Published in final edited form as:

Annu Rev Microbiol. 2013 ; 67: 459–475. doi:10.1146/annurev-micro-092412-155642.

Experimental Approaches for Defining Functional Roles of Microbes in the Human Gut

Gautam Dantas[1], **Morten O.A. Sommer**[2,3], **Patrick H. Degnan**[4], and **Andrew L. Goodman**[4]

Gautam Dantas: dantas@wustl.edu; Morten O.A. Sommer: msom@bio.dtu.dk; Patrick H. Degnan: patrick.degnan@yale.edu; Andrew L. Goodman: andrew.goodman@yale.edu

[1]Center for Genome Sciences & Systems Biology and Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, Missouri 63108

[2]Department of Systems Biology, Technical University of Denmark, DK-2800 Lyngby, Denmark

[3]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2970 Hørsholm, Denmark

[4]Microbial Diversity Institute and Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut 06536

Abstract

The complex and intimate relationship between humans and their gut microbial communities is becoming less obscure, due in part to large-scale gut microbial genome-sequencing projects and culture-independent surveys of the composition and gene content of these communities. These studies build upon, and are complemented by, experimental efforts to define underlying mechanisms of host-microbe interactions in simplified model systems. This review highlights the intersection of these approaches. Experimental studies now leverage the advances in high-throughput DNA sequencing that have driven the explosion of microbial genome and community profiling projects, and the loss-of-function and gain-of-function strategies long employed in model organisms are now being extended to microbial genes, species, and communities from the human gut. These developments promise to deepen our understanding of human gut host–microbiota relationships and are readily applicable to other host-associated and free-living microbial communities.

Keywords

microbiome; flora; metagenomics; sequencing; gain-of-function; loss-of-function

INTRODUCTION

Since the beginning of microscopy, we have recognized that the human gut harbors enormous microbial communities. Recent technological advances in microbial genetics and

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

genomics have led to a renewed appreciation that diverse aspects of human health and disease are critically influenced by the human microbiota. This hypothesis is compelling because of at least three striking differences between the genetic capacity of an individual's own genome and the added functional repertoire encoded in his or her gut microbiome. First, unlike the human genome, the gut microbiome is not a composite of maternal and paternal genes. Instead, we are born without any microbes and acquire these organisms through an ongoing process of environmental exposure, selection, and interspecies competition. Second, interpersonal microbial differences far exceed interpersonal genomic differences. Common estimates suggest that individual human genome sequences vary at approximately one nucleotide per every thousand (44); in contrast, gut microbial communities can exhibit drastically different representations of microbes at the phylum level (88). Third, the genetic content of an individual's gut microbial community is dynamic. In mice, changes in diet can restructure community composition in a single day (91). Moreover, these microbial communities outnumber the cells in the human body tenfold (73), and the number of genes in the microbiome exceeds the number in the human genome by more than two orders of magnitude (67). Together these features suggest that a significant component of our genetic repertoire is guided by rules fundamentally different than those that apply to the human genome.

As a result, it is perhaps not surprising that gut microbiome research reflects the legacies of multiple scientific traditions. One approach leverages the power of model systems: Readily cultured human gut species, some of which are genetically tractable, have provided a wealth of information on gene function and adaptations of human gut microbes to their environment. These efforts are complemented by studies that examine gut microbiomes in situ, generally using culture-independent methods to capture microbial diversity and community dynamics in their full complexity. Although these distinctions provide a simple conceptual framework, advances in high-throughput sequencing and other technologies are blurring the divisions between these approaches. The goal of this review is to provide a technique-centric overview of established and emerging experimental strategies that combine direct manipulation and genomics in the context of human gut microbial communities.

FUNCTIONAL INSIGHTS FROM SIMPLIFIED MODELS OF THE HUMAN GUT MICROBIOME

Human gut microbiome research rests on a strong foundation: The most well-studied and paradigm-defining organism in biology, *Escherichia coli*, is derived from this microbial community (64). Almost uniquely among gut bacteria, our understanding of *E. coli* gene function comes largely from direct biochemical, genetic, and genomic analyses rather than homology-based annotation (46). However, it is important to note that *E. coli* and the entire phylum *Proteobacteria* are relatively minor members of the human gut microbiota: Instead, the gram-negative *Bacteroidetes* and the gram-positive *Firmicutes* constitute 80–90% of these communities (42).

After *E. coli*, arguably the next best-studied model gut commensal bacterium is the obligate anaerobe *Bacteroides thetaiotaomicron. B. thetaiotaomicron* and related species are among

the most dominant organisms found in the distal small intestinal and colonic microbiota (97). They contribute to the transition from nutrition from breast-milk to plant-derived polysaccharides (6), as well as to the prevention of pathogen invasion by mediating the development of the intestinal mucosal barrier (98). *B. thetaiotaomicron* possesses an expanded capacity to sense, harvest, and metabolize complex plant-derived dietary polysaccharides that cannot natively be digested by the host (5, 72, 97). Mechanistic insights into these key functions have come from careful genetic and biochemical analyses of the relevant *B. thetaiotaomicron* metabolic enzymes and their structural complexes, such as the starch-utilizing genes (75, 76), as well as from system-wide -omic analyses (97).

Few other gut commensals have received anywhere near the degree of deep multidisciplinary analytic treatment as *E. coli* or *B. thetaiotaomicron*, especially for the purposes of modeling host-microbiota commensalism (5, 98). Mahowald et al. (57) made headway in this respect, reporting the complete genome sequences of *Eubacterium rectale* and *E. eligens*, two important representatives of the fastidious, dominant gut *Firmicutes*. By coupling this genomic information with transcriptomic analyses of *E. rectale* during growth with *B. thetaiotaomicron* in gnotobiotic mice, the authors were able to model niche specialization and functional redundancy between members of these two major bacterial phyla and to define host-derived glycans as a nutritional source that determines microbiota ecosystem stability (57). Gene catalogs from additional representative gut symbionts will continue to expand as a result of large-scale multi-investigator efforts such as the NIH-funded Human Microbiome Project (89).

Caveats of Studies in Model Systems

Renewed efforts to model the human gut microbiota in simplified systems are revealing several important caveats to these studies. For instance, microbial communities removed from their native environment can exhibit altered compositions. This is clearly the case in extreme examples: Transplantation of mouse microbiota into germ-free zebrafish, or zebrafish microbiota into germ-free mice, reshapes the community to more closely resemble that of the recipient host (68). This host effect is likely reduced (but not eliminated) in studies of human microbial communities grafted into another mammal, such as a germ-free mouse (15, 33, 49, 91). In addition to the microbiota responding to the host, model systems may disrupt the response of the host to the microbiota. For example, germ-free mice exhibit reduced $CD8^+$ and $CD4^+$ T cell counts in the small intestine that are restored upon colonization with a complete mouse microbiota (81). Transplantation of human gut microbial communities into these animals, in contrast, fails to restore T cell counts and other microbiota-dependent immune responses despite reaching the same density as a transplanted mouse microbiota (15).

