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REVIEW ARTICLE

Decoding molecular interactions in microbial communities

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One sentence summary: Understanding how microbes interact is key to deciphering microbial community assembly and stability; approaches that span whole communities to single isolate analyses, as well as sequence-function and synthetic approaches, lead to a deeper understanding of microbial interactions.

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ABSTRACT

Microbial communities govern numerous fundamental processes on earth. Discovering and tracking molecular interactions among microbes is critical for understanding how single species and complex communities impact their associated host or natural environment. While recent technological developments in DNA sequencing and functional imaging have led to new and deeper levels of understanding, we are limited now by our inability to predict and interpret the intricate relationships and interspecies dependencies within these communities. In this review, we highlight the multifaceted approaches investigators have taken within their areas of research to decode interspecies molecular interactions that occur between microbes. Understanding these principles can give us greater insight into ecological interactions in natural environments and within synthetic consortia.

Keywords: microbial community; microbiome; molecular interactions; metagenomics; synthetic biology; corrinoids

INTRODUCTION

Microbial communities dominate every niche on our planet. From oceans to soil to our own bodies, nearly all environments are populated with diverse communities of microorganisms that drive earth's biogeochemical cycles and influence plant and animal health. Diverse communities of microbes perform activities that can be beneficial or harmful to their surroundings. They form dynamic relationships with their environment, by both shaping and being shaped by environmental fluctuations. The collective metabolic activities of microbial communities have been implicated in a wide range of phenomena, from the production and sequestration of greenhouse gases to the protection from disease in wildlife, crop plants and humans.

Until recently, methods of investigation limited our understanding of microorganisms, as experimental studies were primarily performed on single organisms isolated from their environment and examined in the laboratory. The advent of new DNA sequencing technologies in the last decade has revolutionized our understanding of microbial communities. Coupled with advances in imaging and analytical methods, these technologies have provided a new view of microbial diversity and composition, and have inspired new approaches for probing microbial community structure and function (Box 1).

Box 1. Microbiome tool kit: techniques for investigating microbial communities.

Sequencing-based techniques

Metagenomics

Metagenomics assesses the genetic content of a community. Next-generation sequencing platforms are used to generate the sequence data from either amplicon sequencing

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(16S rRNA for bacteria or internal transcribed spacer for fungi) to assess phylogenetic diversity or whole genome shotgun sequencing to examine the entire genetic content. These data are then computationally analyzed to characterize communities based on relative sequence abundance and predicted gene function (Mardis 2013; Jones *et al.* 2015).

Metatranscriptomics
Metatranscriptomic approaches utilize sequencing platforms to measure the RNA levels in a given sample. This is done by converting the RNA in a sample into cDNA, which is then sequenced using a next-generation platform. The sequence data are computationally analyzed to identify genes that are expressed in the sample by methods similar to metagenomics analysis (Simón-Soro, Guillen-Navarro and Mira 2014; Franzosa *et al.* 2015).

Metaproteomics
Metaproteomic approaches profile the protein content in a microbial community sample. The proteins are enzymatically digested and analyzed by liquid chromatography-mass spectrometry. Mass data from protein fragments are then mapped to the metagenome sequence to identify specific gene products (Bergen *et al.* 2013).

Metabolomics
Metabolomics is the analysis of metabolites in communities. Metabolic products of community samples are analyzed by liquid or gas chromatography coupled with mass spectrometry to profile the small molecules produced in the community. Currently, the structures of only a fraction of metabolites can be determined by this approach, but comparative analysis of multiple samples can identify differences in metabolite production between samples (Marcobal *et al.* 2013; Ursell *et al.* 2014).

Single cell genome sequencing
To sequence the genome of single cells in the absence of culturing, individual microbial cells are separated from each other by microfluidics, and genomic DNA is amplified prior to sequencing (Gawad, Koh and Quake 2016). Although the genome sequences are often incomplete, this technique provides sequence data for uncultured microorganisms and provides links between a cell's localization within the community, its cellular morphology, and the metabolic pathways encoded in its genome.

Imaging techniques

Fluorescence in situ hybridization
FISH utilizes fluorescently labeled oligonucleotide probes that hybridize with target RNAs in permeabilized cells. The use of FISH probes specific to bacterial 16S ribosomal RNA is often used to provide spatial information for bacteria in multispecies aggregates such as biofilms. The use of FISH in combination with fractionation or PCR techniques can further classify the organisms based on the presence of functional genes and can aid in culture-independent isolation of organisms (Nikolakakis *et al.* 2015).
Conventional FISH imaging has limitations in the number of different bacterial taxa that can be discriminated from each other. A modified technique called CLASI-FISH uses a multifluorophore approach. By combining two or more labels for a microbe of interest, an expansion of the labeling capabilities can be achieved (Valm *et al.* 2011; Valm, Mark Welch and Borisy 2012; Mark Welch *et al.* 2016). Another approach, catalyzed reporter deposition (CARD)-FISH uses a tyramide signal that is deposited using peroxidase activity. This enables a higher level of FISH sensitivity. Using paramagnetic beads coated with an antibody targeting the CARD-FISH epitope, magneto-FISH enables the physical capture of microbial complexes for further study (Pernthaler *et al.* 2008; Orphan 2009). Another method called HCR-FISH uses DNA strands that do not hybridize without exposure to an initiator strand. This causes a chain reaction of hybridization that builds a DNA structure (Dirks and Pierce 2004). Using a multiplexed approach, many different types of microbes can be identified simultaneously (Yamaguchi *et al.* 2015).

Imaging mass spectrometry
Imaging mass spectrometry approaches enable the detection of small molecules based on their mass paired with information on their spatial distribution. The image contains a group of mass spectra that are obtained at spatial locations throughout a sample. MALDI-IMS and SIMS imaging are two techniques that use this approach. These methods are reviewed in detail elsewhere (Phelan *et al.* 2012; Bodzon-Kulakowska and Suder 2016).

Secondary ion mass spectrometry
A primary ion beam is used to bombard the sample to release secondary ions containing labeled isotopes, which are measured by mass spectrometry. A mass spectrometer capable of scanning over two dimensions is used to detect compounds produced in a spatial pattern. A modified version of SIMS called nanoSIMS enables the detection of molecules at a finer scale (lateral resolution better than 100 nm) (Carpenter *et al.* 2013).

Stable isotope probing
Stable isotopes are added to a sample, and the incorporation of these isotopes into proteins and nucleic acids is subsequently measured to track the metabolic activity of cells (Bergen *et al.* 2013).

Nanospray desorption electrospray ionization (NanoDESI) mass spectrometry
NanoDESI is a technique that enables detailed molecular characterization of compounds from a specific location on a sample. This technique enables the study of live cells in a colony without damaging them (Traxler *et al.* 2013). Two capillary tubes are used to form a solvent bridge that contacts the sample and carries analytes to the mass spectrometer; one capillary applies the solvent and the other transports the dissolved analytes from the bridge to the mass spectrometer (Roach, Laskin and Laskin 2010).

As knowledge of microbial communities has grown, heightened interest has inspired studies of the mechanisms of interactions between microbes in the laboratory. With the technological advances that have coincided with this recent surge in interest, the scientific community is now uniquely positioned to make new discoveries about microbial communities and develop methods to manipulate them. Here, we take an integrated view of the study of microbial communities (Fig. 1), highlighting examples of multifaceted approaches that have led to novel insights and introducing the laboratory techniques and technologies that serve as tools for ongoing research.

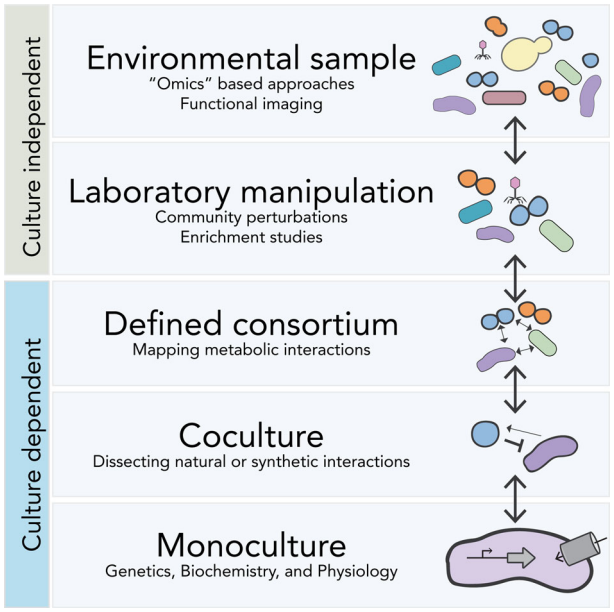

Figure 1. Integrated, multilayered approaches needed to decode microbiomes. Whole communities can be studied by culture-independent approaches performed directly on environmental samples or on communities cultivated in the laboratory. Culture-dependent approaches, in which defined consortia, cocultures or single microbes are examined by a variety of methods in the laboratory, enable more detailed studies about molecular interactions in a subset of the community.

