

# Obesity alters gut microbial ecology

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**We have analyzed 5,088 bacterial 16S rRNA gene sequences from the distal intestinal (cecal) microbiota of genetically obese *ob/ob* mice, lean *ob/+* and wild-type siblings, and their *ob/+* mothers, all fed the same polysaccharide-rich diet. Although the majority of mouse gut species are unique, the mouse and human microbiota(s) are similar at the division (superkingdom) level, with Firmicutes and Bacteroidetes dominating. Microbial-community composition is inherited from mothers. However, compared with lean mice and regardless of kinship, *ob/ob* animals have a 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes. These changes, which are division-wide, indicate that, in this model, obesity affects the diversity of the gut microbiota and suggest that intentional manipulation of community structure may be useful for regulating energy balance in obese individuals.**

energy balance/obesity | host-microbial interactions | intestinal bacterial diversity | *ob/ob* mice | phylogenetics

The 10 trillion to 100 trillion microorganisms that populate our adult intestines benefit us in a number of ways (1). One benefit is that they allow us to extract calories from otherwise indigestible common polysaccharides in our diet. This benefit occurs because components of the microbiota are able to adaptively deploy a large array of glycoside hydrolases and polysaccharide lysases that we humans do not encode in our genome (2, 3) (<http://afmb.cnrs-mrs.fr/CAZY/>). Furthermore, studies using germ-free and colonized normal and knockout mice fed a standard, polysaccharide-rich rodent-chow diet indicate that this mutualistic host-microbe relationship allows the extracted energy to be stored in adipocytes through a pathway that involves microbial regulation of the intestinal epithelial expression of fasting-induced adipocyte protein (Fiaf), a circulating inhibitor of lipoprotein lipase (LPL) (4). Microbial fermentation of dietary polysaccharides to monosaccharides and short-chain fatty acids in the distal gut and their subsequent absorption stimulate *de novo* synthesis of triglycerides in the liver. Microbial suppression of Fiaf in the gut epithelium results in reduced levels of this circulating LPL inhibitor, increased LPL activity in adipocytes, and enhanced storage of liver-derived triacylglycerols in fat cells (4).

Although the root cause of obesity is excess caloric intake compared with expenditure, differences in gut microbial ecology between humans may be an important factor affecting energy homeostasis; i.e., individuals predisposed to obesity may have gut microbial communities that promote more efficient extraction and/or storage of energy from a given diet, compared with these communities in lean individuals. This hypothesis raises a number of basic questions about gut microbial ecology in humans and mice. For example, how do the distal-gut microbiotas of the two hosts compare? Does kinship play an important role in the composition of the microbial community? Does adiposity affect community structure, and, if so, at what taxonomic level do these effects occur, and do they reflect a heretofore unappreciated form of homeostatic feedback between the microbiota and host energy balance?

Although information is limited, a current conceptualization of bacterial diversity in the human gut is that there is a restricted suite of highly adapted bacteria, likely inherited from the immediate family and, possibly, filtered by host genotype (5).

Studies are needed to characterize the rules controlling microbial diversity in the human gut. Remarkably, a comprehensive enumeration of the gut microbiota has not yet been reported for *Mus musculus*, even though this mammalian species provides a very attractive model for systematically exploring the roles of host genotype, maternal exposure, diet, and energy balance on intestinal microbial ecology. Therefore, in this report, we use C57BL/6 mice, homozygous for a mutation in the leptin gene (*ob/ob*) that produces a stereotyped, fully penetrant obesity phenotype (6, 7), and their lean *ob/+* and *+/+* siblings, to show that microbial-community composition in the distal intestine changes at a division-wide level in response to increasing adiposity. This finding provides another perspective about the link between the gut microbiota and host energy balance.

## Materials and Methods

**Animals.** C57BL/6J *ob/+* mothers and their *ob/ob*, *ob/+*, and *+/+* offspring were raised under a 12-h light cycle, in a specified pathogen-free state. Weaning and adult mice were fed PicoLab chow diet (Purina) ad libitum. All experiments involving mice were performed under protocols approved by the Washington University Animal Studies Committee. All animals were killed at the same time of day.