FUNCTIONAL INSIGHTS FROM IN SITU ANALYSES

In addition to sequencing individual microbial genomes, massively parallel, low-cost DNA sequencers have also enabled deep cataloging of community diversity through the selective sequencing of informative phylogenetic markers or unbiased shotgun sequencing of metagenomic DNA (65, 67, 89). Phylogenetic surveys have focused almost exclusively on sequencing select regions of the small-subunit (16S) ribosomal RNA (rRNA) gene, which is

present in all microbes (16, 96). The 16S rRNA genes from a microbial community can be amplified using primers complementary to highly conserved regions of the gene and sequenced in parallel to generate community diversity profiles independent of culturing bias (48, 55). The identity and abundance of each community member can be inferred by comparison of each unique sequence to reference 16S rRNA databases (16), and phylogenetic profiles of different microbial samples can be compared by various statistical clustering metrics as well as ecological and evolutionary measures of diversity (55, 89).

While 16S rRNA-based surveys attempt to reveal “who’s there?” in a particular microbial community, shotgun metagenomic sequencing attempts to answer the complementary question of “what can they do?” (37). Metagenomic sequencing experiments aim to catalog all the genes from a community without the need for initial culturing, by random sequencing of all DNA extracted from the sample (31, 67, 88). The polypeptides predicted from these sequences are annotated by homology to gene function databases (31, 67, 88). Analogous to 16S rRNA statistical analyses, metagenomes can be computationally compared to infer differences in composition and relative abundance of encoded community genetic functions (54, 55).

Defining Normal

By using these in situ approaches, a substantial effort has been undertaken to define the normal (healthy) human gut microbiota, across a range of timescales, geography, host genetics, environments, and cultural traditions. In an elegant example of how metagenomic surveys can promote functional insight, Hehemann et al. (39) identified genes encoding porphyranase and agarase enzymes in the gut microbiomes of Japanese individuals but not North Americans. These functions were acquired likely by lateral gene transfer from marine *Bacteroidetes* (associated with seaweed consumed in the Japanese diet) to resident commensals in Japanese individuals and selected for by regular seaweed consumption characteristic of this diet (39). In recent years, reduced sequencing costs have allowed interrogation of increasing numbers of individuals, with ever-greater temporal resolution and coupled metagenomic analyses (48, 67, 88, 99). These studies, which are the subject of several recent reviews (56, 93), were inspired by earlier surveys performed on marine and other environmental microbiomes, and indeed the computational methods that were critical for analyzing these data were developed largely by the environmental microbiology community (37, 70).

For example, Yatsunenko et al. (99) used both 16S rRNA sequencing and metagenomics to describe the microbial communities from 531 samples from healthy children and adults from rural Venezuela, rural Malawi, and metropolitan areas in the United States. This large-scale study highlighted a pronounced, age-independent clustering of the microbiota of US metropolitan inhabitants that differed from the Malawi and Amerindian cohorts (99). On the basis of these data, the authors selected 110 individuals for shotgun sequencing, which revealed that the dynamics observed in infant microbiota is neither a consequence of a lower diversity of encoded functions nor an indicator of functions that are uniquely specialized to this early host developmental period (99). In a separate study, less than 50% of the metagenomic sequences recovered from 124 European individuals had high sequence

identity to the ~200 reference complete gut microbial genomes available at the time, illuminating the importance of culture-independent metagenomic sequencing in uncovering compositional and functional novelty in the human microbiota (67). This observation also emphasizes the need for increasing the diversity of organisms included in reference gut genome projects (65, 89).

Defining the Effects of Microbiome Perturbations

In situ surveys of healthy humans provide a critical foundation for studies of perturbed gut microbial communities. Perhaps one of the most dramatic modifications to the gut ecosystem is that caused by antibiotic treatment; as a result, in situ studies of the response of the microbiome to antibiotics highlight general themes that apply to other, more subtle perturbations.

Most culture-based studies have focused on specific indicator organisms to understand the effect of antibiotic treatment on the gut microbial community. These studies showed that resistance can emerge during treatment and persist for years after treatment is concluded (18, 78, 79). Culture-based analyses complement emerging culture-independent studies of the effects of antibiotic treatment on gut microbial communities. For example, Dethlefsen et al. (21) demonstrated that ciprofloxacin treatment perturbs the abundance of over one-third of all taxa in the human gut microbiota and causes a significant reduction in diversity. Temporal analysis reveals that the recovery after antibiotic treatment is often incomplete and does not follow common trajectories across individuals (22).

Furthermore, in situ studies have revealed that antibiotic exposure can create vacancies in the gut microbiota that become occupied by new strains (18). For instance, mice treated with streptomycin are increasingly susceptible to enteric infection (29), and humans treated with various antibiotics show increased risk of *Clostridium difficile* diarrhea (101). How these vacancies occur, and how the combination of recovery and invasion leads to emergence of the posttreatment communities, remains largely unclear (18). More subtle perturbations, such as changes in diet, also rapidly and broadly restructure gut microbial community composition in humans. This relationship is discussed in several recent reviews (23, 25, 50) and will have substantial implications for our ability to re-engineer the gut microbiota to address health issues (36). Specifically, medical tests often require fasting or other dietary normalization (a glucose meal prior to an oral glucose tolerance test, for example); a corresponding standard meal may prove essential for separating diet from other factors in shaping the microbiome (32).

Caveats of In Situ Metagenomics

It is increasingly clear that a wide range of human pathologies are associated with an altered gut microbiota (19), but determining whether these alterations observed by 16S rRNA sequencing are a driving factor (cause) or a consequence of disease (correlation) is not straightforward. Even in cases in which the gut microbiota does contribute to disease, the species that exhibit a change in abundance in diseased individuals are not necessarily the disease-causing agents within the community. For example, mice lacking the immunoregulatory cytokine interleukin-10 (IL-10) spontaneously develop colitis. This

pathology is not observed in animals maintained in the germfree state or treated with antibiotics, suggesting that microbes are required for disease pathogenesis (74). Although members of the *Enterobacteriaceae* are strongly enriched in gut microbial communities of IL-$10^{-/-}$ mice, isolates from this group do not cause colitis even though the microbiota is required for disease (8). Members of a different bacterial phylum (*Bacteroides*), however, do specifically induce disease in IL-$10^{-/-}$ animals, even though they do not show a change of abundance as a result of this host mutation (8).

Also, the success of metagenomic studies is closely tied to the quality of the underlying functional annotations of metagenomic sequence fragments. As discussed above, 40–60% of the gene content of a human gut microbiome consists of protein-coding sequences that lack obvious homologs in current reference databases (66, 99). Further, annotations based on overall sequence homology (rather than conservation of key protein folds, active sites, DNA binding residues or other features) affect the accuracy of functional assignments that lack experimental validation. Stepwise perpetuation of such incorrect assignments across proteins increasingly dissimilar to an experimentally validated reference sequence has been termed function creep (26, 87).