DISSECTING MICROBIAL COMMUNITY INTERACTIONS FROM THE TOP DOWN, BOTTOM UP AND EVERYWHERE IN THE MIDDLE

Integrated approaches to the study of microbial communities

An ideal model community would be able to assemble reproducibly in the laboratory, and every microbe (and the host, if applicable) would be cultured, sequenced and genetically tractable. With such capabilities, the functions of individual microbes and genes could be elucidated, and hypotheses about interactions between organisms could be tested. Clearly, we are far from realizing this ideal because of limitations in culturing and genetic manipulation of the majority of microbes. However, the availability of fast, inexpensive sequencing of single genomes and metagenomes, coupled with transcriptomics, proteomics and metabolomics, has revolutionized the microbial ecology field by enabling comprehensive community-level analysis, both *in situ* and in laboratory settings. Such studies have led to valuable insights about microbial diversity and community structure, and have informed models of metabolic networks. In order to make full use of these new technologies, however, these top-down approaches must be coupled with analyses of individual microbes from these communities, which enables the characterization of their metabolic requirements and capabilities. In addition, investigating metabolic interactions between individual microbes in cocultures and larger consortia can reveal the types of interactions that occur in a community. We propose that the most successful examples of microbial community analysis are those in which multiple layers of approaches—from culture-independent analysis of the whole community to examination of individual genes—are employed to form a more complete understanding of microbial interactions in the community. A schematic of this overall approach is presented in Fig. 1, and we describe several examples of this multilayered approach below.

Gut communities

Analysis of the human gut microbiota

It is greatly appreciated by the scientific community, and more recently by the general public, that the human gut microbiota plays an important role in health and disease. Major advances in our understanding of the composition and diversity of the gut microbiota in healthy and diseased individuals have resulted from comparative metagenomic analyses of fecal samples across populations and over time. These studies revealed that the human gut harbors over a thousand microbial taxa, largely composed of bacteria, and higher microbial diversity is indicative of a healthy gut in many circumstances (Eckburg *et al.* 2005; Ley *et al.* 2006; Dethlefsen *et al.* 2008; Turnbaugh *et al.* 2008, 2009a; Human Microbiome Project Consortium 2012). We understand now that an imbalance in the microbial composition—termed dysbiosis—in the human gut is associated with obesity, diabetes, neurological disorders, inflammatory bowel diseases and cancer (Ley *et al.* 2005; Turnbaugh *et al.* 2008; Cryan and Dinan 2012; Schwabe and Jobin 2013; Goodrich *et al.* 2014; Hold *et al.* 2014; Lee and Hase 2014; Moreno-Indias *et al.* 2014). Furthermore, unlike other ecosystems, the human gut microbiota lacks a well-defined core set of organisms that spans all human populations, though at the phylum level Firmicutes, Bacteroidetes and Actinobacteria typically dominate (Faith *et al.* 2013). Within individuals, approximately 60% of the gut microbiota is projected to remain stable for decades (Faith *et al.* 2013), yet rapid, temporary shifts in microbiota composition can be induced by lifestyle changes or acute intestinal infection (David *et al.* 2014). In inflammatory bowel diseases, such as Crohn's disease, shifts in the microbiota and abnormal inflammatory responses are hallmarks of disease progression (Hold *et al.* 2014; Buttó and Haller 2016). These insights highlight the power of metagenomics to enhance our understanding of the complex ecosystem of the gut. However, in order to understand the roles of individual members of the community and the mechanisms by which they interact, it is necessary to perform laboratory manipulations of the community.

Major advances in understanding the links between the human gut microbiota and diet, the immune system, obesity and mental health have been made by using the gnotobiotic (germ-free) mouse system, in which mice reared in a sterile environment are inoculated with a single bacterial strain, a defined consortium or a complete community (Gordon and Pesti 1971). Studies of germ-free mice inoculated with fecal samples from human donors, known as 'humanized' mice, have demonstrated the effects of environmental factors such as diet on community composition and host metabolism. For example, humanized mice reared on a 'western' diet (high fat and simple carbohydrates with low fiber) experienced changes in microbial community composition, metabolome and gene expression, in addition to increased adiposity, in comparison to the microbiota of mice fed a diet rich in plant-derived polysaccharides (Turnbaugh *et al.* 2009b; Marcobal *et al.* 2013). Another recent study showed a link between the western diet and reduction in diversity of the gut microbiota. Humanized gut communities in germ-free mice consuming a western diet, but not those consuming a high-fiber, plant polysaccharide-based diet, experienced a decline in bacterial diversity over multiple generations (Sonnenburg *et al.* 2016).

Transitioning the mice from a western diet to a high-fiber diet only restored a subset of the original diversity, whereas a fecal transplant from a donor reared exclusively on a high-fiber diet resulted in the recovery of the lost microbes (Sonnenburg *et al.* 2016). These results suggest that some bacterial taxa are permanently lost from the population over generational time. It is therefore tempting to speculate that the rise in obesity, autoimmune diseases and other afflictions in industrialized countries is a consequence of reduced diversity in the gut microbiota that results from the consumption of a western diet over numerous generations (Sonnenburg *et al.* 2016). However, given that these observations are in an animal model, continued research to explore the relationship between dietary shifts in human populations and loss of microbial diversity is needed.

Given the emergence of these and other links between the composition of the gut microbiota and health, obvious questions are what constitutes a desirable microbiota and how a dysbiotic microbiota can be converted to a healthy one. To address these questions, it is crucial that we understand the ecology, physiology and biochemistry of the community such that the roles of individual members can be better understood. Although culture-based studies can only provide mechanistic details about a small fraction of the population, they can reveal key insights that lead to a better understanding of the whole population (Fig. 1). Recent improved techniques for culturing microbes have been developed that maintain some of the metabolic interactions by allowing the diffusion of nutrients between organisms (Box 2). For example, a recent study challenged the notion that large proportions of human gut bacteria are unculturable by performing large-scale culturing of fecal samples with a single growth medium (Browne *et al.* 2016). Over 70% of the species in the original community were represented in the eight million colonies that were collected. Interestingly, over half of the bacteria were found to produce spores, providing a possible mechanism for transmission of oxygen-sensitive organisms between hosts (Browne *et al.* 2016).

Box 2. New approaches to culturing microbes.

The largest hurdle to overcome in examining the roles of individual members of a microbial community is the limited ability to culture the majority of microbes; over 99% of environmental microbes are thought to be unculturable (Li *et al.* 2014), leaving a tiny, non-representative fraction available for controlled manipulations in the laboratory.

Conventional approaches to cultivating microbes involve the enrichment and isolation of the organism from its environment. Media components such as the carbon and nitrogen source are optimized to encourage the growth of the desired organisms. Even with the addition of numerous nutrients, trace elements, growth factors and biological extracts, this approach often does not satisfy the complete nutritional requirements of the organism. Hence, multiple methods have been developed to encourage growth by allowing for exchange of nutrients between the isolate and native community members, as described below.

- The isolation chip, or iChip, is comprised of several hundred diffusible chambers. The use of agar plugs that contain individual bacteria allows each cell to remain isolated from its neighbors while still enabling the sharing of metabolic compounds and nutrients that support growth (Nichols *et al.* 2010).
- Similar to the iChip, a 'mini-trap' uses microchambers with diffusible membranes embedded into an oral appliance. In one study, the use of this device increased the cultivable population by 11% (Sizova *et al.* 2012).
- Nutrient cross-feeding interactions can be preserved even when isolating microbes on traditional agar plates. Several studies have reported the successful isolation of microbial colonies by plating a diluted sample such that a high density of colonies emerges to allow for cross-feeding of essential nutrients. In one study, this isolation technique resulted in the capture of as many as 50% of the bacterial OTUs present in a stool sample (Goodman *et al.* 2011). Another study used soft agar to facilitate diffusion of small molecules and a membrane filter to enable nutrient exchange (Tanaka and Benno 2015). The nature of the metabolite exchange was identified in another study in which isolates dependent on a 'helper' strain were found to require siderophores produced by the helper (D'Onofrio *et al.* 2010).