**PCR Amplification of 16S rRNA Genes.** Ceca were recovered immediately after the mice were killed. The contents of each intact cecum was recovered by manual extrusion and was frozen immediately ( $-80^{\circ}\text{C}$ ) until use. A frozen aliquot ( $\approx 100$  mg) of each sample was added to tubes containing 500  $\mu\text{l}$  of extraction buffer (200 mM Tris, pH 8.0/200 mM NaCl/20 mM EDTA), 210  $\mu\text{l}$  of 20% SDS, 500  $\mu\text{l}$  of phenol:chloroform:isoamyl alcohol (24:24:1), and 500  $\mu\text{l}$  of 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK). Microbial cells were disrupted mechanically at  $23^{\circ}\text{C}$  with a bead beater (BioSpec Products; instrument set on high for 2 min). Isolated DNA was fractionated by electrophoresis through 1% agarose gels, and the dominant band was purified by using the MiniElute Gel Extraction kit (Qiagen).

For each mouse, five replicate 25- $\mu\text{l}$  PCRs were performed, each containing 100–200 ng of purified genomic DNA, 100 mM Tris (pH 8.3), 500 mM KCl, 20 mM  $\text{MgSO}_4$ , 200  $\mu\text{M}$  dNTPs, 200  $\mu\text{M}$  concentration of the bacteria-specific primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), 200  $\mu\text{M}$  concentration of the universal primer 1391R (5'-GACGGGCGGTG-WGTRCA-3') (8), 1 M betaine, 800  $\mu\text{g}/\text{ml}$  BSA, and 1 unit of Taq polymerase (Invitrogen). Cycling conditions were  $94^{\circ}\text{C}$  for 2 min, followed by 20 cycles of  $94^{\circ}\text{C}$  for 1 min,  $55^{\circ}\text{C}$  for 45 sec, and  $72^{\circ}\text{C}$  for 2 min, with a final extension period of 20 min at  $72^{\circ}\text{C}$ . Replicate PCRs were pooled. The resulting 1.3-kb amplicons were gel-purified by using the Qiagen extraction kit and subcloned into TOPO TA pCR4.0, followed by transformation

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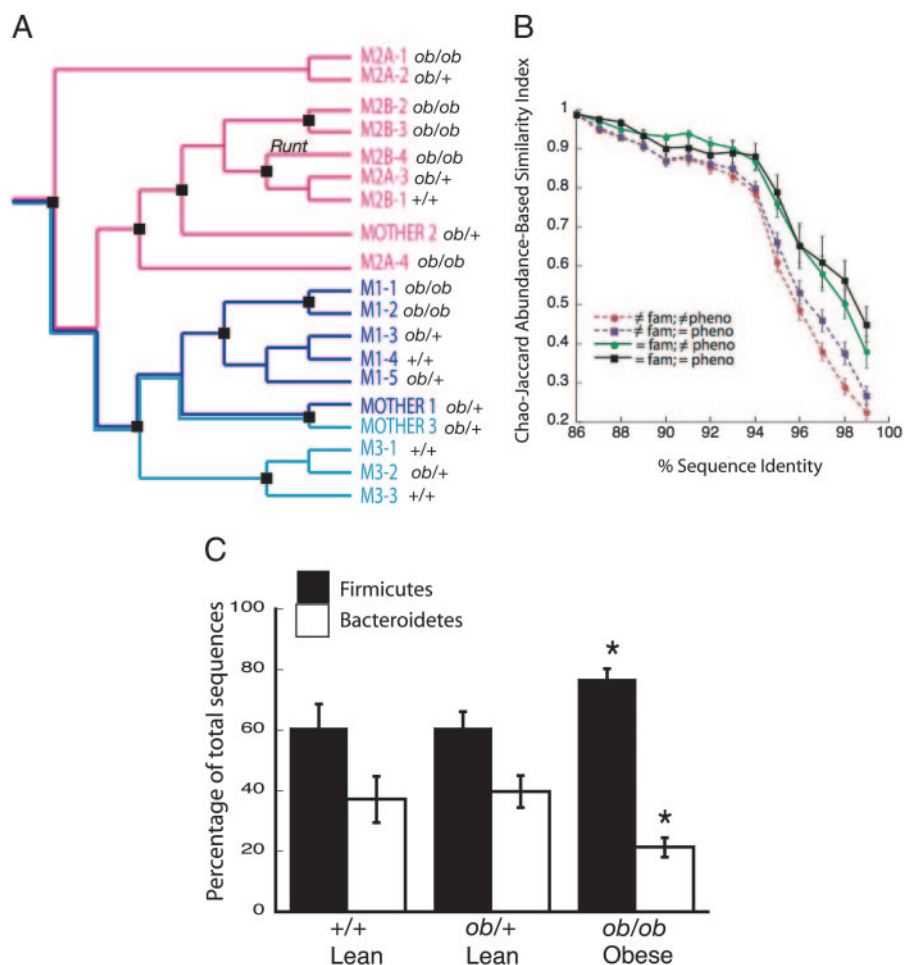
Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ014552–DQ015671 (mothers) and AY989911–AY993908 (offspring)].

