Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome

Peter J. Turnbaugh,1 Fredrik Bäckhed,3 Lucinda Fulton,2 and Jeffrey I. Gordon1,*
1Center for Genome Sciences
2Genome Sequencing Center
Washington University, St. Louis, MO 63108, USA
3Sahlgrenska Center for Cardiovascular and Metabolic Research, Wallenberg Laboratory, Göteborg University, SE-413 45 Göteborg, Sweden
*Correspondence: jgordon@wustl.edu
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SUMMARY

We have investigated the interrelationship between diet, gut microbial ecology, and energy balance using a mouse model of obesity produced by consumption of a prototypic Western diet. Diet-induced obesity (DIO) produced a bloom in a single uncultured clade within the Mollicutes class of the Firmicutes, which was diminished by subsequent dietary manipulations that limit weight gain. Microbiota transplantation from mice with DIO to lean germ-free recipients promoted greater fat deposition than transplants from lean donors. Metagenomic and biochemical analysis of the gut microbiome together with sequencing and metabolic reconstructions of a related human gut-associated Mollicute (Eubacterium dolichum) revealed features that may provide a competitive advantage to members of the bloom in the Western diet nutrient milieu, including import and processing of simple sugars. Our study illustrates how combining comparative metagenomics with gnotobiotic mouse models and specific dietary manipulations can disclose the niches of previously uncharacterized members of the gut microbiota.

INTRODUCTION

Energy balance is an equilibrium between the amount of energy extracted from the diet and the amount expended. Studies in germ-free mice indicate that the structure and operations of the intestinal microbial community (the gut microbiota) should be factored into this equation. Colonization of adult germ-free animals with a distal gut microbiota harvested from the ceca of conventionally-raised donors fed a low-fat polysaccharide-rich diet produces a marked increase in body fat content within 10–14 days (Bäckhed et al., 2004; Turnbaugh et al., 2006). This increase in adiposity reflects the effect of the gut microbiota on both sides of the energy balance equation. The microbiota ferments complex dietary plant polysaccharides that cannot be digested by the host, which lacks the requisite glycoside hydro-
lases in its proteome (Sonnenburg et al., 2005; http://www.cazy.org/). The resulting monosaccharide and short-chain fatty acid (SCFA) products are absorbed by the host and delivered to the liver where they are converted to more complex lipids. The microbiota concomitantly regulates expression of host genes that promote the deposition of this absorbed energy in adipocytes (Bäckhed et al., 2004, 2007; Dumas et al., 2006; Martin et al., 2007).

As in humans, >90% of bacterial phylogenetic types (phylo-
types) comprising the mouse distal gut microbiota are members of two bacterial divisions (phyla): the Bacteroidetes and the Firmicutes (Eckburg et al., 2005; Ley et al., 2005, 2006b; Frank et al., 2007). Studies of genetically obese C57BL/6J mice homozygous for a mutation in the leptin gene (ob/ob) and their lean ob/+ and +/+ littermates revealed that obesity in this model is associated with a division-wide increase in the relative abundance of the Bacteroidetes and a corresponding division-wide decrease in the relative abundance of the Firmicutes (Ley et al., 2005). Shotgun sequencing of DNA prepared from the cecal microbiota of ob/ob, ob/+ , and +/+ littermates indicated that the obesity-associated gut “microbiome” (genes present in the microbiota) had an increased capacity for fermenting polysaccharides relative to the lean-associated microbiome (Turnbaugh et al., 2006). These in silico predictions were supported by assays of intestinal contents. Furthermore, when adult wild-type germ-free mice fed a standard polysaccharide-rich chow diet were colonized with a microbiota harvested from ob/ob or lean (+/+ ) donors, adiposity in recipients of the obese microbiota increased to a significantly greater degree than in recipients of a lean microbiota, supporting the conclusion that the obese gut microbiota has an increased and transmissible capacity to promote fat deposition (Turnbaugh et al., 2006).

The linkage between adiposity and gut microbial ecology in mice appears to apply to humans. Culture-independent, 16S rRNA sequence-based methods have been used to survey the distal gut microbiota of 12 unrelated obese men and women before and after being randomly assigned to a fat-restricted (FAT-R) or a carbohydrate-restricted (CARB-R) low-calorie diet (Ley et al., 2006b). Obese subjects had a lower relative abundance of the Bacteroidetes and higher relative abundance of the Firmicutes than lean controls. Over time, the relative abundance of the Bacteroidetes progressively increased, proportional to the degree of
The Gut Microbiome and Diet-Induced Obesity

