primer IS4908S (5′-ATCAGTATGCTTTGTGTGTG) and either AR7 [5′-GGGCAACGCCTGCTGACTACGTACGTCTGTAAT] or AR8 [5′-GGGCAACGCCTGCTGACTACGTCTGTCGATTAGTTGTTGTGGCTGTAAT].

**pGGERM-Directed Mutagenesis of CPS Loci**

The first gene in every CPS locus was targeted using the pGERM suicide vector (Xu et al., 2003; Sonnenburg et al., 2005). Mutagenesis was performed using protocols described by Hooper et al. (1999). The site of insertion of pGERM was verified by PCR (see Table S5 for a list of primers), and by sequencing.

**Backpack Experiments**

Hybridomas were grown to 50%–75% confluency in DMEM and washed three times in pyrogen-free saline. Cells were introduced into the gnotobiotic isolators in a way that preserved sterility (see Crawford and Gordon, 2005 for details) and then injected subcutaneously into 6-to 8-week-old male B6 Rag1−/− recipients (2 x 106 cells/mouse; n = 16–20 mice/experiment; three independent experiments). Ten days after implantation, each mouse was colonized with a single gavage of 108 CFU of *B. thetaiotaomicron* (with or without the ΔCPS4 isogenic mutant). Mice were then sacrificed 10 days following colonization, and cecal contents were recovered. The small intestine was rapidly divided into 16 equal length segments, and segments 9–12 and 14 were snap frozen in liquid nitrogen for subsequent isolation of total cellular RNA (see below).

**The Impact of Anti-*B. thetaiotaomicron* on Bacterial RNA Expression 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; selected based on cecal IgA levels contents [range of cecal IgA, 9–153 μg/ml; geometric mean, 38 μg/ml]) and stored at –80°C until use. An aliquot (~200 mg) was thawed in 2–3 volumes of...
RNAProtect (QIAGEN) and centrifuged (3000 x g for 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 phenol:chloroform:isoamyl alcohol (125:24:1; pH 4.5; Ambion). Acid-washed silica beads (Sigma; 212–300 μm diameter; 250 mg) were introduced, and bacteria were lysed (Mini-Beadbeater; Biospec; “high” setting for 5 min at room temperature). Following centrifugation (13,000 x 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 cold isopropanol, and RNA was purified (RNEasy kit; QIAGEN; note that residual genomic DNA was subsequently removed by treatment with DNase-free; Ambion). The relative proportion of bacterial versus host RNA in each preparation was defined by qRT-PCR, using primers di-

Supplemental Data

The Supplemental Data include six supplemental figures and five supplemental tables and can be found with this article online at http:// www.cellhostandmicrobe.com/cgi/content/full/2/5/328/DC1.

ACKNOWLEDGMENTS

We thank David O’Donnell and Maria Karlsson for invaluable help with husbandry of gnotobiotic mice, Sabrina Wagoner for expert technical assistance, Jennifer Gill and Abigail Salyers for contributions to transposon mutagenesis of B. thetaiotaomicron, and Emil Unanue and members of the Gordon lab for many helpful discussions. This work was supported by grants from the NIH (DK30292, T32HD07409, T32CA09547).

Received: June 19, 2007
Revised: August 17, 2007
Accepted: September 19, 2007
Published: November 14, 2007

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Accession Numbers
GeneChip data sets are available in the Gene Expression Omnibus repository under the accession numbers GSE9018, GSE9019, and GSE6504.