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**Fig. 2.** Effects of kinship and obesity on gut microbial ecology. (A) Unweighted-pair-group method with arithmetic-mean (UPGMA) tree, based on pairwise differences between the cecal microbial communities of each mouse (UniFrac metric, based on 5,088 sequences). Dark blue, mother 1 and her offspring (M1-); pink, mother 2 and offspring; light blue, M3 and offspring. mothers 1 and 3 are siblings from different litters. Mother 2 had two sequential litters (M2A- and M2B-). All nodes are robust with respect to the specific mice included (jackknife values  $>0.95$  for all nodes, representing the percentage of time the node was present when a randomly chosen mouse was removed from the distance matrix for  $n = 1,000$  replicates). Nodes denoted by a square are robust to sequence number (jackknife values  $>0.67$ , representing the number of times the node was present when 200 sequences were randomly chosen from each mouse for  $n = 100$  replicates, excluding mice M2A-1 and M2A-2, which had  $<200$  sequences). (B) Community similarity between the microbiota of mouse pairs belonging to different families ( $\neq$  fam), the same family ( $=$  fam), different phenotypes [obese or lean, ( $\neq$  pheno)], or the same phenotype ( $=$  pheno). Community similarity was calculated for taxa (defined by percentage of conserved sequence identities, ranging from 86% to 99%, excluding hypervariable regions of the gene in the alignment) by using the Chao-Jaccard abundance-based similarity index (16). This index estimates the similarity of communities, based on the probability (taking into account community coverage) of a taxon belonging to one community or another or both. Identical communities have an index of 1. Values shown are means for each category. Error bars are standard errors among the similarity indices between all pairs of mice in each category. (C) Proportion of Bacteroidetes and Firmicutes in the cecal microbiota of lean vs. obese mice. Mean values  $\pm$  SEM are plotted. \*,  $P < 0.05$  within the division.

three healthy adults (11), combined with a review of 16S rRNAs in GenBank (1), had revealed nine divisions in the human colonic mucosa and feces (Fig. 1B). As in humans, the two most abundant bacterial divisions in mice were the Firmicutes (60–80% of sequences) and the Bacteroidetes (20–40%; also known as the Cytophaga-Flavobacterium-Bacteroides or CFB) (Fig. 1B). Greater than 75% of the Firmicutes are in *Clostridium* cluster XIVa, a common clade in humans that includes butyrate producers (21) and *Eubacterium eligens*. The majority ( $>88\%$ ) of the Bacteroidetes belong to Bacteroidetes 4b, which lacks a cultured representative (11). Proteobacteria and Actinobacteria, present at low levels in the human colonic microbiota, and TM7, previously identified in the human mouth (gingival) microbiota, each comprised  $\leq 1\%$  of the mouse cecal bacterial communities.

**A Deep-Branching Clade of the Cyanobacteria in the Guts of Mice and Other Animals.** In addition to the divisions described above, we found members of a group of bacteria that had been detected

previously in human and other animal guts (Fig. 1C) but not characterized phylogenetically. Our analysis revealed a coherent gut-associated clade rooted deep in the Cyanobacteria, a division whose members perform oxygenic photosynthesis. This group may represent descendants of nonphotosynthetic ancestral cyanobacteria that have adapted to life in animal gastrointestinal tracts. Several sequences from other lightless environments were associated with the gut clade. Sequencing the genomes of these deep-rooting Cyanobacteria could shed light on the great oxygenation event that occurred  $\approx 2.2$  billion years ago, when Cyanobacteria profoundly changed the chemistry of the Earth's atmosphere.

**Kinship Has a Strong Effect on Microbial Diversity.** We next compared bacterial-community composition between mice by using recently developed computational methods that determine the fraction of branch length within a phylogenetic tree that is

unique to each animal (UNIFRAC metric; see *Materials and Methods*). We used two permutations of the UNIFRAC metric: The first considers only taxa that are present, regardless of their abundance; the second weights branch lengths based on the relative abundance of sequences from each mouse (see *Materials and Methods, Supporting Results*, and Figs. 3 and 4, which are published as supporting information on the PNAS web site and which demonstrate the application of UNIFRAC to the 11,831-member human colonic data set).