A significant step toward understanding the metabolic capabilities of human-associated bacteria was the Human Microbiome Project, which provided genome sequences of hundreds of cultured bacteria from the gut and other sites in the body (Human Microbiome Project Consortium 2012). *In silico* analysis of metabolic pathways in these sequences, coupled with empirical data on the metabolic capabilities of the bacteria, led to the development of models of the carbon and energy flow throughout the gut community. These models show a metabolic hierarchy (Fig. 2) in which a subset of the bacteria degrade complex carbohydrates to sugars which are fermented by other organisms to form organic acids such as butyrate, acetate, propionate, succinate and lactate (Fischbach and Sonnenburg 2011). The H_2, CO_2 and formate formed in these reactions are further converted to acetate by acetogens, and are also used as substrates by methanogens and sulfate-reducing bacteria (SRB) in

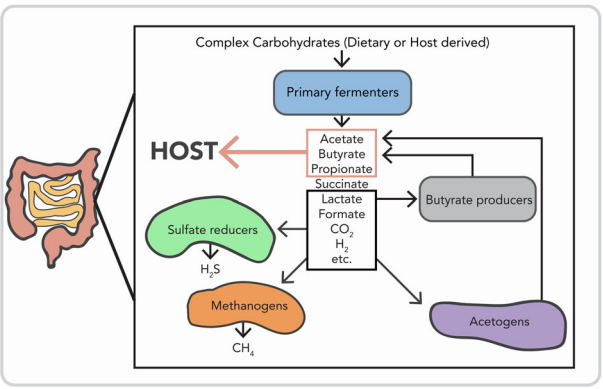

Figure 2. Simplified model of carbon flow in the human gut. Complex carbohydrates obtained from the diet or host mucin are broken down by saccharolytic primary fermenters, usually *Bacteroides* and *Bifidobacterium* spp (Willis *et al.* 1996). Some end products of this fermentation (acetate, butyrate and propionate) are absorbed by the host. Other products such as glucose (not shown) and lactate are fermented to butyrate by bacteria such as *Clostridium*, *Eubacterium* and *Fusobacterium* spp. (Bourriaud *et al.* 2005; Belenguer *et al.* 2006). Consumption of formate, CO_2 and H_2 by acetogens (like *Blautia hydrogenotrophica* and *Marvinbryantia formatexigens*) prevents their buildup and provides additional acetate. Methanogens (such as *M. smithii*) and SRBs (such as *D. piger*), which are present in 30% and 50% of individuals (Hansen *et al.* 2011; Rey *et al.* 2013), also consume fermentation products to generate methane and hydrogen sulfide, respectively (Loubinoux *et al.* 2002; Samuel and Gordon 2006).

microbiota that harbor these organisms (Loubinoux *et al*. 2002; Samuel and Gordon 2006) (Fig. 2).

Coculture studies of primary and secondary fermenters have revealed the ability of some organisms to customize their metabolism to complement the metabolic activities occurring in their environment. The metabolism of the primary fermenter *Bacteroides thetaiotaomicron* changes depending on which secondary fermenters are present in the gut. Co-association of *B. thetaiotaomicron* with the methanogen *Methanobrevibacter smithii* in germ-free mice resulted in higher adiposity compared to mice monoassociated with *B. thetaiotaomicron*. Transcriptomics and biochemical analysis of *B. thetaiotaomicron* showed that co-association with *M. smithii* had a dramatic impact on the expression and activity of its fructofuranosidase enzymes (Samuel and Gordon 2006). In contrast, co-association of *B. thetaiotaomicron* and the SRB *Desulfovibrio piger* had no effect on adiposity or fructofuranosidase expression (Samuel and Gordon 2006). Sulfate cross-feeding is an important component of the interaction between these two bacteria, as growth of *D. piger* in the gut was reduced when co-associated with a mutant *B. thetaiotaomicron* that lacked sulfatase activity (Rey *et al*. 2013). These studies demonstrate that associations with different partners can dramatically impact gene expression and metabolism of bacteria in ways that directly influence host metabolism and health.

Genetic analysis of model gut bacteria such as *B. thetaiotaomicron* in conjunction with community interaction studies *in vitro* and within the germ-free mouse has been instrumental in identifying genes necessary for survival within the gut community (Salyers, Pajeau and McCarthy 1988; Tancula *et al*. 1992). In one approach—a modern version of signature-tagged mutagenesis called INSeq—germ-free mice were inoculated with a pooled collection of ∼35 000 transposon insertion mutants to identify genes in *B. thetaiotaomicron* that influence competitive survival within the gut (Goodman *et al*. 2009). The mutants that dropped out of the population included those with defects in importing vitamin B_{12} (cobalamin). Notably, these mutants were more competitive when co-inoculated with a consortium of cobalamin-producing bacteria and less competitive when co-inoculated with cobalamin-scavenging bacteria (Goodman *et al*. 2009). Thus, examining mutants in the context of communities with different compositions further demonstrates the effect of metabolic interactions on competitive survival.

Other model systems

The human gut is a challenging system to investigate specific microbe–microbe interactions because of its complexity, variability between individuals and costs associated with the germ-free mouse model. Invertebrate gut systems with reduced complexity and cost have emerged as models, though the community composition and host environment differ considerably from the human gut. For example, the *Drosophila melanogaster* (fruit fly) gut is orders of magnitude less complex than the human gut, harboring only 5–20 individual species in laboratory-reared flies (Chandler *et al*. 2011; Wong, Ng and Douglas 2011). With a less costly germ-free system and high percentage of the organisms cultivable and genetically tractable, the fly gut can be used to investigate ecological interactions in a natural gut community (Wong, Ng and Douglas 2011).

In contrast to the fly gut, whose simplicity, tractability and culturability make it an attractive model microbial ecosystem, some microbial communities that have proven to be far less tractable are nevertheless appealing to study because of their ecological or industrial importance. The wood-feeding termite gut microbiota harbors numerous unusual microbes that are capable of degrading lignocellulose and are therefore of interest for their potential cellulosic biofuel applications, yet very few of the dominant organisms from this niche have been successfully cultured. Two notable exceptions are the bacteria *Treponema primitia* and *T. azotonutricium*, which were isolated from the termite *Zootermopsis angusticollis* and were found to colocalize in the gut (Rosenthal *et al*. 2011). Physiological studies of monocultures and cocultures, in conjunction with transcriptomics analysis, revealed metabolic codependence between these bacteria and with the insect host (Rosenthal *et al*. 2011).

The gut tract of 'lower' termites such as *Zootermopsis* spp. is home to a complex community consisting of microbes from all three domains including phylogenetically unique protists that themselves contain bacterial and archaeal endosymbionts (Ohkuma 2008). The limited ability to culture these microbes or manipulate the host has led to the development and use of cutting-edge technologies to examine the roles of the microbes *in situ* (Box 1). For example, unculturable bacterial cells from the termite gut were isolated and underwent single-cell genome sequencing, which led to the metabolic characterization of new phyla endemic to this niche (Hongoh *et al*. 2008a). Additionally, functional imaging techniques including nanoscale secondary ion mass spectrometry (nanoSIMS) and focused ion beam-scanning electron microscopy were applied to examine carbon flow in termites that were fed ^{13}C-labeled cellulose (Hongoh *et al*. 2008b; Carpenter *et al*. 2013). This analysis showed an enrichment of ^{13}C within Oxymonad protist cells and, to a lesser extent, in prokaryotic cells on the surface of the protist cells, suggesting that the [^{13}C]cellulose is first phagocytosed by the protists, and degradation products are transferred from protist to prokaryote cells within the gut (Carpenter *et al*. 2013).

