

Metabolic interactions in microbial communities: untangling the Gordian knot

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Metabolic exchanges are ubiquitous in microbial communities. However, detecting metabolite cross-feedings is difficult due to their intrinsically dynamic nature and the complexity of communities. Thus, while exhaustive description of metabolic networks operating in natural systems is a task for the future, the battle of today is divided between detailed characterizations of small, reduced complexity microbial consortia, and focusing on particular metabolic aspects of natural ecosystems. Detecting metabolic interactions requires methodological blend able to capture species identity, dependencies and the nature of exchanged metabolites. Multiple combinations of diverse techniques, from metagenomics to imaging mass spectrometry, offer solutions to this challenge, each combination being tailored to the community at hand.

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Introduction

Microbial communities are intertwined by metabolic links, whether viewed as narrowly as a pair of symbionts, or as broadly as the earth-wide ecosystem lined up with trophic chains. Understanding metabolic interactions at the global level is thus indispensable in microbial ecology and evolution. However, shifting attention from isolated metabolism of pure cultures to that of microbial communities is challenging and requires new tools and methods. And, as in the case of any complex network, when choosing a focus point in the large web of metabolic interactions, we have to compromise between resolution of detail and coverage.

Seeing microbial metabolism in the community context (as opposed to pure cultures) reveals new phenotypes [1•], helps designing synthetic communities for biotechnology [2,3],

and enables cultivating the ‘uncultivables’ [4]. Accumulating examples of metabolic cross-feeding [5,6•] and evidence from metabolic modeling [7] create an anticipation of many more to be discovered. Within the broad range of metabolic interactions, here we concentrate primarily on nutrient exchange. We aim to show how studying complex communities is shifting the paradigm of microbial metabolism and what methods and challenges await for those trying to disentangle inter-species connections.

Metabolite exchanges provide group advantage

Multiple studies show that metabolite exchanges form a strategy for group success [6•,8–11]. Metabolic interactions frequently contribute, through division of labor, to the emergent abilities at community level, such as biodegradation [12,13], faster growth [10] or increased virulence [9,14]. Outsourcing metabolic functions to fellow members embeds each pathway in a specialized micro-environment, hence avoiding biochemical conflict [15]. Moreover, under nutrient-poor conditions species can be readily prompted to share metabolites and thus complement each other’s biosynthetic capabilities [16,17••,18]. Metabolic specialization can be found even within the same species, for example, filamentous cyanobacteria with specialized heterocyst cells for nitrogen fixation [19].

Despite benefits associated with cross-feeding, its evolution remains controversial, especially in case of metabolic cooperation [20,21]. Emergence and maintenance of metabolic exchanges depends on particular circumstances, such as spatial structure of microbial community, nutrient availability, diffusion constraints and cost effectiveness of concerned biosynthetic processes [22–24]. For example, aggregating or forming a biofilm maximizes efficiency of nutrient transfer and stimulates otherwise thermodynamically unfavorable metabolic processes [25]. In extreme cases, metabolic dependency results in endosymbiotic relationship, a popular solution for hydrogen-producing ciliates that harbor methanogenic archaea for H₂ outflow [26].

Microbial metabolism is plastic and responsive to social cues

Microorganisms can often utilize and secrete a large number of metabolites [27,28]. This plastic network is readily adapted and regulated in response to nutrients, for example, to optimize resource allocation [29,30], but also in response to cues from other microorganisms [31•]. Certain bacterial species can modulate yeast metabolism,

to reduce secretion of toxic ethanol, by deploying chemical signaling [1•]. Transcriptional response of *Streptococcus* species shows metabolic adaptations to other members of community [32].

Discovering metabolic interactions

Meta-omics analyses guide interaction discovery

Meta-omics technologies are culture independent and scalable in space/time. Metagenomics is a particularly powerful tool for discerning species identity and for detecting patterns of interspecies associations. These in turn can generate verifiable hypotheses about metabolic (and other) interactions between community members. Genotyping of associated microbes can reveal their functional palettes [33] and task distribution among community members [12]. For example, individual genomes of a co-aggregated pair of archaea showed that one of the symbionts is dependent on another for lipid, cofactor, amino acid, and nucleotide biosynthesis [34]. Following a specific community over time can also reveal metabolic dependencies as one species dynamically responds to change in abundance of the other, as shown in an activated sludge community [35]. Overlaying taxonomic data with other information, such as spatial distribution and geochemical profiles [36] or specific enzymatic function [37], can deepen insight into community co-metabolism. Beyond individual communities, metagenomics has allowed the identification of species co-occurrence structure across different habitats/samples [38,39]—associations that hint at interspecies interactions [7•,40].