Diet-Induced Obesity Alters Gut Microbial Ecology

Ten germ-free male C57BL/6J mice were weaned onto a low-fat chow diet rich in structurally complex plant polysaccharides ("CHO" diet) and then gavaged at 12 weeks of age with a distal gut (cecal) microbiota harvested from a conventionally raised donor (see Table S1 available online for the percentage of calories derived from protein, carbohydrate, and fat). This process of "conventionalization" was designed to insure that all recipients inherited a similar microbiota. All recipients were subsequently maintained in gnotobiotic isolators. Four weeks later, five of the conventionalized mice were switched to a "Western" diet high in saturated and unsaturated fats (41% of total calories), and the types of carbohydrates commonly used as human food additives (sucrose [18% of chow weight], maltodextrin [12%], plus corn starch [16%]; Tables S1 and S2). The remaining five mice were continued on the CHO diet. All mice were sacrificed 8 weeks later (24 weeks after birth) (Figure 1A). Mice on the Western diet gained significantly more weight than mice maintained on the CHO diet (5.3 ± 0.8 g versus 1.5 ± 0.2 g; p < 0.05, Student's t test) and had significantly more epididymal fat (3.7 ± 0.5% versus 1.7 ± 0.1% of total body weight; p < 0.01, Student's t test).

Cecal microbial community structure was defined in each mouse in each of the two groups by sequencing full-length 16S rRNA gene amplicons produced by PCR of community DNA (see Supplemental Experimental Procedures; Table S3). Communities were then compared using the UniFrac metric (Lozupone et al., 2006). The premise of UniFrac is that two microbial communities with a shared evolutionary history will share a threshold cutoff, we identified 132 "strain"-level phylotypes represented within the Mollicute bloom; the bloom was dominated by uncharacterized members of the microbial community.

RESULTS

Diet-Induced Obesity Alters Gut Microbial Ecology

Figure 1. Experimental Design
(A) Diet-induced obesity (DIO) in germ-free mice colonized with a complex microbial community.
(B) Conventionally-raised (CONV-R) wild-type mice fed a Western or CHO diet.
(C) Specific dietary shifts after 2 months on the Western diet.
(D) Microbiota transplantation experiments from donor mice on multiple diets to lean germ-free CHO-fed recipients. Numbers in parentheses refer to the age of mice at each step in the protocol. Mouse diets are labeled Western, FAT-R, CARB-R, and CHO (see Tables S1 and S2).

weight loss. These changes occurred in both the CARB-R and FAT-R groups and were division-wide; i.e., they were not due to blooms or extinctions of specific phylogenetic types (phylootypes). Correspondingly, overall levels of diversity in the microbiota of each individual remained constant as these changes occurred in their gut microbial ecology (Ley et al., 2006b).

The leptin deficient, ob/ob mouse model of obesity established a correlation between host adiposity, microbial community structure, and the efficiency of energy extraction from a standard, low-fat rodent chow diet that was rich in plant polysaccharides, but it did not allow us to investigate the effects of manipulating diet or diminishing host adiposity on the gut microbiota and its microbiome. Furthermore, leptin deficiency is extremely rare in diet or diminishing host adiposity on the gut microbiota and its microbiome. Consequently, overall levels of diversity in the microbiota of each individual remained constant as these changes occurred in their gut microbial ecology (Ley et al., 2006b).

The gut microbial ecology also differed: the division-wide changes in Bacteroidetes and Firmicutes produced by weight loss were a shared feature, but the collections of microbial lineages represented in each division in each individual were distinctive. In addition, despite general compliance to their assigned diets, the type of food consumed varied between individuals assigned to a given treatment group (FAT-R or CARB-R) and within individuals assigned to time (Ley et al., 2006b). Therefore, in this report, we have turned to a mouse model of diet-induced obesity (DIO) produced by consumption of a prototypic high-fat/high-sugar Western diet, where all animals were genetically identical, with all animals “inherited” a similar microbiota, and where, once an obese state was achieved, specified diets could be imposed to reduce adiposity. Comparative metagenomic and functional analyses of the distal gut microbiota of these mice have allowed us to test the hypothesis that the relationship between diet and the representation of microbial lineages and genes in the microbiome is dynamic and adjustable and impacts the capacity of the microbiota to promote host adiposity. This study also illustrates how the marriage of gnotobiotic mouse models and metagenomics can reveal heretofore unappreciated roles (niches/professions) played by previously uncharacterized members of the microbial community.
Other Mollicutes phylogenetically related to this clade have been cultured from the human gut (e.g., *Eubacterium dolichum*, *E. cylindroides*, and *E. biforme*) and observed in 16S rRNA data sets generated from the fecal microbiota of obese humans (Ley et al., 2006b). However, there are no reported cultured representatives of the dominant phylotypes observed in the DIO mouse model (Figure 4).