Coupling *in situ* imaging and nanotechnology with metatranscriptomics can create functional links between metabolic processes and the organisms performing them in the absence of culture-based analysis. In one study of termite gut metabolism, an uncultured, unsequenced Deltaproteobacterium was found to be the dominant producer of transcripts for the formate dehydrogenase and formyl-tetrahydrofolate synthetase enzymes, which are markers of acetogenic and related metabolisms (Rosenthal *et al*. 2013). The link between the transcripts and the organism producing them was made by two methods. Microfluidic multiplex digital PCR was used to amplify both the 16S rRNA gene and the functional genes, providing both phylogenetic and metabolic information for individual cells. Hybridization chain reaction (HCR)-fluorescence *in situ* hybridization (FISH) was also used to visualize cells containing both the transcripts of interest and phylogenetic marker RNAs (Rosenthal *et al*. 2013). This study further showed that the newly identified Deltaproteobacterium is physically associated with protist cells, indicative of interdomain metabolic relationships between the two (Rosenthal *et al*. 2013).

Oral communities

Another example of a microbial community for which research has spanned multiple layers of complexity is the human oral microbiome. The study of oral microbial communities has a rich history, beginning in 1684 with the observations of dental plaque by Leewenhoeck (1684). Since that time, a large body of work on the oral microbiome has emerged. The oral cavity contains several anatomically and chemically distinct niches including the teeth, gum, tongue, palate, cheek and tonsils that each select for communities with distinct features (Human Microbiome Project Consortium 2012). For example, microbes form biofilms

on the tooth pellicle, a saliva-based glycoprotein that coats the tooth surface, and are exposed to saliva, while a distinct dental plaque community within the gingival (gum) crevice is exposed to crevicular fluid, which is chemically distinct from saliva.

Sequence-based analysis of the microbes that inhabit healthy and diseased human oral niches showed that these environments are comprised of several hundred bacterial taxa (Paster *et al.* 2001; Dewhirst *et al.* 2010; Griffen *et al.* 2012; Wade 2013). These studies also revealed that, in contrast to the gut microbiota where high bacterial diversity is associated with a state of health, higher bacterial diversity in the mouth correlates with a higher frequency of dental caries and oral diseases such as periodontitis (Costalonga and Herzberg 2014). A temporal analysis of the formation of dental caries showed that increased microbial diversity is followed by a decrease in diversity at the site of the caries due to acid production by Streptococcal species, which both degrades the tooth's enamel and inhibits the growth of other microbes (Simón-Soro *et al.* 2013).

Dental plaque formation is an especially informative model for the study of succession in microbial communities because the community reassembles reproducibly following the cyclical removal of oral biofilms with dental hygiene procedures. Additionally, microbial succession can be analyzed by implanting sterile disks into the oral cavity and removing them at different time points to monitor the formation of a plaque community on its surface. 16S amplicon sequencing of disk implants over a 7-day period demonstrated that facultative anaerobes of the genera *Streptococcus*, *Neisseria*, *Abiotrophia*, *Gemella* and *Rothia* were early colonizers of the plaque bacterial community (Takeshita *et al.* 2015). Later colonizers consisted of obligate anaerobes such as *Porphyromonas*, *Fusobacterium*, *Prevotella* and *Capnocytophaga* (Takeshita *et al.* 2015). In plaque scraping samples, an ordered spatial distribution of specific bacterial taxa was visualized by combinatorial labeling and spectral imaging (CLASI)-FISH (Fig. 3) (Mark Welch *et al.* 2016).

The ability to culture microbes from enrichments from human plaque samples paved the way for the development of model systems to investigate the assembly of oral biofilms *in vitro* (Carlsson 1967; Socransky, Dzink and Smith 1985). These studies revealed that initial colonizers such as Streptococci express adhesins that mediate attachment to the tooth pellicle (Human Microbiome Project Consortium 2012). Interestingly, the identity of the initial colonizing bacteria significantly influences downstream health outcomes. For example, initial colonization with *Streptococcus mutans* is correlated with cariogenic activity, whereas the abundance of *S. gordonii* is positively correlated with healthy teeth (Kolenbrander *et al.* 2010). If hygienic standards are not maintained, colonization by Streptococci allows for the next wave of bacteria to adhere, one of which, *Fusobacterium nucleatum*, serves as a physical tether for multiple other species (Kolenbrander, Andersen and Moore 1989). The quorum-sensing molecule AI-2 is secreted by *F. nucleatum*, which induces changes in expression of adhesion molecules in the 'red complex' of bacteria—*Porphyromonas gingivalis*, *T. denticola* and *Tannerella forsythia*—which are highly associated with chronic and aggressive periodontitis (da Silva-Boghossian *et al.* 2011; Jang *et al.* 2013). Later colonizers and oral pathogens, such as *Aggregatibacter actinomycetemcomitans*, interact with *F. nucleatum* via their O-polysaccharide (Rupani *et al.* 2008). The abundance of *A. actinomycetemcomitans* and *P. gingivalis* is highly correlated with aggressive periodontits (Casarin *et al.* 2010).

The metabolic characteristics of the commensal bacterium *S. gordonii* and the pathogen *A. actinomycetemcomitans* in monoculture, as well as the molecular interactions between the two

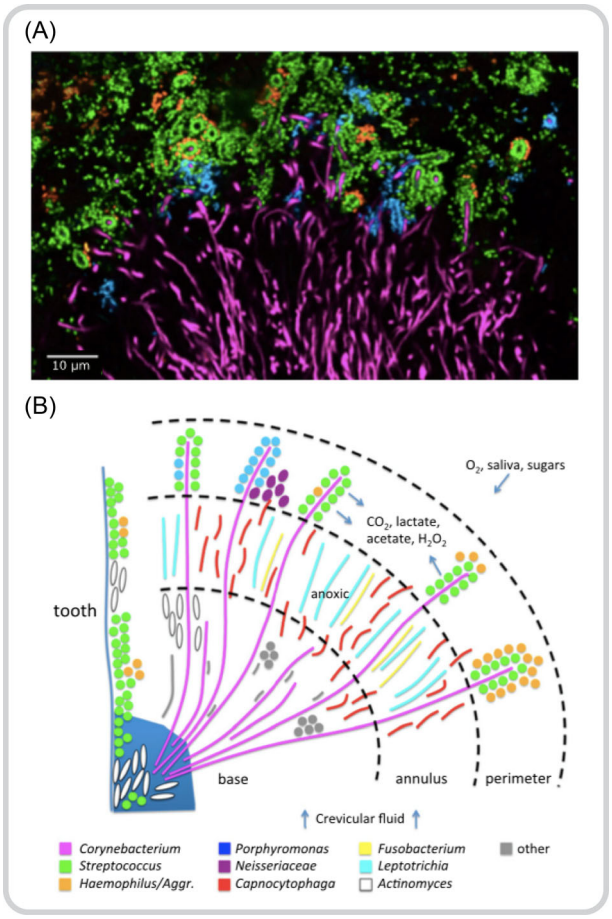

Figure 3. Localization of oral microbial taxa in dental plaque. (**A**) CLASI-FISH imaging of plaque microbial communities. (**B**) Schematic of the spatial distribution of bacteria based on CLASI-FISH images. (Reprinted with permission from Mark Welch *et al.* 2016.)

organisms in coculture, have been examined in detail. Pure-culture studies of *A. actinomycetemcomitans* determined that lactate is its preferred carbon source (Brown and Whiteley 2007). *Streptococcus gordonii* produces lactate as a by-product of its metabolism of carbohydrates such as glucose, fructose, mannose and galactose, commonly available carbon sources in the oral cavity (Tong, Zeng and Burne 2011). When cultured together, lactate production from *S. gordonii* promotes the growth of *A. actinomycetemcomitans* both *in vitro* and in a mouse abscess model (Brown and Whiteley 2007; Ramsey, Rumbaugh and Whiteley 2011). Lactate cross-feeding is not unique to this pair or oral microbes; lactate produced by *F. nucleatum* or other Streptococci can also support Veillonellae (Periasamy and Kolenbrander 2009). *Streptococcus gordonii* is not purely beneficial to *A. actinomycetemcomitans*; however, *S. gordonii* secretes hydrogen peroxide, an antimicrobial compound that prevents colonization of other microbes, at millimolar levels (Barnard and Stinson 1996, 1999; Liu *et al.* 2011). In response, *A. actinomycetemcomitans* induces the expression of a catalase encoded by *katA*, which detoxifies hydrogen peroxide, and is necessary for survival in the presence of high levels of this antimicrobial, via the transcriptional regulator OxyR (Thomson *et al.* 1999; Ramsey and Whiteley 2009). Expression of another gene in *A. actinomycetemcomitans*, *dspB*, is also induced by OxyR upon hydrogen peroxide exposure. *dspB* encodes a glycosyl hydrolase that is necessary for biofilm dispersal