Transcriptomics and proteomics are commonly used to complement metagenomics, to deduce what genome encoded metabolic potential is being used [41••,42]. For instance, analysis of transcriptional patterns in co-culture of a marine bacterium and a diatom, as well as ocean samples, pinpointed cross-feeding of 2,3-dihydroxypropane-1-sulfonate, a new link in marine microbial food web [41••]. Metabolic applications of meta-proteomics are more commonly used for relatively simple systems—it was used to demonstrate metabolic adjustments made by three species comprising a model oral biofilm [43] or to show how the presence/absence of *Aggregatibacter actinomycetemcomitans* modulates metabolism of other bacteria in a 10-species biofilm [44]. Although not distinguishing between species, these results give a sense of the complexity and scale of metabolic adjustments that happen in ‘real-world’ communities. On a larger scale, meta-proteomics, in combination with meta-genomics, allowed proposing differential flow of nitrogen, sulfur and hydrogen among the abundant taxa of marine microbial communities in response to oxygen availability [45].

Isotope labeling for tracing community-scale pathways

Tracing of isotope labeled substrates, a standard approach in pathway discovery, can also be adapted to reveal flow of metabolites in microbial consortia. Although this is the

most conclusive method for showing metabolite exchange, the major challenge is to distinguish labeling fingerprints of different populations. To do so, one can use an artificially expressed reporter protein [46], species-specific peptides [47], or detect labeled DNA or RNA in conjunction with metagenomics analysis [48]. To give some examples, ¹³C labeling served to experimentally prove bacterial feeding on fungal exudates [49], to suggest a chain of toluene degraders in methanogenic enrichment culture [13] and to identify key naphthalene-degrading bacteria *in situ* [50].

Imaging community structure—clues from the neighbors

Efficient mass transfer between organisms is a prerequisite of successful metabolic interaction, therefore it is not uncommon for microbial partners to form tight aggregates and develop special structures that facilitate metabolite exchange. Microscopic detection of these structures can be a powerful tool in identifying interacting microorganisms. Illustrative is an example of nanotubes formed by cross-feeding *Escherichia coli* auxotrophs [51] or variety of formations in acid mine drainage community, such as cytoplasmic bridges, pili, and ‘synaps like connections’ [52].

Fluorescence *in situ* hybridization (FISH) based methods reveal spatial distribution of interacting partners, for instance showing stratification and co-aggregation patterns in biofilms [53] or bacterial groups attached to phytoplankton host [54]. In addition to resolving spatial structure, imaging, for example, based on fluorescent dyes, can be used to assess general metabolic state of community members [55,56•].

Exploration using metabolomics

Mass spectrometry (MS) based methods can detect a broad spectrum of compounds and are being developed rapidly. This technique has a wide range of modifications, varying in application from a single cell to multiple colonies on a petri dish (reviewed by Watrous *et al.* [57]). Interestingly, MS can be used in an imaging set-up to study metabolic interactions [58]. The potential of imaging-MS unfolded, for example, in a study of chemical interactions on actinomycete bacteria, showing interactions through spectra of secondary metabolites [59•]. Application of MS to microbial interactions is, however, currently limited by various challenges in data analysis and compound identification [1•,59•,60,61]. Other methods that can facilitate interrogation of metabolic space of the community are reviewed by Maurice *et al.* [56•] and Wessel *et al.* [62•].

Metabolomics alone usually does not provide sufficient resolution to pinpoint exchanged molecules. Elucidating cross-feeding in a complex nutritional environment is possible only in combination with other techniques such

as stable isotope labeling and FISH. Such methodological blend allowed detecting nitrogen transfer from cyanobacteria to their symbiotic diatoms [63] or from methane-oxidizing archaea to sulfate-reducing bacteria in marine seeps [64,65]. Several examples of metabolic interactions detected through combination of different methods are described in Table 1.

Synthetic communities as model systems

While natural consortia are still difficult to scrutinize, enrichment cultures offer a compromise between natural and synthetic communities. These are cultures obtained from natural samples by promoting growth of organisms of interest, typically by manipulating medium composition. Synthetic microbial communities provide further reduction in the complexity, creating a more tractable system for discovering metabolic exchanges [66,67]. Communities constructed with the isolates from the same environment maximize resemblance to the natural community and preserve indigenous interactions shaped by co-adaptation/evolution [68].

The pre-requisite for common history of member species can be relaxed when addressing fundamental questions like emergence and evolution of metabolic interactions [17^{••},69]. To this end, one might also turn to engineered dependencies through genetic manipulation and/or laboratory evolution [16,18,70[•],71]. Despite being less ‘natural’, engineered interactions have the obvious advantage of knowing the identity of the transmitted metabolite (or at least of the involved pathways), as well as being easier to obtain, monitor, and control. Engineered communities are most common object to study synergistic growth effects of metabolic cross-feeding [10,70[•]].

