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Creating and characterizing communities of human gut microbes in gnotobiotic mice

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Microbiology labs are laden with flasks, plates, and freezer stocks containing axenic cultures and their products. In contrast, virtually every other habitat on Earth is filled with microbial communities of varying degrees of complexity. In this context, microorganisms are interdependent components of ecosystems; deciphering this dynamic requires a move from microbial organisms studied in isolation to model microbial communities studied under conditions that mimic those encountered by their members in their native habitats. Here we focus on model communities consisting of microbes that inhabit the human body habitat containing our largest collection of organisms – the gut.

The adult human gastrointestinal tract is a microbial bioreactor, containing all three domains of life. This ecosystem is teeming with microorganisms at its distal end (10^{11}-10^{12} cells/ml luminal contents in the colon) and less so at its proximal end (an estimated 10^3-10^4/ml luminal contents in the duodenum). The gut microbiota affects myriad aspects of our systems physiology, ranging from processing and harvesting of macro- and micronutrients (and xenobiotics!) from our diets, to shaping the features of our innate and adaptive immune system. Recently, deep sampling of the fecal microbial community has revealed that each of us harbors a collection of a several hundred bacterial phylotypes (Qin *et al.*, 2010; Turnbaugh *et al.*, 2010). The exact set of microbes differs from person to person although there is a greater degree of similarity between family members (Turnbaugh *et al.*, 2009a; Turnbaugh *et al.*, 2010). A catalogue of several million genes present in the fecal microbiome has been assembled (Qin *et al., 2010*) from analysis of a 577 Gbp dataset obtained from shotgun sequencing of fecal community DNA prepared from 124 Europeans and a 10.1 Gbp dataset generated from a set of deeply sampled obese monozygotic co-twins living in the USA. These datasets provide a starting point for making *in silico* predictions about functions that can be attributed to the gut microbiota. Measurements of expressed mRNAs (Turnbaugh *et al.*, 2010), proteins (Verberkmoes *et al.*, 2009) and metabolites (Hoverstad *et al.*, 1984; Li *et al.*, 2008; Martin *et al.*, 2008) in fecal samples represent a first step towards testing these predictions.

Generating germ-free mice via embryo transfer

Germ-free (GF) mice provide a complementary approach for characterizing the properties of the human gut microbiome. Methods for establishing and propagating inbred strains of mice under GF conditions were established more than 50 years ago by several groups. Re-derivation was based on Caesarian section of a conventionally-raised (i.e. microbe-laden) mother, passaging the uterine horns containing the pups through a germicidal bath, and

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delivery of her pups in the GF isolator where they were suckled by a lactating foster mother (the original GF foster mothers were generated by Caesarian delivery of litters, hand feeding of pups in an GF isolator with an autoclaved artificial liquid diet until a male and female reached reproductive maturity; colonies were established from these GF progenitors and their offspring distributed to other gnotobiotic facilities; e.g. see http://gordonlab.wustl.edu/SuppInfo/Reyniers_Sacksteder_1957.pdf). This approach requires precise timing and is also inefficient: in our experience, 0-5 wild-type pups survive to weaning age/re-derivation (n=100 C-sections performed between 1998-2007). Therefore, we have supplanted this method with embryo transfer: embryos are harvested 1d after mating, and transferred under sterile conditions to a pseudo-pregnant GF mother generated by mating to a vasectomized GF male (Table 1). This technique yields 5-8 live born animals/25 embryos transferred/recipient mother (n >250 procedures). GF status is verified by PCR of feces using universal bacterial 16S rRNA gene primers and by culturing fecal and skin swabs under conditions that support growth of a broad range of anaerobic and aerobic bacterial species and fungi.