To determine whether these diet-induced changes in gut microbial ecology also occur in mice exposed to microbes starting at birth, we conducted a follow-up study using a different experimental design. In this case, conventionally raised C57BL/6J mice were weaned onto a Western or a CHO diet and then maintained, in separate cages, on those diets for 8 to 9 weeks (n = 8 animals/group). All animals were sacrificed after 12 weeks of age (Figure 1B). Those on the Western diet gained significantly more weight (13.8 ± 0.9 g versus 10.9 ± 0.9 g; p < 0.05, Student’s t test) and had significantly greater adiposity (epididymal fat pad weight was 3.0 ± 0.2% of total body weight in the Western diet group versus 1.6 ± 0.1% in the CHO group; p < 0.001, Student’s t test). The cecal microbiota of these conventionally raised mice fed the Western diet was dominated by the same Mollicute lineage that had been identified in the earlier conventionalization experiment involving germ-free animals (Figure S1).

The immune system is one of the host factors that influences gut microbial ecology (Suzuki et al., 2004; Ley et al., 2006a; Lupp et al., 2007; Peterson et al., 2007). However, this bloom occurred in all mice fed the Western diet and did not require a functional innate or adaptive immune system; i.e., the Mollicute bloom was present at a significantly higher abundance in the cecal microbiota of conventionally raised Western diet-fed C57BL/6J wild-type mice with a cecal microbiota harvested from mice maintained on CHO or Western diet (n = 14 mice/treatment group). Mean values ± SEM are shown. Asterisks in panels (A)–(D) indicate that the differences are statistically significant (Student’s t test, p < 0.05) after using the Bonferroni correction to limit false positives.

To directly test whether the DIO-associated gut microbial community possesses functional attributes that can increase host adiposity to a greater degree than a CHO diet-associated gut microbial community, we transplanted the cecal microbiota harvested from obese, conventionally-raised wild-type donors who had been on the Western diet for ≥8 weeks since weaning...
biota exhibited a significantly greater percentage increase in body fat as defined by dual energy X-ray absorptiometry (DEXA) than mice who had been gavaged with a microbiota from CHO-fed donors (43.0 ± 7.1% versus 24.8 ± 4.9% percentage increase; p < 0.05, Student’s t test based on the combined data from all three experiments) (Figure 3D). Importantly, there were no statistically significant differences in chow consumption (14.5 ± 0.3 versus 14.7 ± 0.8 kcal/d) or initial weight (22.9 ± 0.3 versus 23.8 ± 0.7g) between recipients of the obese and lean cecal microbiotas.

To test the impact of defined shifts in diet on the body weight, adiposity, and distal gut microbial ecology of obese mice, we designed two custom chows that were modifications of the Western diet: one with reduced carbohydrates (CARB-R); the other with reduced fat (FAT-R) (see Tables S1 and S2 for information about the composition and caloric density of these diets).

Sixteen conventionally raised C57BL/6J mice, representing two families derived from two mothers who were sisters to ensure that they all inherited a similar microbial community (Ley et al., 2005), were weaned onto the Western diet and maintained on it for two months. A subset of mice from each family was subsequently continued on the Western diet for 4 weeks (n = 5; control group) while the remaining siblings were switched to the CARB-R (n = 6) or FAT-R diets (n = 5) for 4 weeks (Figure 1C).

Mice switched to the FAT-R or CARB-R diet consumed significantly fewer calories (12.5 ± 0.1 kcal/day [FAT-R] and 12.0 ± 0.2 kcal/d [CARB-R] versus 14.1 ± 0.2 kcal/day [Western]; p < 0.0001, ANOVA), gained significantly less weight (0.6 ± 0.3 g [FAT-R] and 0.0 ± 0.3 g [CARB-R] versus 2.0 ± 0.3 g [Western]; p < 0.01, ANOVA), and had significantly less fat (epididymal fat pad weight: 1.9 ± 0.3% of total body weight for FAT-R and 1.9 ± 0.2% for CARB-R versus 2.8 ± 0.2% [Western]; p < 0.05, ANOVA) than those maintained on the Western diet (Figure S2).

This provided us with the animal model we had sought: diet-induced obesity followed by weight stabilization and reductions in adiposity in genetically identical mice consuming defined diets who had inherited a similar microbiota from their mothers.

16S rRNA gene sequence-based surveys revealed that weight stabilization was accompanied by (1) a significant reduction in the relative abundance of the Mollicutes (31.9 ± 11.6% of all bacterial sequences for FAT-R and a significantly more pronounced decrease to 6.1 ± 3.6% for CARB-R versus 50.3 ± 6.1% for the Western diet; p < 0.05, ANOVA) and (2) a significant division-wide increase in the relative abundance of Bacteroidetes (2.8-fold on the FAT-R, and 2.2-fold on the CARB-R diets; p < 0.05, ANOVA) (Figure S3).