Another group of model systems for microbial interactions emerge from microbiota of fermented food [72]. These associations typically have reduced complexity compared with most environmental or host-associated systems, and can be grown in well controlled environment without loss of tractability, for example, cheese rinds [73]. Spatial organization, species succession, stability, resilience, and co-evolution history, such as those of water and milk kefir grains [74,75], create a rich ground in the search for metabolic interaction mechanisms.

Divide and conquer through temporal/spatial compartmentalization

Using a defined assembly of microorganisms opens opportunities to employ methods inapplicable to complex systems. For example, species quantification can be done with selective plating, quantitative PCR or flow cytometry. However, for better control over metabolite production and consumption, as well as for discerning metabolic roles of different populations, modification to mixed cultures can be made. One of the simplest techniques is based on the cell-free culture filtrate — the so-called

conditioned or spent medium. This approach is frequently used to assay activity of secretome of the donor microorganism(s) by adding its conditioned medium to the recipient culture. This allows identifying non-induced dependencies such as an interaction network between seven gut symbionts knitted by polysaccharide degradation products [76].

Other approaches try to preserve real time molecule diffusion between species, but keep symbionts physically separated, for example, by means of a semi-permeable membrane [68], structuring their microenvironment in a microfluidics device [77], encapsulating cells in hydrogels [78], or co-culturing in a Petri dish [79]. Artificial barriers provide better control over conditions and more convenient quantification, separation and analysis of interacting populations, also in a high-throughput manner [80]. It is important to note that the co-culture conditions can have a profound impact on community metabolism [81] and hence caution is warranted when extrapolating the conclusions to other contexts.

In silico hypothesis generation

Mathematical models of community metabolism are expanding the toolbox for discovering metabolic dependencies in microbial communities [82–84]. Although still in development, community models hold a distinct appeal due to broad applicability and scalability to the ecosystem level [85]. For identifying potential exchanged metabolites, steady-state models are of particular interest as these can be applied with as little information as the identity of community members and their genome sequences [7[•]]. These can be further extended, albeit for small communities and with additional information on the metabolic physiology of the community and its members, to address more complex problems such as community dynamics [86].

Community models so far have been largely devoted to understanding general principles of community structure [24,83,87–89], but also have accurately captured experimentally observed metabolic dependencies [7[•],18,89]. The next frontier for the models will be to provide hypotheses verifiable with the experimental approaches discussed above. In particular, models hold a great potential to suggest cross-feeding scenarios and thus to narrow down the set of metabolites to be tested.

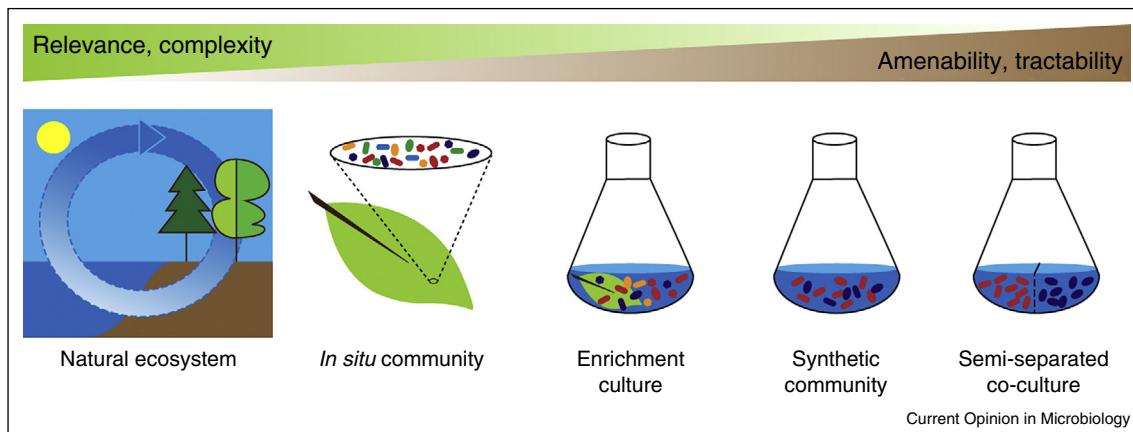
Untangling the Gordian knot

Enumerating metabolic exchanges, being difficult even for small communities, becomes overwhelming for natural communities with hundreds of species living in fluctuating environment. One of the main underlying reasons for this difficulty is that metabolites cannot be directly attributed to a particular species or abiotic source. Furthermore, a large fraction of microbial diversity still remains largely undiscovered or uncharacterized for their