The extent of function creep in a metagenomic analysis is expected to scale with the evolutionary distance between the members of a microbiota and the model organisms that provide the experimental basis for the functional assignments. The extensive interpersonal variation observed in the human gut microbiome adds a further complication. As a simplified illustration of this challenge, we queried the metagenomes of 39 unrelated individuals (4) against the EcoCyc database of all *E. coli* genes whose functions have been assigned by direct experimental study (46). Notably, the percentage of reads that show significant similarity to these experimentally validated reference genes varies widely across these metagenomes, even though all the samples are from the same environment (Figure 1). Use of the MetaCyc database (11), which includes experimentally validated genes from diverse organisms, largely addresses this challenge (Figure 1). However, MetaCyc is restricted to metabolic pathways and it is likely that many important functions of the gut microbiota (e.g., transport, regulation, host interaction) are encoded in genes that do not belong in this category.

EMERGING INTERFACES BETWEEN MODEL SYSTEMS AND IN SITU STUDIES

Sequence-based interrogations enable extremely detailed in situ snapshots of compositional and functional diversity of gut microbial ecosystems (65, 67, 99). However, a complete understanding of microbiota functions must also involve complementary approaches to genetically and functionally manipulate, model, and perhaps engineer these communities. Importantly, the same high-throughput sequencing and computational methods that have produced the wealth of in situ studies described above can also be leveraged to allow new experimental manipulations (33, 34, 61, 82). Novel mechanistic insights gleaned from experimental studies can then be used to better understand in situ sequencing surveys, enabling iterative cycles of observation, perturbation, and analysis (33). In the following sections, we highlight some of the experimental models that have been developed for both

gain-of-function and loss-of-function assays of the gut microbiota at various scales. As an organizing principle, we attempt to categorize model experimental systems in terms of their prime applicability to three hierarchical levels of biological complexity: genes, species, and communities.

Loss-of-Function Approaches at the Gene Level

New techniques have allowed both targeted and untargeted loss-of-function genetic studies in prominent human gut symbionts in the mammalian host environment. For example, conversion of otherwise intractable polysaccharides into useable calories is a major component of human-symbiont interaction, but these processes have been difficult to mechanistically dissect in vivo because of the diverse range of saccharide linkages present in the gut and the enormous numbers of polysaccharide utilization loci present in single gut microbial species (97). To address this challenge, Martens et al. (58) developed a markerless gene deletion system for *B. thetaiotaomicron* that enabled creation of a quintuple-mutant strain unable to express the genes encoding each of the polysaccharide utilization systems that target mucin *O*-glycan linkages. This strain was rapidly outcompeted in vivo under conditions where mucin *O*-glycans are the preferred carbon source, providing new insight into the mechanism of bacterial glycan foraging in vivo (58).

Other techniques are extending genetic loss-of-function approaches to the genome scale. Signature tag mutagenesis and other negative selection strategies are widely used for identifying virulence factors of pathogens in vivo (13), but they have only recently been applied to human gut symbionts (34, 35). This approach, called insertion sequencing (INSeq), involves three steps. First, the target organism is mutagenized with a randomly inserting *mariner* transposon (51) that has been modified to encode recognition sites for the Type IIS restriction enzyme MmeI (62). Mutant populations are passed through a selective condition (colonization of the gastrointestinal tract of a germ-free mouse, for example), with samples collected before, during, and after selection. Genomic DNA is isolated from each sample and digested with MmeI, which cleaves its target 20 bp away from its binding site. This captures short fragments of genomic DNA adjacent to each transposon insertion in the population. Sequencing these fragments on a high-throughput DNA sequencer identifies insertions that change in abundance over the course of selection, highlighting genes required for fitness in these conditions. This strategy has revealed hundreds of genes that mediate colonization of human gut symbionts specifically in the mammalian gut environment: For example, *B. thetaiotaomicron* requires vitamin B_{12} transporters in certain community contexts but not others, suggesting that availability of these small molecules has a previously unrecognized role in shaping community composition in vivo (34). Because germ-free mice can be readily colonized with $>10^8$ human gut symbionts and this population rapidly grows to 10^{11} cells/g or higher in the distal gut, complex populations (10^5 or more different mutants) can be studied in a single animal without the population bottlenecks that complicate signature-tagged mutagenesis studies of pathogens. Related techniques have been applied to pathogenic bacteria (28, 30, 52, 92).

Gain-of-Function Approaches at the Gene Level

INSeq identifies gene functions by probing phenotypic consequences of removing one gene from a genome: Functional metagenomics invokes the opposite concept by adding genetic fragments to a heterologous host (Figure 2). This approach involves the extraction, shotgun cloning, and expression of metagenomic DNA within a host strain of interest followed by a selection for a specific function. Clones capable of surviving the selection as a result of a gain of function are sequenced and analyzed (17, 61, 83). Thus, metagenomic functional selections can be considered a targeted sequencing approach that facilitates the identification of parts of the metagenome encoding experimentally validated functions. Like INSeq, an advantage of this strategy is that genes can be associated with functions without relying on sequence homology.

Enzymatic functions involved in tolerance toward toxic compounds or substrate utilization are well suited for functional metagenomic selections, because they can be readily selected for by exposing the cell library to concentrations of an inhibitor, which is lethal to the wild-type host cell, or by subjecting the library to growth on an energy or nutrient source that does not support growth of the wild-type host cell. This approach has been used for close to a decade to study the antibiotic resistance genes encoded by environmental microbial communities (1, 69). Functional selections were first applied to study the human gut microbiota in 2009 (84). In that study, we mapped antibiotic resistance genes encoded by the gut microbiota of two unrelated individuals and identified several hundred resistance genes, including a large number of novel sequences. Many of these novel resistance genes were encoded primarily in the strict anaerobes in these communities (82). Even though subsequent studies have identified additional antibiotic resistance genes from the human gut microbiota (12, 20), comprehensive profiling of hundreds of individuals is still needed to better estimate the pool of resistance genes in the human gut microbiome. The human gut microbiota has long been implicated in the dissemination of antibiotic resistance genes to human pathogens, although the typical frequencies of genetic exchange in an unperturbed gut ecosystem are thought to be rather low (45, 53, 94).

Further developments within the field of metagenomic functional selections will likely involve the engineering of more complex genetic networks within the host cell to facilitate the identification of biological functions other than antibiotic tolerance. For instance, functional metagenomic clone libraries have been interrogated for their ability to utilize a variety of dietary fibers to identify novel β-glucanase, hemicellulase, galactanase, amylase, or pectinase activities (86). Additionally, host strains containing intracellular biosensors allow the identification of clones containing metagenomic DNA involved in the production of quorum-sensing inducers (95).

Loss-of-Function Approaches at the Species Level

Loss-of-function and gain-of-function approaches have also been applied at the level of species rather than genes. For example, the duration of host immune responses to the gut microbiota has been obscure because microbial colonization causes continual stimulation with microbial antigens. In an elegant study, Hapfelmeier et al. (38) used a species-level loss-of-function strategy to colonize germ-free mice with an auxotrophic *E. coli* strain that

grows readily in the lab but does not persist in vivo. These reversibly colonized animals return to the germ-free state unless continually inoculated with bacteria, which provides new insight into the dynamics and persistence of host-commensal interactions. Specifically, the authors demonstrated a long-lasting IgA response that persists months after exposure to gut microbes. This loss-of-function approach could be used to determine the dynamics of other host responses to the microbiota, including gut development, pathogen resistance, or even behavior (10, 81, 85).