To test if these alterations in gut microbial ecology had an effect on the ability of the microbiota to promote host adiposity, we colonized germ-free, CHO-fed recipients with a cecal microbiota harvested from conventionally raised donors who had been on the Western diet since weaning (8 weeks) and then switched to a FAT-R or CARB-R diet (n = 1 donor and 4 to 5 germ-free recipients/treatment group/experiment; n = 3 independent experiments; Figure 1D). Unlike recipients of the DIO-associated microbiota, there was no statistically significant difference in the amount of fat gained between mice colonized with the FAT-R or CARB-R communities, compared to mice colonized with a cecal microbiota from lean CHO-fed donors (33.6 ± 8.7%, 37.4 ± 10.6%, and 24.8 ± 4.9% increases, respectively; p = 0.2, ANOVA).
Combined, these results indicate that both the FAT-R and CARB-R diets repress multiple effects of Western diet-induced obesity; i.e., they decrease adipose tissue mass, diminish the bloom in a single uncultured Mollicute lineage, increase the relative abundance of Bacteroidetes, and reduce the ability of the microbiota to promote fat deposition.

The Western Diet-Associated Gut Microbiome
To further investigate the linkage between diet-induced obesity and the Mollicute bloom, we performed capillary sequencing of seven cecal samples obtained from seven mice: (1) three samples were from animals fed the Western diet (one that had been conventionalized and two that were conventionally raised), (2) two were from conventionally raised mice that had been switched from the Western to FAT-R diet for 4 weeks, and (3) two were from conventionally raised mice that had been switched to the CARB-R diet for 4 weeks (one mouse/family/diet; as noted above, the conventionally raised mice were from two mothers who were sisters; Table S4). A total of 48 Mb of high-quality sequence data was generated (average of 7 Mb/cecal DNA sample; Table S5).

Taxonomic Assignments
All seven data sets were dominated by sequences homologous to known bacterial genomes (49.97 ± 2.52%), followed by sequences with no significant homology to any entries in the nonredundant (NR) database (34.82 ± 1.89%) or that could not be confidently assigned (10.28 ± 0.45%), followed by sequences homologous to eukarya (4.56 ± 1.02%), archaea (0.27 ± 0.05%), and viruses (0.10 ± 0.01%) (BLASTX assignments performed with MEGAN [Huson et al., 2007]; for further details, see Supplemental Experimental Procedures) (Figure S4A). The sequences homologous to eukarya could be assigned to two principal groups: metazoa (largely derived from host cells) and apicomplexa.

Consistent with the PCR-based 16S rRNA data, the largest group of sequences in all seven cecal microbiomes was homologous to the Firmicutes division of Bacteria. Analysis of 16S rRNA gene fragments culled from the metagenomic data sets confirmed the presence of the Mollicute bloom in the Western diet-associated cecal microbiome (Figure S4D). However, all of the data sets, including those from mice on the Western diet, had a low relative abundance of sequences homologous to previously sequenced Mollicute genomes (Figure S4C). These results support the conclusion that the genetic makeup of the DIO-associated Mollicute bloom is distinct from that of previously sequenced Mollicutes.

Analysis of 16S rRNA gene fragments and NR-based taxonomic assignments confirmed that both the FAT-R and the CARB-R diets resulted in an increased relative abundance of sequences homologous to the Bacteroidetes (Figures S4B and S4D). To focus on the microbiome’s bacterial and archaeal gene content, all sequences that could be confidently assigned to eukarya were removed before conducting the analyses described below.

Functional Predictions
Metagenomic sequencing reads were subsequently assigned to orthologous groups from the STRING-extended COG database (von Mering et al., 2007) and the Kyoto Encyclopedia for Genes and Genomes (KEGG; Kanehisa et al., 2004). KEGG pathway-based metabolic reconstructions of cecal microbiomes harbored from mice fed the Western, CARB-R, or FAT-R diets revealed a variety of differences associated with the various diets (Table 1). Notably, the Western diet microbiome is significantly enriched for KEGG pathways involved in the import and fermentation of simple sugars and host glycans, including “fructose and mannose metabolism” and “phosphotransferase system” (p < 0.05 based on bootstrap analysis of pathway relative abundance in the Western versus CARB-R microbiome; Rodriguez-Brito et al., 2006).