Studies of complex microbial communities in gnotobiotic mice

GF mice can be colonized with microbial communities of varying complexity and origin at defined stages of their life. For example, gut microbial communities can be harvested from various body habitats of conventionally-raised mice with defined genotypes and physiological phenotypes, and introduced into GF recipients (possessing a desired genotype) to determine how much of the donor phenotype is transferable to the resulting conventionalized mice via the microbiota. If complete or even partial phenotypic transfer occurs, follow-up studies can be performed to define composition of the donor community, the mechanisms by which the donor community impacts host physiology, and how the recipient affects the transplanted microbiota/microbiome. These types of studies have typically been performed using gut contents (e.g. Turnbaugh *et al.*, 2006), but in principle can be extended to communities harvested from any body habitat

We have developed procedures for subjecting GF and conventionalized mice to a variety of surgical and non-surgical manipulations while maintaining their gnotobiotic environments: these procedures include (i) endurance training through swimming in a warmed sterile water tank placed within the isolator (a ‘gnotatorium’; Crawford *et al.*, 2009); (ii) using a plexiglass gnotobiotic transporter to bring mice to an irradiator for whole body irradiation; (iii) bone marrow transplantation following whole body irradiation using marrow harvested from animals of varying genotypes (Crawford and Gordon, 2005); (iv) employing specialized transporters that fit inside a magnetic resonance imager to determine adiposity; and (v) techniques for generating aggregation chimeras using morula-stage embryos (Wong *et al.*, 2000).

We have also validated procedures for transplanting human fecal microbial communities into GF mouse recipients that are then fed diets that do or do not resemble those of the human donors (Turnbaugh *et al.*, 2009b). We have found that a remarkable proportion of human fecal microbial diversity can be transferred in this fashion even if the donor specimen had been frozen at −80°C for 1–2 years (all bacterial phyla, up to 90% of class-level and genus-level taxa, and 60-90% of species level-phylotypes in donor samples are identifiable in recipient mice using 16S rRNA-based pyrosequencing). Once engrafted, the transplanted human microbial communities are remarkably stable, can be reliably transmitted across generations of animals and exhibit well defined and reproducible biogeographical features along the length of the mouse gut (Turnbaugh *et al.*, 2009b). Efficient intergenerational transfer of transplanted human fecal microbiota allows the microbiota and the host's innate/adaptive immune system to co-evolve beginning at birth in ‘second generation’ mice.

'Humanized' gnotobiotic mice can be used for proof-of-mechanism studies that cannot be readily conducted in humans where potentially confounding variables, including variations in host genotype, diet, and antibiotic consumption are notoriously difficult to control. A derivative of this procedure is to capture as much diversity as possible by culturing a donor's fecal microbiota, and then transferring this culture collection *en masse* to wild-type or genetically engineered GF recipients (culturable 'humanized' mice).

Assembling defined model communities in vivo *using gnotobiotic mice*

As more members of the human gut microbiota are cultured and their genomes sequenced (Nelson *et al.* 2010), an opportunity exists to create model human gut communities in gnotobiotic mice where all community members and their complement of microbial genes are known. Members present in these synthetic human gut microbial communities can be selected from culture collections based on various criteria, including their consistent association with specific human physiologic or pathophysiologic states, their representation in a fecal microbiota that when transferred *en masse* confers a phenotype to recipient germ-free mice, their phylogenetic features, and/or by the results of *in silico* predictions of their functions based on inspection of their genomes. These communities can be used to address a number of basic questions in the field: e.g., (i) to what extent do priority effects, where established species are able to sequester limiting space or resources and are thereby able to exclude potential colonizers, determine community composition; (ii) what is the strength of inter-specific interactions (a key to generating predictive models of community structure and dynamics; Trosvik *et al.*, 2010); (iii) what are the genetic predictors of niche overlap; (iv) how robust are the assembled communities to various environmental perturbations; and (v) what is the microbial host range of viruses and the determinants of viral lifecycles in various regions of the gut ecosystem (Reyes *et al.*, 2010).