Personalized, arrayed culture collections (33) provide an additional approach for targeted exclusion of single species from a microbial community. Arrayed culture collections consist of thousands of isolates from a single human donor, clonally archived in multi-well format. The 16S rRNA sequence of each isolate is determined using barcoded sequencing primers on a high-throughput DNA sequencer. These communities can then be reassembled in germ-free mice, with key species (or groups of species) excluded for loss-of-function studies. This strategy is based on the observation that 99% of the 16S rRNA reads derived from an individual's gut microbiome correspond to phylum-, class-, and order-level taxa that can be readily cultured from that individual's microbial community, and 70% of these reads belong to bacterial genera that can be readily cultured from the original sample (33). Furthermore, metagenomic sequencing reveals that 90–95% of annotated functions (e.g., KEGG orthology and pathway annotations, Enzyme Commission assignments) identified in complete, uncultured human gut microbiomes are also detected in culture collections derived from these communities. Although some functions of a complete microbial community will undoubtedly require its uncultured members, personalized culture collections should allow experimental dissection of many aspects of the host-microbiome relationship at the species level.

Gain-of-Function Approaches at the Species Level

Colonization of germ-free mice with a single bacterial species represents a simple gain-of-function microbiome study. This approach has been used to address diverse questions, including whether a single species is sufficient to restore host transcriptional or developmental responses induced by a complete microbiota (40, 85) or directly test whether the impacts of an unhealthy microbiota can be attributed to particular members of the community (3). Importantly, examples are emerging in which gain-of-function phenotypes can be observed by adding a single species to existing microbial communities: In mice, development of T helper cells that express IL-17 is triggered by segmented filamentous bacteria (SFB) that naturally occurs in some mouse facilities (27, 43). Although SFB cannot currently be cultured in the laboratory, it can be maintained in pure culture in gnotobiotic mice. Co-housing and feeding experiments demonstrate that this single species is sufficient to induce intestinal Th17 differentiation in mice that carry a complete (SFB-negative) community of their own.

Loss-of-Function Approaches at the Community Level

Community loss-of-function experiments seek to understand and model the impact of altering community structure on the host-commensal ecosystem (7). Experimental models involve either directed reduction or removal of subpopulations from a complete microbiota,

such as the antibiotic-induced perturbations described above (14, 101), or artificial construction and study of defined minimal microbiota subcommunities, where the effective loss-of-function is all the normal microbiota members not included in the artificial community (33, 60). Murine models have been particularly effective in understanding how antibiotic dose, target spectrum, and duration influence the selective reduction or removal of gut microbial phylotypes, and defining the potential functional consequences of such changes (83). In these models, host genotype, diet, and other variables that may affect baseline microbiota composition can be controlled, and the impact of antibiotic perturbation can be assayed by high-resolution phylotype or metagenomic sequencing (2).

For example, mice treated with the glycopeptide vancomycin show a decrease in the normally abundant *Firmicutes* and *Bacteroidetes*, with concomitant increase in the *Proteobacteria* and *Tenericutes*, with no reduction in overall microbiota biomass (71). The phylotype-specific effect of vancomycin is explained at least partially by its mechanism of action: The drug targets a peptidoglycan precursor needed for normal cell wall synthesis in gram-positive bacteria and is thus ineffective against the gram-negative *Proteobacteria* because it cannot penetrate their outer membrane, and the *Tenericutes* cell wall is not composed of peptidoglycan.

However, community loss-of-function due to antibiotic action can also proceed through more complex and indirect ecosystem perturbations. Mice treated with a combination of metronidazole, neomycin, and vancomycin were significantly compromised in their ability to clear pathogenic vancomycin-resistant enterococci (9). The drug combination suppresses the commensal microbiota, which in turn causes downregulation of intestinal expression of RegIII-γ, a secreted host innate immune defense molecule. Cho et al. (14) recently used murine models to understand the impact of so-called subtherapeutic concentrations of antibiotics on the microbiota and the host during infancy. Mice exposed in infancy to subtherapeutic concentrations of penicillin, vancomycin, penicillin and vancomycin, or chlortetracycline showed significantly elevated ratios of *Firmicutes* to *Bacteroidetes* compared with no-antibiotic controls (14). These differences parallel relative increases in the *Firmicutes* population in *ob/ob* mice, which are genetically prone to obesity (90), and indeed the authors observed that the antibiotic-treated mice had significantly increased adiposity relative to the controls (14). This community loss-of-function analysis is particularly significant, considering the disproportionately high level of antibiotic exposure in humans during infancy and early childhood, and the worsening obesity epidemic in children.

Gain-of-Function Approaches at the Community Level

Community-level gain-of-function experiments provide an important strategy for addressing whether an altered microbiome is the cause or the consequence of an observed host phenotype. In these studies, the gut microbiota of a host displaying a certain trait is transferred into a naïve recipient. Transmission of the phenotype is consistent with the donor microbial community composition being a causative factor. Although this approach is not new, recent studies demonstrate that such gain-of-function tests are particularly powerful when coupled with 16S rRNA and metagenomic analyses. For example, Turnbaugh et al.

found that the gut microbiome of leptin-deficient *ob/ob* mice is enriched in genes involved in caloric extraction, and used a microbiome transplant to determine whether this altered genetic capacity has functional consequences (90). To this end, the authors introduced the gut microbial communities of wild-type and *ob/ob* mice into wild-type, germ-free mice and measured weight gain in these recipient animals, demonstrating that the increased capacity for caloric extraction encoded in the microbiome was transmissible. Recent studies have shown that human gut microbial communities can be transplanted into germ-free mice largely intact, with little loss of diversity (91). Communities from individual human donors remain readily distinguishable for months after transplantation into germ-free recipients (33). In an elegant study, Koren et al. (49) transplanted the gut microbial communities of women at varying stages of pregnancy into germ-free mice to connect pregnancy-dependent reconfigurations of community structure with functional consequences for energy balance.

These gain-of-function studies are not limited to the use of germ-free animals. Conventional (non-germ-free) mice can be used as recipients if the microbes responsible for a given trait can successfully invade a preexisting microbial community (24, 43). It is also important to recognize that certain functions of the microbiota may manifest only in specific host genetic contexts and thus would not display a transmissible phenotype. For example, mice lacking IL-10 show increased levels of *Proteobacteria* that are associated with colon cancer, but these microbes do not cause disease in wild-type hosts (potentially because IL-10 also protects against disruption of the epithelial barrier) (3).