The UNIFRAC methods were used to test the effects of kinship and genotype on diversity, in concert with standard multivariate and parametric statistics (principal-coordinates analysis and the unweighted-pair-group method with arithmetic mean and ANOVA applied to principal coordinates). We corroborated the results with an analysis of community similarity that takes into account sampling coverage and “unseen” taxa. This approach, the Chao–Jaccard abundance-based similarity index (16), was applied to taxa delimited by 16S rRNA sequence identity thresholds ranging from 86% to 99%.

The results revealed that mothers and their offspring shared cecal microbiotas with similar community membership, regardless of their *ob* genotype. This characteristic held for both generations; i.e., mothers that were siblings (M1 and M3) shared microbiotas of similar composition, and the microbiotas of their offspring (cousins) were similar. Moreover, the composition of the microbiotas of two litters from the same mother (M2) was not significantly different, whereas the composition of the M2 family was significantly different from the M1/M3 family (the effect of kinship is significant to  $P < 0.001$ ) (Fig. 2*A* in the text; and see Fig. 5*A*, which is published as supporting information on the PNAS web site). The differences in composition between the M1/M3 and M2 families occurred at the level of genera (Fig. 2*B*).

**Obesity Correlates with a Shift in the Abundance of Bacteroidetes and Firmicutes.** Regardless of family membership, obesity was associated with a large shift in the relative abundance of the specific taxa present ( $P < 0.01$ ; see Fig. 5*B*). The change in community structure occurred at the division level. The cecal microbiota of obese mice had a statistically significant 50% reduction in Bacteroidetes, relative to lean mice, and a significantly greater proportion of Firmicutes ( $P < 0.05$ , Fig. 2*C*). The changes were division-wide (there was no specific subgroup that was preferentially lost or amplified within these divisions) and occurred independently of kinship and gender.

Our study included a single runt animal with an *ob/ob* genotype. Its body mass (20 g) was equivalent to that of lean *ob/+* and *+/+* siblings, but the ratio of its epididymal fat-pad mass to total body mass was significantly greater (1.6% vs. 0.7% of total body mass;  $z = 6.64$ ;  $P < 0.0001$ ). This individual consumed

significantly less chow than its lean siblings, yet the relative abundance of Firmicutes (71.2%) and Bacteroidetes (26.1%) was similar to other *ob/ob* animals, suggesting that alterations in the representation of these divisions cannot be simply ascribed to differences in chow consumption or total body mass.

**Prospectus: Contribution of the Population Structure of the Gut Microbiota to Obesity.** Together, these results show that the development of obesity in *ob/ob* mice affects the relative abundance of the major gut bacterial divisions derived from a maternal inoculum. The mechanisms responsible for directing these changes in microbial diversity remain to be defined, although broad division-level effects are not typical of an immune response to bacteria (22).

*ob/ob* mice are more efficient at harvesting energy from food than are lean *+/+* animals (23, 24). The observed alterations in community structure within the distal gut microbiota of *ob/ob* animals may represent an unheralded contributing factor to their pattern of fuel partitioning. In domestic animals, antibiotics are commonly used to increase the efficiency of conversion of fuel in the diet to body mass (25), although the influence of these antibiotics on gut microbial ecology has not been determined with large-scale molecular phylogenetic surveys.

To date, 73 genomes of members of Firmicutes have been fully or partially sequenced (<http://img.jgi.doe.gov>). Most sequenced Firmicutes are in the class Bacillus, which is rare in the human or mouse gut microbiota; none is a member of the *Clostridium* XIV class, which contains the most abundant representatives of this division in the distal guts of mice and humans. Hence, the physiological contributions of Firmicutes to the intestinal ecosystem, and to fuel partitioning, are unclear. The increased ratio of Firmicutes to Bacteroidetes in *ob/ob* mice may help promote adiposity or, alternatively, could represent a host-mediated adaptive response to limit energy uptake/storage (e.g., by reducing the capacity to ferment polysaccharides). One way to address this question is by comparing gene content in the Firmicutes-enriched cecal microbiota of *ob/ob* mice with the Bacteroidetes-enriched microbiota of their lean *ob/+* and *+/+* littermates by using a metagenomics approach (26, 27). The results should provide insights about what metabolic attributes are provided to the host by these division-enhanced communities and how changes in divisional representation may effect energy homeostasis. The idea that gut microbial diversity is linked to obesity deserves exploration in humans, because it may yield new treatment strategies for this growing worldwide threat to our health.

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