To date, these model communities have consisted of as few as 2 and as many as 15 members and have been used to explore some of the metabolic interactions that take place in the distal gut (both microbial-microbial and microbial-host; e.g., Denou *et al.*, 2009; Mahowald *et al.*, 2009; Rey *et al.* 2010; Sonnenburg *et al.*, 2006). These communities have also been extremely useful for technology development. For example, if the complete genome sequence of each member is known, then the relative abundance of each member can be used to infer the proportional representation of genes encoding various functions (e.g., metabolic and signaling pathways) in that community using quantitative metagenomic methods (Morgan *et al.*, 2010). With the current capacity of the Illumina GAIIx DNA sequencer (~30 million reads per lane), relative and absolute species abundance is quantifiable for all microbes representing at least 0.01% of the community, while allowing ≥100 barcoded samples to be pooled in a single lane of the 8-lane flow cell for multiplex sequencing. These inexpensive assays of community member abundance support the large sample sizes needed for computational modeling of the responses of a model community to various perturbations (including systematic alterations in macro- and micro-nutrient composition of the diet), across time.

Understanding how different gut communities modulate their gene expression in response to changes in diet, host physiological status, or invasion with microbial species is another key step in understanding the operations of the gut microbiota. RNA-Seq allows quantification of transcriptomes at high resolution and dynamic range. In the case of synthetic communities, where all the species and genes are known, this high-resolution data can be used to verify gene structure/operons, generate *in silico* reconstructions of expressed metabolic pathways for each member in the community, and make predictions concerning the metabolic niches of each species. These predictions can be informed by RNA-Seq analysis of individual community members during mono-culture under highly defined

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conditions (e.g., minimal medium supplemented with systematically varied carbon sources), then be validated using quantitative mass spectrometry-based analyses of products of microbial metabolism. These studies can prompt follow-up, hypothesis-based studies of metabolic niches where the investigator systematically manipulates the species used to construct these model communities, or uses other approaches to perturb the activities of key members in ways that provide proof-of-principle tests for affecting community function and host physiology (e.g., devising ways to manipulate the hydrogen economy of the gut to affect the efficiency of fermentation and host energy extraction; (Rey *et al.* 2010). In addition, gnotobiotic mice harboring defined collections of sequenced organisms provide an opportunity to further develop methods for extracting and characterizing, by LC-MS, the proteins expressed by their model microbiota (i.e., peptides can be readily mapped since all genes are known; Mahowald *et al.*, 2009).

Community genetics provides another powerful technology to dissect the operations of microbial communities and to identify potential avenues (targets) for microbiome-directed therapeutics. Addition or removal of organisms prior to gavaging the model microbiota into GF mice provides the simplest genetic perturbation to identify species that confer a benefit or detriment to other community members or the host. Another method is insertion-sequencing (INSeq), which combines genome-wide transposon mutagenesis with massively parallel sequencing (Goodman *et al.*, 2009). In this approach, complex populations of tens of thousands of transposon mutants of a given sequenced community member are generated and simultaneously introduced into wild-type or genetically manipulated GF mice in the presence or absence of other (sequenced) microbes. The representation of each mutant in the input community is determined by targeted, sequencing of transposon-adjacent chromosomal DNA and compared to their representation in the output community recovered from the mouse. Differences in mutant representation in input versus output communities indicate which microbial genes confer a fitness advantage as a function of whatever selective pressure is intentionally applied to the system (Goodman *et al.*, 2009).