FUTURE DIRECTIONS

The development of methods that integrate metagenomics and experimental approaches is in its infancy, and the types of questions that can be answered with these techniques are still poorly defined. However, there are several important steps forward in the short term. On the one hand, functional metagenomics highlights novel capacities at the genetic level from fragments of environmental DNA cloned into *E. coli* or another heterologous host, but the microbe that naturally encodes the target activity can be difficult or impossible to identify. Arrayed culture collections, on the other hand, can be used to find microbes that carry out certain functions, but the genetic basis for this behavior remains unknown. Combining these techniques will capitalize on the strengths of each: Members of an arrayed culture collection could be pooled, community DNA harvested, and subcloned into *E. coli* for functional metagenomic analysis. Primers specific to genes of interest could then be used to identify the native source for the gene in the arrayed culture collection (33). In this way, connections can be made between gene-, species-, and community-level functional studies of the microbiome.

Further, current methods of studying the gut microbiota rely on well-mixed samples in which most if not all information on the structure of the microbial community is lost. A promising avenue for studying the gut microbiota relies on engineered microenvironments that mimic the natural ecosystem yet allow detailed and continuous monitoring of the recreated microbiota. It is now feasible to fabricate microchannels that are suitable for long-term cultivation of human epithelial cells and isolates from the human microbiota (41, 80). Indeed, so-called gut-on-a-chip devices have been created in which differentiated cell lines

can be cultivated over several days with isolates from the gut microbiota (47). These devices offer the promise of in situ monitoring of the interactions between members of a microbial community as well as between the microbiota and host cell lines. Advanced imaging coupled to engineered reporter cell lines would likely contribute to our knowledge of processes such as signaling and genetic exchange in the context of a structured community.

These in vitro experimental approaches will be strengthened by the continued development of animal models for studying human gut microbial communities. For example, the term humanized has been used to describe mice implanted with human genes or cells (63, 77, 100), but it also describes gnotobiotic animals carrying human gut microbial communities (59, 91). It is likely that mice humanized with both the genetic/cellular and microbial features of humans will provide important new insights into this host-microbiome relationship. Notably, both the current work described in this review and the future directions outlined above rely enormously on the same foundation of accurate, mechanistic understanding of the functional capacities of the human gut microbiome. If new experimental studies keep pace with the incredible surge in genomics and metagenomics, both approaches will benefit.

Acknowledgments

Support for this work was provided in part by the Children's Discovery Institute of Washington University and St. Louis Children's Hospital (MD-II-2011-117), the March of Dimes Foundation (6-FY12-394), the Kenneth Rainin Foundation (12H1), and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (DP2-DK098089) to G.D.; the EU FP7-Health Program Evotar (282004), the Novo Nordisk Foundation, The Lundbeck Foundation, Ib Henriksens Foundation, and The Danish Free Research Councils to M.O.A.S.; and the Global Probiotics Council, the Crohn's and Colitis Foundation, and the National Institutes of Health Center for Advancing Translational Science (UL1 RR024139), NIDDK (DP2-DK-089121), and National Institute of General Medical Sciences (R01 GM103574) to A.L.G.

Glossary

Microbiota	a microbial community
Gnotobiotic	germ-free animals colonized with specific microbes or groups of microbes
Germ-free	lacking any microbes
Metagenome (or microbiome)	the composite genetic material encoded by all members of the microbiota
Metagenomic functional selections	methods for shotgun, heterologous, expression cloning of microbial community DNA in model organisms to interrogate specific functions
Conventional	describes animals that carry a normal microbial community (e.g., specific-pathogen-free)

LITERATURE CITED

1. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 2010; 8:251–259. [PubMed: 20190823]
2. Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, Young VB. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect. Immun. 2009; 77:2367–2375. [PubMed: 19307217]
3. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science. 2012; 338:120–123. [PubMed: 22903521] Shows how effects of the microbiota can depend on host genotype.
4. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, et al. Enterotypes of the human gut microbiome. Nature. 2011; 473:174–180. [PubMed: 21508958]
5. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005; 307:1915–1920. [PubMed: 15790844]
6. Bjursell MK, Martens EC, Gordon JI. Functional genomic and metabolic studies of the adaptations of a prominent adult human gut symbiont, *Bacteroides thetaiotaomicron*, to the suckling period. J. Biol. Chem. 2006; 281:36269–36279. [PubMed: 16968696]
7. Blaser MJ, Falkow S. What are the consequences of the disappearing human microbiota? Nat. Rev. Microbiol. 2009; 7:887–894. [PubMed: 19898491]
8. Bloom SM, Bijanki VN, Nava GM, Sun L, Malvin NP, et al. Commensal *Bacteroides* species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. Cell Host Microbe. 2011; 9:390–403. [PubMed: 21575910] Demonstrates how gut microbes can impact disease independent of disease-associated changes in community composition.
9. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature. 2008; 455:804–807. [PubMed: 18724361]
10. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, et al. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in amouse via the vagus nerve. Proc. Natl. Acad. Sci. USA. 2011; 108:16050–16055. [PubMed: 21876150]
11. Caspi R, Altman T, Dreher K, Fulcher CA, Subhraveti P, et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res. 2012; 40:D742–D753. [PubMed: 22102576]
12. Cheng G, Hu Y, Yin Y, Yang X, Xiang C, et al. Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. FEMS Microbiol. Lett. 2012; 336:11–16. [PubMed: 22845886]
13. Chiang SL, Mekalanos JJ, Holden DW. In vivo genetic analysis of bacterial virulence. Annu. Rev. Microbiol. 1999; 53:129–154. [PubMed: 10547688]
14. Cho I, Yamanishi S, Cox L, Methe BA, Zavadil J, et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature. 2012; 488:621–626. [PubMed: 22914093]
15. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, et al. Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 2012; 149:1578–1593. [PubMed: 22726443]
16. Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, et al. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res. 2007; 35:D169–D172. [PubMed: 17090583]
17. Dantas G, Sommer MO. Context matters—the complex interplay between resistome genotypes and resistance phenotypes. Curr. Opin. Microbiol. 2012; 15:577–582. [PubMed: 22954750]
18. de Lastours V, Cambau E, Guillard T, Marcade G, Chau F, Fantin B. Diversity of individual dynamic patterns of emergence of resistance to quinolones in *Escherichia coli* from the fecal flora of healthy volunteers exposed to ciprofloxacin. J. Infect. Dis. 2012; 206:1399–1406. [PubMed: 22930806]
19. de Vos WM, de Vos EA. Role of the intestinal microbiome in health and disease: from correlation to causation. Nutr. Rev. 2012; 70(Suppl. 1):S45–S56. [PubMed: 22861807]