### Table 1. Metabolic Pathways Enriched or Depleted in the Western Diet Microbiome

<table>
<thead>
<tr>
<th>Enriched</th>
<th>Metabolic Pathway</th>
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<tr>
<td>phosphotransferase system (PTS)</td>
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<tr>
<td>fructose and mannose metabolism</td>
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<tr>
<td>glycolysis/gluconeogenesis</td>
<td></td>
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<tr>
<td>glutamate metabolism</td>
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</tr>
<tr>
<td>carbon fixation</td>
<td></td>
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<tr>
<td>unclassified (nonenzyme)</td>
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<tr>
<td>pyrimidine metabolism</td>
<td></td>
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<tr>
<td>protein export</td>
<td></td>
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<tr>
<td>phenylalanine, tyrosine, and tryptophan biosynthesis</td>
<td></td>
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<tr>
<td>oxidative phosphorylation</td>
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<tr>
<td>Depleted</td>
<td></td>
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<tr>
<td>ABC transporters</td>
<td></td>
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<tr>
<td>bacterial chemotaxis</td>
<td></td>
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<tr>
<td>bacterial motility proteins</td>
<td></td>
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<tr>
<td>flagellar assembly</td>
<td></td>
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<tr>
<td>protein kinases</td>
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<tr>
<td>two-component system</td>
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<tr>
<td>pentose and glucuronate interconversions</td>
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<tr>
<td>other amino acid metabolism</td>
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<tr>
<td>starch and sucrose metabolism</td>
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<td>ribosome</td>
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Metabolic pathways from KEGG (Kyoto Encyclopedia of Genes and Genomes) found at significantly higher relative abundance (enriched) or lower relative abundance (depleted) in the Western diet microbiome relative to the CARB-R microbiome.

*Assignments to this KEGG pathway include a number of genes that are also involved in glycolysis, pyruvate metabolism, fructose/mannose metabolism, and other pathways. Genes encoding ribulose bisphosphate carboxylase, which catalyzes the primary step in carbon fixation, were not found in the microbiome data sets.

Based on bootstrap analysis of pathway relative abundance in the Western versus CARB-R microbiome (p < 0.05; Rodriguez-Brito et al., 2006).
produced though glycolysis, can be used to generate ATP (via pyruvate kinase) or used to drive the import of additional sugars through transfer of a phosphoryl group to EI of the PTS (Figure 5). PTS genes are found in multiple divisions of bacteria, including Proteobacteria such as *E. coli*, as well as multiple sequenced Firmicutes (e.g., the Mollicutes *Mycoplasma genitalium*, *M. pneumoniae*, *M. pulmonis*, *M. penetrans*, *M. gallisepticum*, *M. mycoides*, *M. mobile*, *M. hypopneumoniae*, *M. synoviae*, and *M. capricolum*; KEGG version 40; Kanehisa et al., 2004). The PTS also plays a role in regulating microbial gene expression through catabolite repression, allowing the cell to preferentially import simple sugars over other carbohydrates (Deutscher et al., 2006).

Multiple components of the PTS are present in the Western diet microbiome (EI and HPr plus EIIC), which could allow the import of simple sugars (e.g., glucose and fructose that together comprise sucrose, an abundant component of the Western diet), as well as sugars associated with the host gut mucosa (N-acetyl-galactosamine) (Figure 5). The Western diet microbiome also contains genes that support metabolism of these phosphorylated sugars to various end-products of anaerobic fermentation (e.g., lactate and the short-chain fatty acids butyrate and acetate; Figure 5). In addition, the Western diet microbiome is enriched for genes encoding beta-fructosidase, a glycoside hydrolase capable of fermenting beta-fructosides such as sucrose, inulin, or levans (p < 0.05 based on a 2 test of Western versus CARB-R microbiome).

Additionally, unlike the more diverse Firmicutes–enriched ob/ob and CARB-R microbiomes, the Western diet-associated...
microbiome is depleted for genes assigned to KEGG pathways involved in motility, including (1) "bacterial chemotaxis," (2) "bacterial motility proteins," and (3) "flagellar assembly" (Table 1). This observation suggests that the Mollicute bloom is either nonmotile or utilizes a mechanism for gliding motility, such as that found recently in other Mollicutes, that is independent of the known pathways for bacterial chemotaxis and flagellar biosynthesis (Jaffe et al., 2004; Hasselbring and Krause, 2007).

Assembly and Analysis of Contigs
All seven microbiome data sets were assembled individually and as one pooled data set using the program ARACHNE (Batzoglou et al., 2002). As expected, the reduced diversity of the Western diet microbiome produced the largest contiguous “genome fragments” (Table S6). Manual inspection of genome fragments from the combined assembly (N50 contig length = 1738 bases; Figure S5) revealed multiple contigs containing genes that were enriched in the Western diet microbiome, including those involved in the degradation of beta-fructosides such as sucrose, inulin, and levan (fructan beta-fructosidase) and the import of simple sugars (PTS genes for fructose and glucose transport). A large contig was also found that contained multiple genes involved in the import of amino acids (ABC transporters) (Figure S5). Interestingly, the two genome fragments containing PTS genes were each flanked by another gene involved in carbohydrate metabolism: in one case, an alpha-amylase (starch degradation) and in the other fragment, fructose-bisphosphate aldolase (glycolysis). These genome fragments are likely derived from the expanded uncultured Mollicute clade: they are composed of reads from microbiomes with a high relative abundance of the bloom and share the highest degree of homology with Bacillus and Mollicute genomes (Table S7).