Creating more realistic defined microbial communities: the challenges ahead

A look into the near future reveals a number of pressing needs. With genome sequences available for almost 200 human gut isolates from 8 bacterial phyla and Archaea, our ability to move towards larger model communities in gnotobiotic mice is limited by our ability to grow microbes in parallel; therefore, we need to identify media capable of supporting growth of diverse sets of microbes, scale up methods for growing anaerobic cultures in parallel (e.g. move from tubes to 96-well plates or microfluidic chips with individually addressable strains), and modify sequencing pipelines to allow for rapid assays of purity of single cell-derived cultures. The current set of sequenced human gut bacteria isolates are largely from different individuals. Using microbial communities obtained from a single individual is desirable for reasons described above, including the fact that co-existing microbial species have co-evolved, creating distinct collections of strain-level phylotypes. Thus, to move towards increasingly realistic communities, we need high-throughput methods to isolate and array in multi-well plates, single cell-derived cultures of hundreds of bacteria from a single individual. Sequencing capacity will likely be available to many individual labs in the next few years to generate draft genomes from hundreds of these arrayed organisms. In the context of human microbiome projects, the ultimate informative model microbiota would contain microbes isolated from single individuals that confer the donor's phenotype to the recipient gnotobiotic mice. The full model community ‘tool kit’, both experimental and computational (including application of existing and new methods for modeling) could be applied to these communities in an attempt to expedite understanding of how their component organisms and genes confer a donor phenotype. However, for these

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efforts to benefit and build the field, we also need to create the infrastructure necessary to readily share both communities and their associated data between labs. The knowledge-base for model microbial community biology (e.g., conditions for culturing its members, microbial genome sequences, quantitative data about community membership as a function of various perturbations, associated meta-transcriptome, meta-proteome, and metabolomic datasets; information about their impact on host physiology) requires systems for data deposition, annotation, and retrieval, that combine computer automation and error checking with as little human curation as necessary to ensure data quality. Finally, currently license agreements, biological safety regulations, and shipping procedures are designed to distribute individual strains or multiple variants of the same strain. We must streamline the regulatory and infrastructure hurdles for multi-species distribution to ensure that the best model communities developed over the coming years have the opportunity to earn their “model” designation as they follow the path of *E. coli* and *B. subtilis* as facilitators of biological discovery.

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Table 1

Procedure for generating GF mice via embryo transfer

Step 1: A 6-10 week old female GF Swiss-Webster mouse is checked for estrus and mated to a vasectomized GF Swiss Webster male; the female is checked for a vaginal plug the next day; if plugged, the mouse is transferred to a 2×2×2 foot flexible film gnotobiotic isolator containing a sterilized transfer pod, a sterilized anesthetic solution, and sterilized surgical equipment (all sterilizations are performed in the port of the gnotobiotic isolator by fogging the materials with a solution of chlorine dioxide (Clidox-S)).
Step 2: Embryos are harvested from 3-4 week old conventionally-raised, super-ovulated females of the desired genotype 1d after they have been mated to conventionally-raised males of the desired genotype. Using a laminar flow tissue culture hood, embryos are obtained by flushing the fallopian tubes with Brinster's BMOC-3 medium supplemented with penicillin (10,000 units/ml) and streptomycin (10 mg/ml). They are then cleaned by serial washes in drops of the same medium (6-10 wash cycles of 2-4 min each; note that these drops are submerged in embryo-tested sterile mineral oil (Sigma))
Step 3: The pseudopregnant GF Swiss-Webster female from step 1 is anaesthetized, placed in the transfer pod, brought into a laminar flow hood, and an incision made along the back to expose ovary and oviduct. The oviduct is then pierced with a 22-gauge needle. The needle is withdrawn and embryos are transferred (25/recipient mother) in the BMOC-3/antibiotic solution into the oviduct using a microtransfer pipette, analogous to the ones used for pronuclear injections of oocytes when generating transgenic animals.
Step 4: The ovary and oviduct are placed back into the abdomen, the dorsal incision is closed, the anaesthetized animal is placed in the transfer pod, and the pod is put into the port of the gnotobiotic isolator. The pod is then chemically sterilized with chlorine dioxide for 15-20 min, re-introduced into the gnotobiotic isolator, and the mother is allowed to revive (a warm heating pad is placed below the plastic isolator). One to two pseudopregnant mice can be processed at the same time using this procedure.