20. de Vries LE, Vallès Y, Agersø Y, Vaishampayan PA, García-Montaner A, et al. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. PLoS One. 2011; 6:e21644. [PubMed: 21738748]
21. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 2008; 6:e280. [PubMed: 19018661]
22. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. USA. 2011; 108(Suppl. 1):4554–4561. [PubMed: 20847294]
23. Dutton RJ, Turnbaugh PJ. Taking a metagenomic view of human nutrition. Curr. Opin. Clin. Nutr. 2012; 15:448–454.
24. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell. 2011; 145:745–757. [PubMed: 21565393]
25. Flint HJ. The impact of nutrition on the human microbiome. Nutr. Rev. 2012; 70(Suppl. 1):S10–S13. [PubMed: 22861801]
26. Friedberg I. Automated protein function prediction—the genomic challenge. Brief Bioinform. 2006; 7:225–242. [PubMed: 16772267]
27. Gaboriau-Routhiau V, Rakotobe S, Lécuyer E, Mulder I, Lan A, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009; 31:677–689. [PubMed: 19833089]
28. Gallagher LA, Shendure J, Manoil C. Genome-scale identification of resistance functions in *Pseudomonas aeruginosa* using Tn-seq. mBio. 2011; 2:e00315–e00310. [PubMed: 21253457]
29. Garner CD, Antonopoulos DA, Wagner B, Duhamel GE, Keresztes I, et al. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a *Salmonella enterica* serovar Typhimurium murine model of infection. Infect. Immun. 2009; 77:2691–2702. [PubMed: 19433544]
30. Gawronski JD, Wong SM, Giannoukos G, Ward DV, Akerley BJ. Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for *Haemophilus* genes required in the lung. Proc. Natl. Acad. Sci. USA. 2009; 106:16422–16427. [PubMed: 19805314]
31. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006; 312:1355–1359. [PubMed: 16741115]
32. Goodman AL, Gordon JI. Our unindicted coconspirators: human metabolism from a microbial perspective. Cell Metabol. 2010; 12:111–116.
33. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, et al. Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc. Natl. Acad. Sci. USA. 2011; 108:6252–6257. [PubMed: 21436049]
34. Goodman AL, McNulty NP, Zhao Y, Leip D, Mitra RD, et al. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe. 2009; 6:279–289. [PubMed: 19748469]
35. Goodman AL, Wu M, Gordon JI. Identifying microbial fitness determinants by insertion sequencing using genome-wide transposon mutant libraries. Nat. Protoc. 2011; 6:1969–1980. [PubMed: 22094732]
36. Haiser HJ, Turnbaugh PJ. Is it time for a metagenomic basis of therapeutics? Science. 2012; 336:1253–1255. [PubMed: 22674325]
37. Handelsman J. Metagenomics: application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev. 2004; 68:669–685. [PubMed: 15590779]
38. Hapfelmeier S, Lawson MA, Slack E, Kirundi JK, Stoel M, et al. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science. 2010; 328:1705–1709. [PubMed: 20576892] Uses microbial genetics to define the duration of host responses to the microbiota.
39. Hehemann JH, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature. 2010; 464:908–912. [PubMed: 20376150]

40. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001; 291:881–884. [PubMed: 11157169]
41. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. Science. 2010; 328:1662–1668. [PubMed: 20576885]
42. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012; 486:207–214. [PubMed: 22699609]
43. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009; 139:485–498. [PubMed: 19836068] Combines in situ analyses and experimental study to identify key microbes that induce immune response.
44. Jobling, MA.; Hurles, ME.; Tyler-Smith, C. Human Evolutionary Genetics. New York: Garland Sci; 2004.
45. Karami N, Martner A, Enne VI, Swerkersson S, Adlerberth I, Wold AE. Transfer of an ampicillin resistance gene between two *Escherichia coli* strains in the bowel microbiota of an infant treated with antibiotics. J. Antimicrobiol. Chemother. 2007; 60:1142–1145.
46. Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, et al. EcoCyc: fusing model organism databases with systems biology. Nucleic Acids Res. 2012; 41:D605–D612. [PubMed: 23143106]
47. Kim HJ, Huh D, Hamilton G, Ingber DE. Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. Lab Chip. 2012; 12:2165–2174. [PubMed: 22434367]
48. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc. Natl. Acad. Sci. USA. 2011; 108(Suppl. 1):4578–4585. [PubMed: 20668239]
49. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell. 2012; 150:470–480. [PubMed: 22863002] Demonstrates transmission of a phenotype from humans to germ-free mice via their gut microbiota.
50. Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat. Rev. Microbiol. 2012; 10:323–335. [PubMed: 22491358]
51. Lampe DJ, Akerley BJ, Rubin EJ, Mekalanos JJ, Robertson HM. Hyperactive transposase mutants of the Himar1 *mariner* transposon. Proc. Natl. Acad. Sci. USA. 1999; 96:11428–11433. [PubMed: 10500193]
52. Langridge GC, Phan MD, Turner DJ, Perkins TT, Parts L, et al. Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. Genome Res. 2009; 19:2308–2316. [PubMed: 19826075]
53. Lester CH, Frimodt-Møller N, Sørensen TL, Monnet DL, Hammerum AM. In vivo transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. Antimicrob. Agents Chemother. 2006; 50:596–599. [PubMed: 16436715]
54. Lozupone CA, Hamady M, Cantarel BL, Coutinho PM, Henrissat B, et al. The convergence of carbohydrate active gene repertoires in human gut microbes. Proc. Natl. Acad. Sci. USA. 2008; 105:15076–15081. [PubMed: 18806222]
55. Lozupone C, Hamady M, Knight R. UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinforma. 2006; 7:371.
56. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012; 489:220–230. [PubMed: 22972295]
57. Mahowald MA, Rey FE, Seedorf H, Turnbaugh PJ, Fulton RS, et al. Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. Proc. Natl. Acad. Sci. USA. 2009; 106:5859–5864. [PubMed: 19321416]
58. Martens EC, Chiang HC, Gordon JI. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe. 2008; 4:447–457. [PubMed: 18996345]

59. Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, et al. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. Mol. Syst. Biol. 2008; 4:157. [PubMed: 18197175]
60. McNulty NP, Yatsunenko T, Hsiao A, Faith JJ, Muegge BD, et al. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Sci. Transl. Med. 2011; 3:106ra06.
61. Moore AM, Munck C, Sommer MOA, Dantas G. Functional metagenomic investigations of the human intestinal microbiota. Front. Microbiol. 2011; 2:188. [PubMed: 22022321]
62. Morgan RD, Bhatia TK, Lovasco L, Davis TB. MmeI: a minimal Type II restriction-modification system that only modifies one DNA strand for host protection. Nucleic Acids Res. 2008; 36:6558–6570. [PubMed: 18931376]
63. Muruganandan S, Sinal CJ. Mice as clinically relevant models for the study of cytochrome P450-dependent metabolism. Clin. Pharmacol. Ther. 2008; 83:818–828. [PubMed: 18388875]
64. Neidhardt, FC.; Ingraham, JL.; Low, KB.; Magasanik, B.; Schaechter, M.; Umbarger, HE., editors. Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology. 1st. Washington, DC: ASM; 1987. p. 1,654
65. Nelson KE, Weinstock GM, Highlander SK, Worley KC, Creasy HH, et al. A catalog of reference genomes from the human microbiome. Science. 2010; 328:994–999. [PubMed: 20489017]
66. Qin J, Li Y, Cai Z, Li S, Zhu J, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012; 490:55–60. [PubMed: 23023125]
67. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010; 464:59–65. [PubMed: 20203603]
68. Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell. 2006; 127:423–433. [PubMed: 17055441]
69. Riesenfeld CS, Goodman RM, Handelsman J. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ. Microbiol. 2004; 6:981–989. [PubMed: 15305923]
70. Riesenfeld CS, Schloss PD, Handelsman J. Metagenomics: genomic analysis of microbial communities. Annu. Rev. Genet. 2004; 38:525–552. [PubMed: 15568985]
71. Robinson CJ, Young VB. Antibiotic administration alters the community structure of the gastrointestinal microbiota. Gut Microbes. 2010; 1:279–284. [PubMed: 20953272]
72. Salyers AA, O'Brien M, Kotarski SF. Utilization of chondroitin sulfate by *Bacteroides thetaiotaomicron* growing in carbohydrate-limited continuous culture. J. Bacteriol. 1982; 150:1008–1015. [PubMed: 6804433]
73. Savage DC. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. 1977; 31:107–133. [PubMed: 334036]
74. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect. Immun. 1998; 66:5224–5231. [PubMed: 9784526]
75. Shipman JA, Berleman JE, Salyers AA. Characterization of four outer membrane proteins involved in binding starch to the cell surface of *Bacteroides thetaiotaomicron*. J. Bacteriol. 2000; 182:5365–5372. [PubMed: 10986238]
76. Shipman JA, Cho KH, Siegel HA, Salyers AA. Physiological characterization of SusG, an outer membrane protein essential for starch utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. 1999; 181:7206–7211. [PubMed: 10572122]
77. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. Nat. Rev. Immunol. 2012; 12:786–798. [PubMed: 23059428]
78. Sjölund M, Tano E, Blaser MJ, Andersson DI, Engstrand L. Persistence of resistant *Staphylococcus epidermidis* after single course of clarithromycin. Emerg. Infect. Dis. 2005; 11:1389–1393. [PubMed: 16229767]
79. Sjölund M, Wreiber K, Andersson DI, Blaser MJ, Engstrand L. Long-term persistence of resistant *Enterococcus* species after antibiotics to eradicate *Helicobacter pylori*. Ann. Intern. Med. 2003; 139:483–487. [PubMed: 13679325]

80. Skolimowski M, Nielsen MW, Emneus J, Molin S, Taboryski R, et al. Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies. Lab Chip. 2010; 10:2162–2169. [PubMed: 20571689]
81. Smith K, McCoy KD, MacPherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin. Immunol. 2007; 19:59–69. [PubMed: 17118672] Lists a wide range of phenotypes observed in germ-free animals.
82. Sommer MO, Church GM, Dantas G. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. Virulence. 2010; 1:299–303. [PubMed: 21178459]
83. Sommer MO, Dantas G. Antibiotics and the resistant microbiome. Curr. Opin. Microbiol. 2011; 14:556–563. [PubMed: 21802347]
84. Sommer MO, Dantas G, Church GM. Functional characterization of the antibiotic resistance reservoir in the human microflora. Science. 2009; 325:1128–1131. [PubMed: 19713526]
85. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc. Natl. Acad. Sci. USA. 2002; 99:15451–15455. [PubMed: 12432102]
86. Tasse L, Bercovici J, Pizzut-Serin S, Robe P, Tap J, et al. Functional metagenomics to mine the human gut microbiome for dietary fiber catabolic enzymes. Genome Res. 2010; 20:1605–1612. [PubMed: 20841432]
87. Temperton B, Giovannoni SJ. Metagenomics: microbial diversity through a scratched lens. Curr. Opin. Microbiol. 2012; 15:605–612. [PubMed: 22831844]
88. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, et al. A core gut microbiome in obese and lean twins. Nature. 2009; 457:480–484. [PubMed: 19043404]
89. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007; 449:804–810. [PubMed: 17943116]
90. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006; 444:1027–1031. [PubMed: 17183312] Combines metagenomics and gut microbiota transplantation experiments to define host and microbial contributions to obesity.
91. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci. Transl. Med. 2009; 1:6ra14.
92. van Opijnen T, Bodi KL, Camilli A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat. Methods. 2009; 6:767–772. [PubMed: 19767758]
93. Walter J, Ley R. The human gut microbiome: ecology and recent evolutionary changes. Annu. Rev. Microbiol. 2011; 65:411–429. [PubMed: 21682646]
94. Werner G, Freitas AR, Coque TM, Sollid JE, Lester C, et al. Host range of enterococcal *vanA* plasmids among gram-positive intestinal bacteria. J. Antimicrobiol. Chemother. 2011; 66:273–282.
95. Williamson LL, Borlee BR, Schloss PD, Guan C, Allen HK, Handelsman J. Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. Appl. Environ. Microbiol. 2005; 71:6335–6344. [PubMed: 16204555]
96. Woese CR, Fox GE. Phylogenetic structure of prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA. 1977; 74:5088–5090. [PubMed: 270744]
97. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, et al. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science. 2003; 299:2074–2076. [PubMed: 12663928]
98. Xu J, Gordon JI. Honor thy symbionts. Proc. Natl. Acad. Sci. USA. 2003; 100:10452–10459. [PubMed: 12923294]
99. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, et al. Human gut microbiome viewed across age and geography. Nature. 2012; 486:222–227. [PubMed: 22699611]
100. Yoshizato K, Tateno C, Utoh R. Mice with liver composed of human hepatocytes as an animal model for drug testing. Curr. Drug Discov. Technol. 2012; 9:63–76. [PubMed: 22023259]

101. Young VB, Schmidt TM. Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. J. Clin. Microbiol. 2004; 42:1203–1206. [PubMed: 15004076]