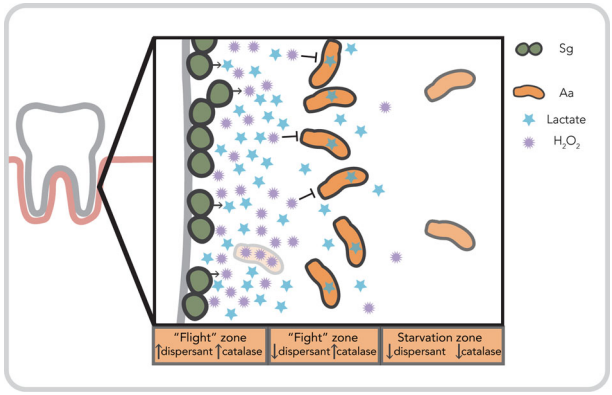

Figure 4. Model of *A. actinomycetemcomitans* (Aa) and *S. gordonii* (Sg) interaction in a mouse infection model. Sg adheres to the tooth pellicle surface and produces lactate, the preferred carbon source for Aa. Sg also produces hydrogen peroxide, which kills Aa at higher concentrations and induces production of biofilm dispersant by Aa ('Flight' zone). In the 'Fight' zone, catalase secreted by Aa detoxifies hydrogen peroxide effectively, and dispersant production is downregulated, allowing biofilm formation. In this zone, Aa has access to lactate. In the starvation zone, the lactate concentration is insufficient to promote growth of Aa (Stacy *et al.* 2014).

(Kaplan *et al.* 2003; Stacy *et al.* 2014). Thus, exposure to hydrogen peroxide produced by *S. gordonii* induces a 'fight or flight' response in *A. actinomycetemcomitans* in which it seeks out a new residence with less hydrogen peroxide while simultaneously working to detoxify its environment (Stacy *et al.* 2014). However, its reliance on *S. gordonii* for carbon means that *A. actinomycetemcomitans* cannot survive far from *S. gordonii* (Fig. 4). *Streptococcus gordonii* does not encode its own catalase enzyme, and in the mouse model, coinfection with the *A. actinomycetemcomitans katA* mutant resulted in lower viability of *S. gordonii*, leading to the interesting suggestion that this relationship is actually mutualistic in nature (Stacy *et al.* 2014). This example underscores the importance of culture-based studies and genetic manipulation in elucidating the intricate molecular details of a naturally evolved microbe–microbe interaction.

Marine ecosystems

The ocean is the largest ecosystem on our planet. Marine microbial communities account for approximately half of earth's photosynthetic biomass and the majority of carbon dioxide fixation, oxygen production and geochemical cycling of nitrogen (Field *et al.* 1998; Partensky and Garczarek 2010). Given that the microbial communities in the ocean are involved in fundamental processes that affect all of the planet's ecosystems, and that climate change is predicted to result in decreased diversity of marine phytoplankton, understanding the interactions between marine microbes is imperative to maintaining the health of our global environments.

Culture-independent, sequence-based approaches have uncovered immense microbial diversity in the oceans, including bacteria (Ferreira *et al.* 2014), fungi (Le Calvez *et al.* 2009) and viruses (Hurwitz, Brum and Sullivan 2015). A metagenomic analysis of 68 oceanic sites spanning all oceans except the Arctic identified a core set of functional gene families found across all sites. Additionally, temperature was identified as the most influential environmental factor on marine microbial community composition (Sunagawa *et al.* 2015). Using culture-independent approaches, over 35 000 operational taxonomic units (OTUs) were found in the ocean, and were predominantly comprised of the bacterial phyla Proteobacteria and Cyanobacteria (Sunagawa *et al.* 2015); the fungal phyla Chytridiomycota, Zygomycota, Glomeromycota, Basidiomycota and Ascomycota (Le Calvez *et al.* 2009); and the bacteriophage families Myoviridae, Podoviridae and Siphoviridae (Sullivan, Waterbury and Chisholm 2003; Hurwitz, Brum and Sullivan 2015).

Interestingly, substantial overlap between core functional genes of ocean microbiomes and human gut microbiomes has been observed (Sunagawa *et al.* 2015). Niche-specific gene functions have also been found; for example, the ocean microbiome has a higher abundance of genes for nutrient transport and energy production, while functions enriched in the gut microbiome included host immune evasion, carbohydrate transport and metabolism, and signal transduction (Sunagawa *et al.* 2015). This comparison highlights the immense taxonomic diversity of marine microbiota while revealing unexpected commonalities between the communities of two very different habitats.

Microbial interactions in the epipelagic zone

Cyanobacteria are the major microbial source of dissolved oxygen in marine ecosystems. These phototrophs are ubiquitous in areas that are penetrated by sunlight and are predominantly found in the upper layers (epipelagic zone) of the ocean (Partensky and Garczarek 2010; Walsh *et al.* 2015). Small both in cell and genome size, bacteria of the genus *Prochlorococcus* are the most abundant photosynthetic organisms in the ocean and are estimated to account for approximately 50% of the total chlorophyll (Partensky, Hess and Vaulot 1999; Partensky and Garczarek 2010). Remarkably, these tiny cyanobacteria fix four gigatons of carbon each year (Biller *et al.* 2015). Single-cell sequencing from environmental samples showed that hundreds of ecotypes of *Prochlorococcus* exist in marine environments, and that all share a set of core genes (Kashtan *et al.* 2014). These ecotypes can be distinguished from one another based on their accessory genes, which contribute to distinct physiologies (Johnson *et al.* 2006). Culture-dependent studies of some of these ecotypes show that they vary in their distribution across the water column. For example, ecotypes adapted to high light are located at shallower depths compared to low light adapted strains, and are affected by factors such as latitude and temperature (Johnson *et al.* 2006). These ecotypes also vary in their ability to grow in coculture with different heterotrophic partners (Sher *et al.* 2011). Although the mechanistic details of these interactions have not yet emerged, it is evident that interactions with heterotrophic microbes in their environment impact the ecology of this influential group of organisms.

Deep sea microbial interactions

In deep ocean environments, communities of microbes that reside in methane seeps are important for the biogeochemical cycling of methane, a potent greenhouse gas. The anaerobic methanotrophic archea (ANME) are responsible for at least 80% of methane consumption in the ocean (Reeburgh 2007). The methane oxidation pathway in ANME is believed to be a reversal of the pathway used by methanogenesis archaea to produce methane (Hallam *et al.* 2004). ANME rely on syntrophic interactions with SRB partners for consumption of the electrons generated by methane oxidation. FISH studies revealed that in some consortia a spherical aggregate of ANME is surrounded by an outer layer of SRB, while other consortia have a mosaic distribution of the two partners (Fig. 5A, B, D and E) (Strous and Jetten 2004; Knittel *et al.* 2005; Dekas, Poretsky and Orphan 2009; Vigneron *et al.* 2013).

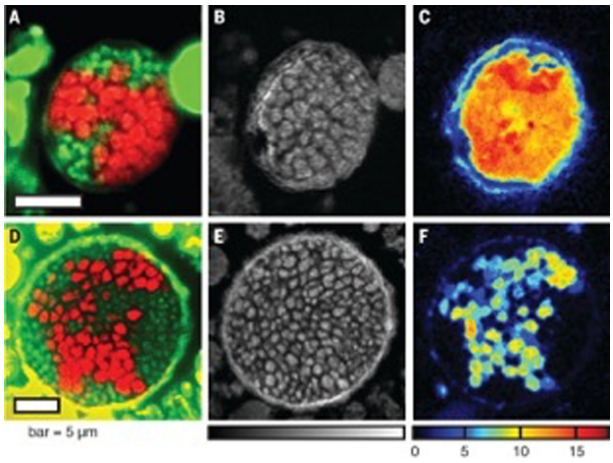

Figure 5. Imaging and metabolic profiling of ANME/SRB aggregates treated with $^{15}N_2$. (**A–C**) ANME/SRB consortium with sulfate. (**D–F**) ANME/SRB consortium with AQDS as the sole electron acceptor. (A and D) FISH images highlighting AMNE in red and Desulfobacteriaceae in green. (**B** and **E**) NanoSIMS detection of $^{12}C^{14}N$, indicating total cellular biomass. (C and F) NanoSIMS detection of ^{15}N. (Reprinted with permission from Scheller *et al.* 2016.)