Validation of PTS Expression
We constructed a cDNA library from mRNA-enriched total community RNA that had been isolated from the cecum of an obese mouse fed the Western diet (see Supplemental Experimental Procedures for information about the mRNA enrichment procedure). Sequence analysis of the inserts in this library confirmed that a gene encoding EII of the fructose, mannose, and N-acetyl-galactosamine specific PTS transporter (COG3716) was expressed. The low representation of mRNA-derived sequences in our library precluded further (cost-effective) characterization of the DIO cecal microbiome’s transcriptome. However, sequencing of 16S rRNA-derived inserts in the library provided further support of the high abundance of the Mollicute bloom: 80.6% of expressed 16S rRNAs had a best-BLAST-hit to Mollicute gene sequences (BLASTN comparisons with the NCBI nucleotide database, e-value < 10\(^{-25}\)).

Validation of Enhanced Fermentation in the DIO Microbiota
To verify our in silico predictions concerning metabolic activities that are enriched in the Western-diet associated gut microbiome, we performed gas-chromatography–mass spectrometric and microanalytic assays of the concentrations of short-chain fatty acids and lactate in aliquots of the same cecal samples that had been used for 16S rRNA surveys and metagenomic sequencing of community DNA (Supplemental Experimental Procedures). As predicted from our metabolic reconstructions, the cecal contents of mice fed the Western diet (on average, 50% Mollicutes) had a significantly higher concentration of multiple end-products of bacterial fermentation, including lactate, acetate, and butyrate compared to the cecal contents of CARB-R mice (on average, 6% Mollicutes) (Figure S6).

Whole Genome Sequencing and Analysis of a Human Gut-Associated Mollicute
We have yet to successfully culture representatives of the Mollicute clade that blooms in the distal gut microbiota of mice fed a Western diet. Therefore, to obtain additional insights about genomic and metabolic features that may allow this lineage to bloom in the cecal habitat of mice fed a Western diet and to validate our comparative metagenomic predictions, we sequenced the genome of *Eubacterium dolichum* strain DSM3991, a related Mollicute (Figure 4) isolated from the human gut microbiota (Table S8). A deep draft assembly of its genome was produced, based on 49-fold coverage with reads from a 454 FLX pyrosequencer (106 Mb) and 9-fold coverage with reads from a traditional ABI 3730xl capillary sequencer (GenBank accession ABAW00000000; http://genome.wustl.edu/pub/organism/).

We first compared this deep draft assembly of the *E. dolichum* genome to eight other deep-draft assemblies of human gut-associated Firmicutes and to fourteen finished Mollicute genomes (Figure 6; Figure S7). The program MetaGene (Noguchi et al., 2006) was used to predict the protein products of these diverse Firmicute/Mollicute genomes and the proteins assigned to the STRING-extended COG database and the KEGG database using BLASTP homology searches (e-value < 10\(^{-5}\); von Mering et al., 2007; Kanehisa et al., 2004).

Principal component analysis (PCA) of KEGG pathway representation in all 23 genomes revealed a clear clustering of the previously sequenced Mollicute genomes and the recently sequenced commensal gut Firmicutes, including *E. dolichum* (Figure 6A). The total size of the *E. dolichum* assembly is over twice the average Mollicute genome (2.2 versus 0.91 Mb) and two-thirds the average size of the recently sequenced gut Firmicute genomes (3.2 Mb). Our analyses revealed that the genome size reduction and corresponding gene loss that has occurred during Mollicute evolution has produced small genomes that are largely restricted to encoding components of metabolic pathways essential for life (Figure S8). Accordingly, bacterial genome size significantly correlates with the clustering results (Figure 6B; R\(^2\) = 0.9, p < 0.05). As expected from its relatively restricted genome size, *E. dolichum* is enriched for many KEGG pathways involved in essential cellular functions such as “Cell division,” “Replication, Recombination, and Repair,” “Ribosome,” and others (Figure S7) but is missing a number of metabolic pathways similar to other “streamlined” genomes (e.g., the mycoplasma and oceanic x-proteobacteria; Jaffe et al., 2004; Giovannoni et al., 2005). Its genome lacks predicted proteins involved in bacterial chemotaxis and flagellar biosynthesis, the tricarboxylic acid cycle, the pentose phosphate cycle, and fatty acid biosynthesis (Figure 6C). It is also significantly depleted for ABC transporters relative to the other gut Firmicutes (Figure S7), and a variety of metabolic pathways for the de novo synthesis of vitamins and amino acids are incomplete or undetectable (Figure 6C).