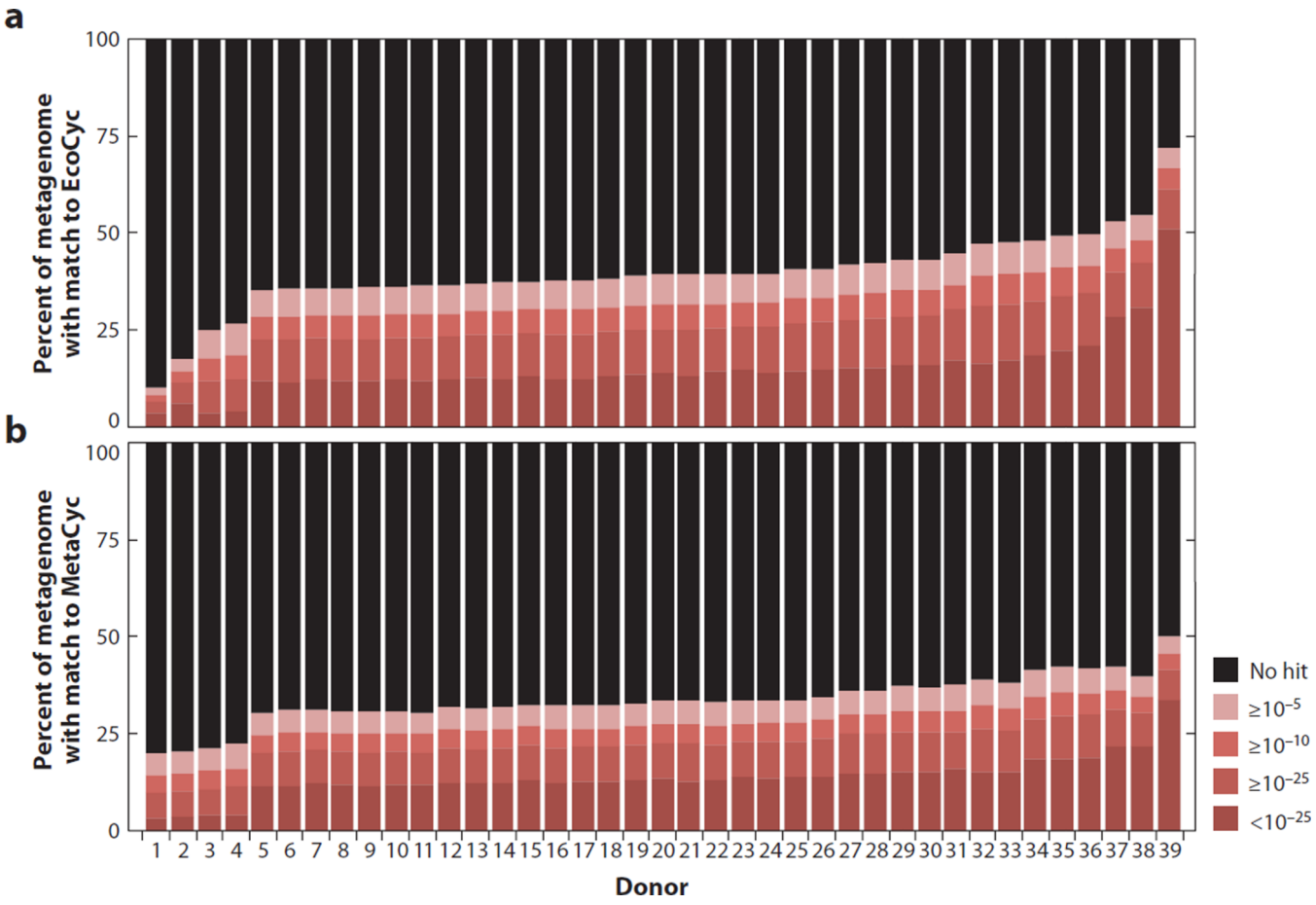

Figure 1.

Experimentally validated gene functions are differentially represented across human gut microbiomes. Sanger-sequenced fecal metagenomes from 39 individual donors (4) were annotated utilizing a database of (*a*) 2,696 *Escherichia coli* proteins with experimentally validated functions (46) or (*b*) 6,977 proteins that are involved in experimentally validated metabolic pathways across 811 species (11). Colored bars represent the proportion of reads from each donor binned according to the e-value of their best BlastX hit.

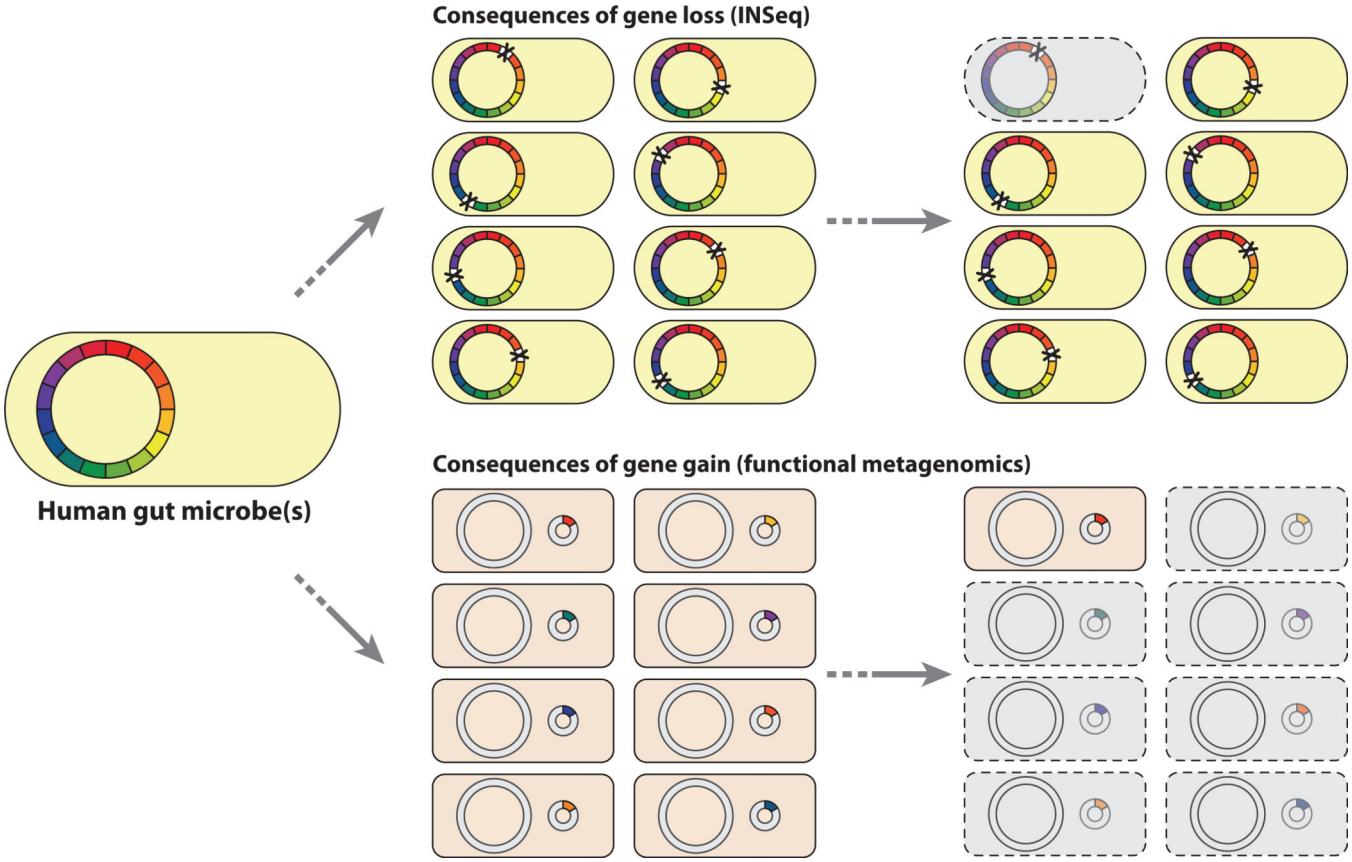

Figure 2.

Complementarity of insertion sequencing (INSeq) and functional metagenomics approaches. INSeq measures the functional consequences of loss of each gene from the genome, and metagenomic functional selections identify phenotypes gained by introduction of each gene into a heterologous host. Gray cells depict gene disruptions resulting in a fitness defect (INSeq), or gene additions that do not confer a selective fitness benefit (functional metagenomics).