The ability to conduct laboratory manipulations of these organisms is hampered by their metabolic codependence which, together with their long generation time (2–7 months), is a barrier to culturing them individually. These constraints on traditional microbiological methods of probing the interactions among ANME and SRB inspired the development of numerous new culture-independent tools, some of which are described in Box 1. One such approach, termed Magneto-FISH, enabled the capture and sequencing of ANME/SRB consortia and showed the presence of bacterial members of the Betaproteobacteria (in the Burkholderiaceae) and Alphaproteobacteria (related to *Sphingomonas*) (Pernthaler *et al.* 2008). Metagenomic analysis of these consortia also revealed the presence of nitrogenase genes necessary for nitrogen fixation in ANME (Meyerdierks *et al.* 2010). Further metabolic studies showed that $^{15}N_2$ was incorporated into the biomass of ANME/SRB consortia (Pernthaler *et al.* 2008). FISH-nanoSIMS imaging of the same aggregates showed that ^{15}N was incorporated at high levels into the ANME and could also be detected in the SRB (Dekas, Poretsky and Orphan 2009).

Although the ANME and SRB cells in these consortia are found in an ordered arrangement, it is possible to decouple them metabolically. Previous studies suggested that the intimate spatial association between the two organisms supports direct electron transfer from ANME to SRB (Meyerdierks *et al.* 2010; McGlynn *et al.* 2015; Wegener *et al.* 2015). In support of this proposed interaction, methanotrophy could be decoupled from sulfate reduction by the addition of the artificial oxidant 9,10-anthraquinone-2,6-disulfonate (AQDS). In consortia treated with AQDS, ANME incorporated ^{13}C-methane into dissolved inorganic carbon independently from sulfate reduction activity, indicating that direct electron transfer normally supports methane oxidation by ANME (Scheller *et al.* 2016). Interestingly, FISH-nanoSIMS analysis showed that fixed nitrogen produced by ANME was no longer found in SRB when electron transfer was decoupled (Scheller *et al.* 2016) (Fig. 5C and F). Thus, the use of multiple functional imaging approaches in conjunction with sequencing and metabolic perturbations has led to the identification of electron transfer and cross-feeding of fixed nitrogen as two key mechanisms of metabolic interaction in this interdomain association.

Unifying principles that shape microbial communities

Though the microbial communities discussed above are distinct from one another in their metabolism, phylogenetic composition and habitat, they are not so unique in the metabolic and physiological 'rules' that they follow. One unifying theme is the division of labor in metabolic processes; rather than single organisms interacting with their environment and fulfilling all of their metabolic needs alone, microbes have distinct roles that contribute to the metabolism of the community. For instance, general patterns are apparent in the flow of carbon across ecosystems; in all cases, a carbon input is converted to carbon intermediates by a primary group of microbes, and these intermediates are further processed to downstream products by other groups of microbes. Furthermore, the majority of sequenced microbes lack some of the biosynthetic pathways for metabolites that they require, underscoring the interdependence of microbes in their environments. It is perhaps because of these interdependencies that microbial communities assemble reproducibly in a given environmental condition. The ecological principles and mechanisms of community assembly remain an ongoing question that can be addressed by examining model ecosystems. For example, the microbiome of cheese rind is a promising emerging model community that assembles reproducibly *de novo* in a laboratory setting (Wolfe *et al.* 2014).

DISSECTING FUNDAMENTAL PRINCIPLES OF COMMUNITY STRUCTURE

The examples described above illustrate the power of combining whole-community analyses such as metagenomics with culture-dependent studies of individual microbes and consortia for understanding the interactions between microbes that contribute to community structure and function. Despite these remarkable advances in technology and knowledge, we are still far from being able to predict the majority of functional interactions between microbes. It is apparent that the establishment of a microbial community through microbe–microbe and microbe–environment (or microbe–host) interactions is encoded in the collective genome sequences of the community members. We now are no longer limited by the ability to generate sequence data for any organism or community, but we are still largely ignorant about how to 'decode' these sequences to understand the suite of interactions that drive the assembly and stability of a microbial community. In this section, we describe recent efforts to decode the relationship between genome sequence and community behavior by examining metabolic connections between organisms and identifying the functions of unknown genes involved in interspecies interactions. We additionally describe synthetic biology approaches that allow researchers to dissect specific, often artificial interactions in such detail that predictive mathematical models of the interactions can be constructed. By dissecting such mechanisms, we do not focus on a particular community of interest; instead, we gain valuable insights into the mechanisms of interactions that apply to many different systems.

The corrinoid model for decoding microbial interactions

The era of facile genome sequencing has brought about a fundamental shift in our ability to understand the metabolic needs of microbes. It is now possible to analyze putative nutritional interactions between organisms based on metabolic pathway predictions, as was highlighted in the examples described in

the previous section. However, such predictions are only possible for known genes and pathways, and the mechanistic details of known interactions cannot be inferred based on sequence. Here, we present an example from our own research of an approach that holds promise for decoding genome sequences to uncover the mechanisms of a particular type of metabolic interaction.

Developing model systems to study specific interactions in detail has proven fruitful in understanding the mechanisms of microbial interactions. One such model metabolite is the cobalamin (vitamin B_{12}) family of cofactors, collectively termed corrinoids. Genome sequence analysis clearly indicates that corrinoids are shared metabolites; based on bioinformatic predictions, over 70% of sequenced bacterial species use corrinoids, while less than 25% synthesize them *de novo* (Zhang *et al.* 2009; Degnan *et al.* 2014). Nearly all of the genetic, biochemical and structural studies of corrinoids to date have focused on a single type of corrinoid, cobalamin (also known as B_{12}), because it is thought to be the only corrinoid that humans can absorb and use as a cofactor (Seetharam and Alpers 1982) (Fig. 6A). However, it has long been known that over a dozen structurally distinct corrinoids with variation in the lower axial ligand are produced and used by various bacteria and archaea (Fig. 6B) (Renz 1999). Although the ecological significance of these structural differences is not known, it is clear that the structure of the lower ligand influences the degree to which a particular corrinoid can be used by a given organism (Mok and Taga 2013; Men *et al.* 2014) (Box 3). Because of this specificity and the fact that corrinoids are shared metabolites, corrinoid cross-feeding can be used as a model for molecular specificity in interspecies metabolic interactions (Seth and Taga 2014). The corrinoid model may contribute to the understanding of other interactions such as cross-feeding of amino acids and other primary metabolites, the use of siderophores to compete for iron or the production of secondary metabolites that influence growth or behavior of other organisms. Moreover, in addition to their utility as a model for microbial interactions, corrinoids impact diverse areas of biology because numerous metabolic pathways in bacteria, archaea, protists and animals rely on corrinoid cofactors (Roth, Lawrence and Bobik 1996; Ryzhkova 2003).

Box 3. Corrinoid structure and function.

- Corrinoids are modified tetrapyrroles that contain a cobalt center. The corrinoid biosynthetic pathway, which involves approximately 30 genes, diverges from a pathway common to other tetrapyrrolic cofactors such as chlorophyll and heme (Warren and Scott 1990; Roth, Lawrence and Bobik 1996).
- The cobalt ion can coordinate an upper and lower axial ligand. In most corrinoid-dependent enzymes, catalysis is achieved by fissure of the cobalt-carbon bond to the upper ligand (labeled as R in Fig. 6). Corrinoids can facilitate radical-mediated reactions (R = 5′-deoxyadenosine) or methyl group transfers ($R = CH_3$), as well as reductive chemistry that proceeds in the absence of a particular upper ligand (Roth, Lawrence and Bobik 1996; Matthews 2009).
- Sixteen corrinoids with structural variability in the lower ligand have been described (Renz 1999). The lower ligand is covalently tethered via the nucleotide loop, and most can coordinate to the cobalt ion (Roth, Lawrence and Bobik 1996; Matthews 2009). Corrinoids with a nucleotide loop lacking the C177 methyl group have also been observed. (Kräutler *et al.* 2003)
- Corrinoids are synthesized only by a subset of prokaryotic species (Roth, Lawrence and Bobik 1996). Most corrinoid-producing organisms produce only a single type of corrinoid (that is, with one specific lower ligand) (Renz 1999).
- Corrinoids are cofactors for mutases, methyltransferases, isomerases, ribonucleotide reductases and reductive dehalogenases. Corrinoid-dependent enzymes function in the utilization of propanediol, ethanolamine and other carbon and nitrogen sources; degradation of certain amino acids, odd chain fatty acids and cholesterols; biosynthesis of methionine, deoxynucleotides and antibiotics; tRNA modification; mercury methylation; acetogenesis; methanogenesis; and halogenated solvent degradation (Matthews 2009).
- Corrinoids with different lower ligands are not functionally equivalent; an organism can only use a subset of the corrinoids that may be present in its environment (Tanioka *et al.* 2010; Yi *et al.* 2012; Mok and Taga 2013).