Table 1**Examples of metabolic exchanges in microbial communities**

Community type	(Eco-) system	Interacting taxa	(Potentially) exchanged metabolite(s)	Methods used to detect/infer			Reference
				Species identity	Inter-species dependency	Exchanged metabolite(s)	
Natural	Anoxic marine sediments	<i>Thioploca</i> (sulphur-oxidizing bacteria), anaerobic ammonium-oxidizing bacteria	NH ₄ , NO ₂	FISH ^a , 16S rDNA sequencing	FISH analysis of spatial association	Inference from N isotope distribution	[90*]
Natural	Deep-sea sediments	ANME-2 archaea group (anaerobic methane-oxidizing archaea), <i>Desulfosarcina/Desulfococcus</i> (sulfate-reducing bacteria)	Reduced N species	FISH	Observed co-aggregation, previously described syntrophic relationship	FISH-coupled nanoSIMS ^c showing ¹⁵ N incorporation across aggregates	[64]
Natural	Ocean plankton	<i>Thalassiosira pseudonana</i> (diatom), Roseobacter clade bacteria	2,3-Dihydroxypropane-1-sulfonate (DHPS)	Metatranscriptome analysis, fractionation of marine biomass	Metabolic exchanges in model bacterial-phytoplankton system	Metatranscriptome analysis, targeted MS metabolomics of the eukaryotic plankton size fraction	[41**]
Enrichment culture ^b	Alkane-degrading methanogenic community	<i>Smithella</i> (bacteria); <i>Methanosaeta</i> and <i>Methanocalculus</i> (methanogenic archaea)	Acetate, electrons	Single-cell genome sequencing, community 16S rDNA analysis	Substrate dependent changes in community composition, <i>a priori</i> knowledge	Analysis of genome sequence and community metatranscriptome	[42]
Enrichment culture	Anaerobic terephthalate-degrading consortium	<i>Pelotomaculum</i> (anaerobic bacteria), <i>Methanosaeta</i> and <i>Methanolinea</i> (hypermesophilic methanogens)	CO ₂ , H ₂ , acetate	16S rDNA profiling, shotgun sequencing	FISH analysis of spatial association, <i>a priori</i> knowledge	Metagenome analysis, thermodynamic considerations	[12]
Synthetic	Isolates from a cellulose-degrading community	<i>Pseudoxanthomonas</i> , <i>Brevibacillus</i> , <i>Clostridium</i>	Acetate, ethanol, saccharides	Known; assessed by real-time PCR	Mixed culture dynamics, conditioned medium experiments	Targeted quantification of cellulose and cellulose degradation products	[91,92]
Synthetic	Isolates from water kefir	<i>Z. florentina</i> , <i>S. cerevisiae</i> , <i>L. hordei</i> , <i>L. nagelii</i>	Amino acids, vitamin B6, unknown factors	Known	Co-culture in transwell plates	Single component exclusion, growth in pairwise cultures	[68]
Synthetic	Human intestinal symbionts	<i>Bacteroides caccae</i> , <i>B. fragilis</i> , <i>B. ovatus</i> , <i>B. thetaiotaomicron</i> , <i>B. uniformis</i> , <i>B. vulgatus</i> , <i>Parabacteroides distasonis</i>	Polysaccharide degradation products (fructose, glucose, among others)	Known	Analysis of species growth in defined media, conditioned media and co-cultures	Assessment of carbohydrate breakdown products released by donors and consumed by recipients	[76]

^a Fluorescence *in situ* hybridization.^b Culture obtained from natural sample by promoting growth of organisms of interest, typically by manipulating medium composition.^c Nanoscale secondary ion mass spectrometry.

Figure 1

Spectrum of microbial community study-systems directed by trade-off between complexity and tractability. Microbial interactions play a central role in biogeochemical cycles in numerous ecosystems, yet are difficult to investigate in molecular detail. In contrast, synthetic communities allow a controlled environment and ease of interpretation. Each study-system in this spectrum offers a choice of resolution to view microbial interactions.

metabolic needs and biosynthetic capabilities. These composite problems necessitate a trade-off between resolution and coverage (Figure 1).

An attractive means to achieve increased resolution of metabolic dependencies is through constructing a smaller manageable model system or by focusing on a particular interaction within a large network. On the other end of the spectrum, one can cover a large system by grouping individual players into higher-order units — guilds (e.g. methanotrophs, sulfur-reducers) and/or metabolite classes (e.g. electron equivalents, fixed nitrogen). Balancing between these two strategies can loosen the tangle and help tracing the main threads in the metabolic knot.

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