*E. dolichum* has a number of genomic features that could promote fitness in the cecal nutrient metabolic milieu created by the host’s consumption of the Western diet. As in the metagenomic
data set generated from the Western diet-associated cecal microbiome, its genome is enriched for predicted PTS proteins involved in the import of simple sugars including glucose, fructose, and N-acetyl-galactosamine (Figure 5; Figure S7). STRING-based protein networks constructed from the _E. dolichum_ genome revealed that many of these PTS orthologous groups are found in the Western diet microbiome, but not in all nine recently sequenced gut Firmicutes (Figure S8). In addition, the _E. dolichum_ genome encodes a beta-fructosidase capable of degrading fructose-containing carbohydrates such as sucrose, genes for the metabolism of PTS-imported sugars to lactate, butyrate, and acetate, plus a complete 2-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis—all genetic features of the Western-diet-associated cecal microbiome (Figure 5; Figure S8).

**DISCUSSION**

The findings presented in this study emphasize the importance of viewing our metabolome in a “supra-organismal” context, where both microbial and host contributions must be considered in order to achieve a fuller understanding of the factors that regulate energy balance. This study also demonstrates the power of combining gnotobiotic mouse models with comparative metagenomics to define relationships between diet, gut microbiota, and the functional attributes associated with a gut microbial community. In a recent study of germ-free and conventionalized mice fed low-fat high-polysaccharide versus Western diets, we examined the effects of the gut microbiota on host metabolism without examining the effects of host adiposity on the structure or metabolic activities of the microbial community (Bäckhed et al., 2007). This study revealed that germ-free C57BL/6J mice were resistant to obesity caused by consumption of the same high-fat/high-sugar Western diet used in the current report. This resistance was associated with increased host metabolism of fatty acids: (1) without a gut microbiota, levels of the active, phosphorylated form of AMP-activated kinase (AMPK) were increased in skeletal muscle and liver as were the activities of AMPK’s downstream targets, acetylCoA carboxylase and carnitine palmityltransferase (Cpt1); and (2) the absence of a microbiota also increased intestinal epithelial expression of angiopoietin-like 4 (also known as fasting induced adipocyte protein), a secreted, circulating inhibitor of lipoprotein lipase in mice and humans that induces peroxisomal proliferator activated receptor coactivator (Pgc-1α) and hence increases mitochondrial fatty acid oxidation (Bäckhed et al., 2007).

We can now add another component to the complex and dynamic interplay between diet, the microbiota, and the energy balance equation: namely, that with administration of a Western diet, there is a restructuring of the distal gut microbial community so that a Mollicute lineage in the Firmicutes, normally present at low abundance in the mouse colon, expands dramatically to resist a Mollicute lineage has an increased fitness not only relative to other Firmicutes but also relative to all other Bacteroidetes identified in the community. By comparing the Western, FAT-R, and CARB-R distal gut microbiomes with a deep draft assembly of a human gut-associated Mollicute closely related to the phytype
that dominates the DIO-associated microbiome, we find that members of this bloom have evolved the capacity to import the type of carbohydrates found in the model Western diet administered to mice as well as in foods commonly consumed by humans living in highly Westernized societies (e.g., glucose, fructose, and sucrose) and to metabolize these imported sugars to short-chain fatty acids that are readily absorbed by the host. While these data are consistent with the hypothesis that the bloom is highly efficient at competing for oligo- and mono-saccharide components of the Western diet, other alternative hypotheses for its success remain to be explored, including changes in the gut environment (e.g., bile acids, motility, pH) and physiology due to alterations in host adiposity or components of the diet.

Gut microbiota transplantation experiments from donors consuming the various diets to lean germ-free recipients, indicate that the significant association between the proportional representation of the Mollicute bloom in the microbiota and host adiposity may be causal rather than usual. The increased adiposity produced by transplantation of a Western versus CHO diet-associate microbiome occurs in germ-free recipients consuming a low fat, polysaccharide-rich CHO diet that has more modest levels of the sugars that are abundantly represented in the Western diet. This raises the possibility that the Mollicute-enriched community not only facilitates transfer of calories from the diet to the host, but also has effects on host metabolism of absorbed calories.

Mollicute members of this bloom have evolved the capacity to import and utilizexylose and fructose into glycogen and to secrete xylose and fructose into the gut environment (e.g., bile acids, motility, pH) and physiology due to alterations in host adiposity or components of the diet.

A recent study explored the relationship between the representation of selected members of the Firmicutes and consumption of high and low carbohydrate diets in 19 obese volunteers over a 4 week period (Duncan et al., 2007). They found that the abundance of a specific group within the Firmicutes that includes Roseburia spp. and Eubacterium rectale decreased with decreased carbohydrate intake, as did fecal butyrate concentrations. These studies of lean and obese humans consuming high-fat/high-sugar Western-type diets will be necessary to determine whether or not the abundance of various Mollicute lineages is significantly linked to diet and/or adiposity.