Significant progress has been made in decoding corrinoid production by identifying and characterizing genes required for the biosynthesis and incorporation of different lower ligands (Table 1). Identifying the genes involved in lower ligand biosynthesis was a key component of decoding corrinoid production. The genes required for the biosynthesis of 5,6-dimethylbenzimidazole (DMB), the lower ligand of cobalamin, were the last components of the cobalamin biosynthetic pathway to be identified (Campbell *et al.* 2006; Hazra *et al.* 2015). The discovery of the *bluB* gene encoding the oxygen-dependent DMB synthase provided a useful marker gene for the biosynthesis of cobalamin in organisms that reside in oxygen-containing environments. The *bluB* gene was initially identified in *Rhodobacter capsulatus* as a factor necessary for the production of photosynthetic pigments, a process that requires cobalamin as a cofactor (Pollich and Klug 1995). A *bluB* mutant of *Sinorhizobium meliloti* was later isolated based on its inability to fix nitrogen in association with plant root nodules, and the function of the BluB enzyme as a 'flavin destructase' that catalyzes the biosynthesis of DMB from flavin mononucleotide was subsequently determined (Campbell *et al.* 2006; Gray and Escalante-Semerena 2007; Taga *et al.* 2007).

The recent discovery of the *bzaABCDE* operon, which encodes the anaerobic biosynthetic pathway for DMB, makes it possible to predict DMB production in anaerobic microbes (Hazra *et al.* 2015). Identifying the *bzaABCDE* genes additionally made it possible to predict the biosynthesis of three other corrinoids, as subsets of this operon are involved in the biosynthesis of other lower ligands that are intermediates on the pathway to DMB (Hazra *et al.* 2015). In contrast to *bluB*, which was discovered serendipitously by researchers studying other biological processes, the *bzaABCDE* genes were first identified by a targeted bioinformatic search of the genome of *Eubacterium limosum*, and the functions of these genes were subsequently elucidated by genetic and biochemical approaches (Hazra *et al.* 2015; Mehta *et al.* 2015).

Unlike benzimidazoles, purines and phenolic compounds have cellular functions apart from their roles as corrinoid lower ligands, and therefore the production of these corrinoids cannot be predicted based solely on the biosynthetic genes for

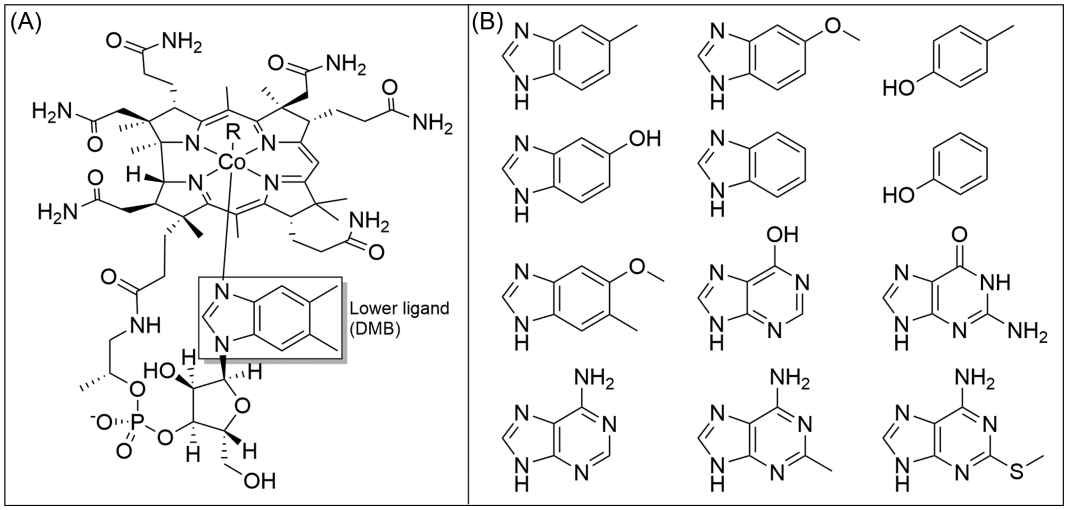

Figure 6. Corrinoid structure. (**A**) The structure of cobalamin is shown with the lower ligand, DMB, boxed. *R* represents the upper ligand (see Box 3). (**B**) Structures of commonly detected lower ligand bases.

Table 1. Marker genes for corrinoid biosynthesis.

Gene	Function	References
hemALBCD cysG cobAIGJMFKLHBNSTOQDCPUV	Corrinoid biosynthesis (aerobic pathway)	Warren *et al.* (2002)
hemALBCD cysG cbiKXLHGFDJETCAPB cobADUTSC	Corrinoid biosynthesis (anaerobic pathway)	Warren *et al.* (2002)
bluB	Cobalamin (DMB lower ligand; aerobic)	Campbell *et al.* (2006); Gray and Escalante-Semerena (2007); Taga *et al.* (2007)
bzaABCDE or bzaFCDE	Cobalamin (DMB lower ligand; anaerobic)	Hazra *et al.* (2015)
bzaABCD or bzaFCD	5-Methoxy-6-methylbenzimidazolyl cobamide	Hazra *et al.* (2015)
bzaABC or bzaFC	5-Methyxybenzimidazolyl cobamide	Hazra *et al.* (2015)
bzaAB or bzaF	5-Hydroxybenzimidazolyl cobamide	Hazra *et al.* (2015)
arsAB	Phenolyl cobamide or *p*-cresolyl cobamide	Chan and Escalante-Semerena (2011)
Absence of *bluB*, *bzaAB* (or *bzaF*) and *arsAB*	Pseudocobalamin (likely)	Crofts *et al.* (2013)

their lower ligands. Instead, substrate specificity in the CobT enzyme, which catalyzes the phosphoribosylation of a lower ligand base prior to incorporation into a corrinoid, can provide clues about the specific corrinoid produced by an organism (Trzebiatowski and Escalante-Semerena 1997; Cheong, Escalante-Semerena and Rayment 1999, 2001, 2002). For example, specific amino acids in the active site of CobT may be markers for the incorporation of adenine (Cheong, Escalante-Semerena and Rayment 2001; Crofts *et al.* 2013; Hazra *et al.* 2013). Additionally, distinct sequences in the *cobT* homologs *arsA* and *arsB*, which are found in bacteria of the *Veillonellaceae* family, direct cells to incorporate phenolic compounds as corrinoid lower ligands (Chan and Escalante-Semerena 2011; Newmister *et al.* 2012). Despite these advances, our ability to predict specificity in corrinoid production is limited by the potential for cells to take up and incorporate lower ligand bases produced by other organisms from the environment (Crofts *et al.* 2013, 2014; Hazra *et al.* 2013). Thus, the potential for lower ligand cross-feeding makes it impossible to rely solely on genome sequence to predict function. However, corrinoid specificity occurs not only at the level of biosynthesis, but also in corrinoid transporters (Degnan *et al.* 2014), in enzymes involved in using corrinoids or modifying them intracellularly (Gray and Escalante-Semerena 2009; Chan and Escalante-Semerena 2011; Mok and Taga 2013) and in proteins or RNA elements that regulate corrinoid-related functions (Fig. 7) (Gallo *et al.* 2008). Thus, examining the relationships between sequence and the structures of preferred corrinoids in all of these elements may make it possible to predict corrinoid preferences on a whole-genome level.

Dissecting microbial interactions by establishing synthetic cocultures coupled with mathematical modeling

Synthetic biology approaches have revealed insights into the detailed mechanisms of metabolic interactions between microbes. Although synthetic approaches use artificial systems in which

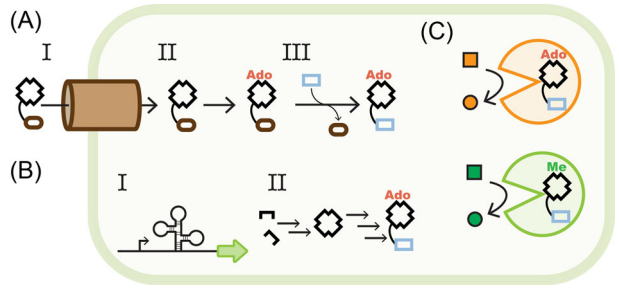

Figure 7. Decoding corrinoid specificity. (**A**) In corrinoid-requiring organisms, specificity for particular corrinoid structures can be encoded in (I) corrinoid transporters, (II) corrinoid adenosyltransferases (Ado represents 5′-deoxyadenosine) and (III) corrinoid remodeling enzymes. (**B**) In corrinoid-producing organisms, specificity can be encoded in (I) the regulation of corrinoid-related genes (e.g. in corrinoid-binding riboswitches) and (II) the corrinoid biosynthetic pathway. (**C**) Corrinoid-dependent enzymes may also be specific for particular corrinoids.

strains are genetically engineered to interact, such controlled systems are often necessary to understand metabolic interactions in sufficient detail to allow mathematical modeling of metabolite exchange.

Creating an obligate synthetic coculture sounds simple in principle, yet it is not as easy as mixing two microbes together. Often the culture is dominated by one microbe because the pair is unable to interact productively. A recent study overcame this challenge by building a coculture based on the exchange of metabolites that are naturally excreted, as identified by exometabolomics, a technique that measures the excreted metabolome (Kosina *et al.* 2016). In that study, metabolites excreted by *Zymomonas mobilis* were identified by exometabolomics, and auxotrophic strains of *Escherichia coli* that depend on these metabolites were constructed. To generate *Z. mobilis* strains that depend on *E. coli* for growth, a forward genetic screen was conducted to identify mutants that have a fitness benefit when cultured with *E. coli*. The construction of stable cocultures of genetically modified strains of the two species confirmed the potential of this approach (Kosina *et al.* 2016).

Mathematical modeling in combination with experimental studies can describe and predict metabolic interactions in microbial communities. For example, flux balance analysis is used to calculate the movement of metabolites based on the known metabolic pathways and enzymes encoded within them, providing information on the abundances of organisms and the metabolic role they serve in their communities (Orth, Thiele and Palsson 2010). An optimized flux balance algorithm was used to predict the relative abundances of bacteria in a three-member synthetic consortium containing *E. coli*, *Salmonella enterica* and *Methylobacterium extorquens* (Harcombe *et al.* 2014). Interestingly, this model successfully predicted that the slowest growing organism, *M. extorquens*, would dominate the triculture because the faster growing organisms (*E. coli* and *S. enterica*) were engineered to be dependent on it for nitrogen.

Synthetic approaches are also used to test and predict metabolic interactions that rely on physical contact and spatial patterning of microbes. In a recent study, 'cooperator' strains of *Acinetobacter baylyi* and *E. coli* were generated which each strain overproduced the amino acid that the other partner required, and partner strains could reciprocally exchange essential amino acids when grown in coculture (Pande *et al.* 2015). This interaction was contact dependent, and nanotubular structures between cells were observed when the pair of microbes was forced

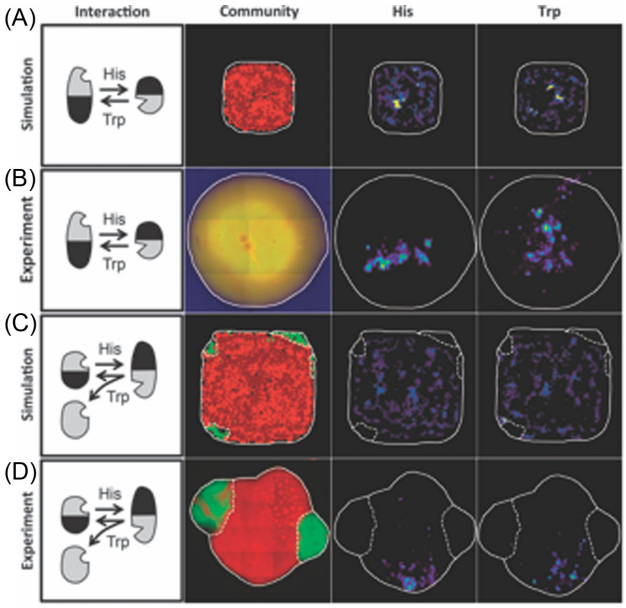

Figure 8. Theoretical model and experimental evidence for spatial heterogeneity of cooperators and non-cooperators. (**A**) Modeling simulation and (**B**) growth experiment of two 'cooperator' strains (labeled with red and green fluorescent proteins, resulting in yellow color) indicate a high degree of intermixing. (**C**) Modeling simulation and (**D**) growth experiment of the two 'cooperator' strains (red) and a 'non-cooperator' strain (green) indicate spatial segregation of non-cooperating bacteria. MALDI-TOF MS analysis of histidine and tryptophan concentrations in colonies illustrates the exclusion of these molecules from non-cooperator regions (reprinted with permission from Pande *et al.* 2016).

to share amino acids (Pande *et al.* 2015). A mathematical model generated for this coculture predicted that cooperatively interacting strains would be spatially well mixed (Fig. 8A). This prediction was verified experimentally, as strains marked with different fluorescent labels were observed to colocalize (Fig. 8B). When non-cooperator strains that do not overproduce amino acids were added to pairs of cooperating bacteria, the model predicted that non-cooperating bacteria would become spatially segregated from the cooperating strains (Fig. 8C). This too was verified experimentally (Fig. 8D). Spatially resolved matrix-assisted laser desorption ionization/ time of flight (MALDI-TOF) mass spectrometry imaging of bacterial cocultures showed that cooperator-rich regions had higher concentrations of the shared amino acids, while these nutrients were sequestered away from non-cooperators (Fig. 8D). Together, these results highlight that spatial structure is an important factor in cooperative and competitive interactions (Pande *et al.* 2016). The ability to generate predictive models of these community behaviors demonstrates the relative simplicity of engineered interactions and is a step toward modeling more complex behaviors in natural settings.

MODULATING MICROBIAL COMMUNITIES

As we gain new insights about microbial interactions, promising handles for modulating communities emerge. Recent efforts have focused on developing new techniques to modulate the composition of microbial communities. For example, one report demonstrated that introducing an *Escherichia coli* strain that overproduces the quorum-sensing autoinducer AI-2 into an antibiotic-treated humanized mouse gut community results in a dramatic alteration in community composition (Thompson *et al.* 2015). Additionally, corrinoids may be used to manipulate

microbial communities; given that corrinoid composition can vary with community composition and metabolism (Men *et al.* 2014), it may be possible to take advantage of the distinct corrinoid specificity profiles of different organisms to modulate community metabolism or composition using corrinoids (Degnan *et al.* 2014; Seth and Taga 2014). More precise tools for 'editing' communities have also been developed, such as lytic phage or CRISPR-Cas systems, that can be programmed to target specific bacterial taxa (Bikard *et al.* 2014; Citorik, Mark and Timothy 2014; Gomaa *et al.* 2014; Ando *et al.* 2015). With these new technologies and more on the horizon, we are now poised to make exciting advances in understanding and manipulating microbial communities that impact human, crop and environmental health.

CONCLUSIONS AND FUTURE OUTLOOK

With a planet experiencing ecological shifts due to climate change and a suite of human ailments linked to alterations in the human gut microbiota, the need for new tools for manipulating microbial communities to restore ecological balance is becoming increasingly urgent. Numerous decades dedicated to the molecular genetic analysis of the best studied microbe, *Escherichia coli*, have resulted in the elucidation of the functions of only half of its genes; how, then, is it be possible to understand a community comprised of thousands of distinct, unknown organisms? Here, we have argued that a combination of approaches is needed that span whole-community analysis, studies of interactions in defined consortia and monoculture studies, incorporating gene discovery, engineered interactions and mathematical modeling to dissect interactions between microbes. Multiple layers of analysis are required because each provides distinct information. Culture-independent studies of complete communities provide metabolic, taxonomic and structural information of the whole community but lack mechanistic detail, while studies of single organisms or defined consortia provide needed mechanistic information for only a small fraction of the community. Additionally, decoding the mechanisms of interactions by investigating model metabolic interactions and synthetic biology approaches could provide generalizable paradigms applicable across systems. A greater understanding of microbial community interactions will be key to developing therapies that modulate community composition and metabolism.

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