Our report describes an initial effort to establish a pipeline that combines gnotobiotic mice, specific dietary manipulations, and comparative metagenomics in order to identify and characterize organisms or groups of organisms that play important roles in nutrient and energy harvest. Some of these organisms may become therapeutic targets for manipulation. This effort is predicated on the concept that the energy and nutrient content of food is a relative term, based in part on the gut microbial ecology of the consumer and is motivated by the fact that obesity (and malnutrition) represents extremely serious global problems.

**Experimental Procedures**

**Animals**
All experiments involving mice were performed using protocols approved by the Washington University Animal Studies Committee.

**Conventionalization**
Germ-free male 8- to 9-week-old C57BL/6J mice were maintained in plastic gnotobiotic isolators under a strict 12 hr light cycle and fed an autoclaved low-fat, polysaccharide-rich chow diet (CHO) ad libitum (Hooper et al., 2002; Backhed et al., 2004). Conventionalization was performed by harvesting cecal contents from conventionally raised animals and introducing them, by gavage, into germ-free recipients, as described in Backhed et al. (2007).

**Conventionally Raised Mice**
Once C57BL/6J littermates were weaned, they were housed individually in microisolator cages where they were maintained in a specified pathogen-free state under a 12 hr light cycle and fed a CHO diet (PicoLab, Purina), a high-fat/high-sugar Western diet (Harlan-Teklad TD96132), a fat-restricted (FAT-R) diet (Harlan-Teklad TD05633), or a carbohydrate-restricted (CARB-R) diet (Harlan-Teklad TD05634) ad libitum.

**Microbiota Transplantation Experiments**
Adult germ-free C57BL/6J mice ≥8 weeks old were colonized with a cecal microbiota obtained from wild-type (+/+)-C57BL/6J donor mice fed CHO, Western, FAT-R, or CARB-R diets. Recipient mice, maintained on a CHO diet, were anesthetized at 0.5 and 14 days postcolonization with an intraperitoneal injection of ketamine (10 mg/kg body weight) and xylazine (10 mg/kg), and total body fat content was measured by dual-energy X-ray absorptiometry (DEXA; Lunar PIXimus Mouse, GE Medical Systems; Bernal-Mizrachi et al., 2002). Recipient mice were housed individually in microisolator cages within gnotobiotic isolators throughout the experiment to avoid exposure to the microbiota of other mice and to allow the direct monitoring of the chow consumed by each mouse. Animals were sacrificed immediately after the final DEXA on day 14.

**Shotgun Sequencing and Assembly of Cecal Microbiomes**
DNA samples were used to construct pOTw13-based libraries (GC10 cells, GeneChoice) for capillary-based sequencing with an ABI 3730xl instrument. Unidirectional (forward) sequencing reads were generated from each library (an average of 10,600 reads/library). Reverse reads were also generated to improve assembly (768-1536 per library; total of 7,680 reads). Sequences were trimmed based on quality score, and vector sequences were removed prior to analysis (Applied Biosystems; KB Basecaller). Each data set was assembled individually, in addition to a combined assembly of all seven data sets, using ARACHNE (Batzoglou et al., 2002; parameters: maxcliq1 = 500; genome size = 1 Gb). ARACHNE was chosen because it has been shown to generate reliable contigs from complex simulated metagenomic data sets (Mavromatis et al., 2007). Genes were predicted from individual sequencing reads and contigs using MetaGene (Noguchi et al., 2006).

**Microbiome Functional Analysis**
NCBI BLAST was used to query the STRING-extended COG database (version 7; von Mering et al., 2007) and the KEGG database (version 40; Kanehisa et al., 2004). COG and KEGG comparisons were performed by using NCBI BLASTX employing default parameters. A cutoff of e-value <10−5 was used for environmental gene tag (EGT) assignments and sequence comparisons. Predicted proteins were searched for conserved domains and assigned functional identifiers with InterProScan (version 4.3; Mulder et al., 2005). Predicted glycosyl hydrolases were confirmed based on criteria used for the Carbohydrate Active Enzymes (CAZY) database (http://www.cazy.org; Bernard Henrissat, personal communication).

**Other Experimental Procedures**
See Supplemental Data for descriptions of protocols used for (1) preparing DNA from the cecal microbiota, (2) performing 16S rRNA sequence-based surveys of the cecal microbiota, (3) making taxonomic assignments of shotgun sequencing reads of the cecal microbiome, (4) transcriptional profiling of the cecal microbiome, (5) sequencing and assembling the E. dolichum genome, (6) assaying metabolic end-products of fermentation, and (7) statistical analyses.
This Whole Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank under Genome Project ID 28755. All reads have been deposited in the NCBI Trace Archive. PCR-derived 16S rRNA gene sequences can be found in GenBank under accession numbers EU503541–EU512027.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, eight supplemental figures, and eight supplemental tables and can be found with this article online at http://www.cellhostmicrobe.com/cgi/content/full/3/4/213/DC1/.

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