Title
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Publication Date
2022

Peer reviewed|Thesis/dissertation

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By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

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Summer 2022

Abstract

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by

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Doctor of Philosophy in Microbiology

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Microbes exist in complex, multispecies communities where they participate in nutritional interactions that shape microbial community structure, function and stability. Nutritional interactions can range from competition for shared nutrients, to mutualisms, where nutrients are reciprocally exchanged in ways that benefit both partners. Not all microbes are able to produce all of their required nutrients, and as a result, rely on receiving nutrients from neighboring microbes. I am interested in understanding mechanisms of nutrient release and its implications in supporting microbial communities.

In Chapter 1, I provide background on how microbes interact in communities, and discuss examples of different types of microbial interactions. I then review how microbes impact their surroundings by releasing by-products and metabolites into their environment, that can then be used by other microbes as nutrients. Next, I explore cooperative and mutualistic behaviors, and theories for how we think cooperative interactions evolve overtime, before discussing auxotrophy, and how this trait may have evolved via adaptive evolution. I end Chapter 1 by discussing cell death, different forms of cell lysis, including bacteriophage-mediated lysis, and review what is known about how these processes are related to nutrient release and nutrient cycling in microbial communities.

In Chapter 2, I discuss two theories for how I think metabolites can be released into the environment to facilitate interdependent metabolite sharing. Microbial communities are composed of complex networks of metabolically interdependent organisms. But it is unclear how these nutritional networks evolve. In particular, the incentive for releasing metabolites, such as amino acids, vitamins and nucleobases is not obvious. I discuss that nutrient release could be a by-product of processes, like cell lysis and regulated metabolite efflux, that could facilitate the emergence of interdependent metabolite sharing.

I experimentally test these predictions in Chapter 3 by hypothesizing that bacteriophage-mediated lysis is a dominant mechanism of nutrient release that can support amino acid auxotrophs. I use bacterial growth assays to investigate how well supernatants, mechanical cell lysates, and phage-generated lysates are able to support a set of amino acid auxotrophs. I found that supernatants and

mechanical lysates minimally support auxotrophs, and phage lysates release a significant amount of bioavailable nutrients, suggesting that in nature, phage are likely to play a large role in providing auxotrophs with their required nutrient.

Chapter 4 explores secretion as a mechanism of nutrient provisioning. More specifically, I test how nutrient overproduction can occur as result of auxotrophic mutations, and the implications of this nutrient overproduction on co-culture growth. To test this I developed an obligate mutualistic synthetic co-culture using two engineered *E. coli* that reciprocally exchange vitamin B_{12} and methionine. I show that co-culture growth is limited by methionine secretion, and reveal how specific auxotrophic mutations are able to increase flux through the methionine biosynthesis pathway to improve co-culture growth.

In addition to exploring mechanisms of nutrient provisioning, I also conducted a sociological project about doctoral students in the biological sciences. In Chapter 5, I investigate key elements of socialization that doctoral students in the biological sciences experience as they navigate their graduate programs. From interviews with over 30 doctoral students, I highlight how informal interactions affect students access to scientific help and expertise.

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Acknowledgements

I would first like to thank and honor Madear, Grandmom Margaret, Pop-pop Tony, Aunt Joan, Jodi and my other ancestors who have protected and guided me personally and spiritually through the last 7 years. I'd like to also thank my newer ancestors and guides: Mr. Nicky, Maurice, Uncle Michael, Uncle Wayne, Aunt Odessa and the many others who were not able to see me finish my degree, but who now surround and guide me.

I want to then extend my appreciation for my PI, Michi, for her support and encouragement throughout the different stages and transitions of my PhD. I watched as Michi shifted to make room for my needs as a graduate student, through family emergencies and conflicting feelings about continuing to pursue my degree. Not many advisors would work with their students to accommodate research interests outside of the lab, and my year doing doctoral education research is what gave me the energy to finish this program, so I am grateful for her help in finding a way to make this PhD my own.

Next, I would like to thank Rocio Sanchez. I truly would not be finishing this PhD without her. After writing cover letters for new jobs, and getting resume feedback from the Career Center, I was ready to submit my "leave of absence" paperwork; fully intending to not return to graduate school. She was the last hurdle I reached before submitting my paperwork. Without Rocio, and our long impromptu conversations, I would have left the program years ago, and I am truly thankful for her continuous support as a mentor and friend.

Members of my PhD lab have been extremely supportive of me throughout this process. I am so happy I was able to begin and end my PhD with a good friend, Kris . We had a joint interview with Michi during our interview weekend for UC Berkeley, joined the PMB department together, rotated in the Taga Lab at the same time, and both decided to join to Taga Lab for our PhD. Kris felt like a stabilizing force during my PhD as people joined and left the lab over the years. And I am looking forward to how our friendship continues to develop! Another good friend from the lab is Alexa. Your words of affirmation throughout this journey have been essential. It is not lost on me that you were one of the first people I vocalized to, that I wanted to quit the program. Your honest and thoughtful advice and feedback over the years has been very sustaining. You have challenged me scientifically and politically in ways that I will continue to cherish now and further into our friendship. Then there is Kenny! You saw my face the most out of everyone in the lab, and I have enjoyed being your benchmate these last 7 years. I've enjoyed getting to know you through our regularly organized lunches, and daily walks to move our cars so we would not get parking tickets on Oxford. Your suggestions and our brainstorming sessions about experimental design have been essential in helping me develop as a scientist. I also want to recognize and acknowledge all of the other wonderfully supportive current and former labmates who have made impacts on my life, both personally and professionally: Amrita, Nicole, Sebastian, Olga, Amanda, Zach, Becky, Zoila, Jong and Joseph. And I will be wishing Eleanor and Dennis the best of luck as new members of the lab!

As I explored science education and sociology, I was mentored and taught by an amazingly supportive set of faculty, students, and friends. Thank you John Matsui, Dax Ovid, Heather

Haveman, Laura Enriquez, Neil Fligstein, Jason Ferguson and my classmates from SOC280Q, SOC280D, and SOC273F.

I want to give a special acknowledgement to my family and extended-family for their continued support through Facetime calls, check-ins, care packages, and visits to the Bay Area. Especially my Mom, Lindsey, Dad, and Donna. And thank you Mom for delivering me in Galloway, NJ on April 19th, 1993, while Waco, Texas was still burning (smiles).

I have so many beautiful friendships that it is hard to begin to list them, but I want to give a special shout out to Yemi and Shannon. Even though they live out of California, I have always felt their love and support. Thank you Iman, for being my introduction to the PMB department and UC Berkeley, as the only other Black graduate student in the department when I first joined. I want to thank all the beautiful friends and family I have made during my time in the Bay Area. Y'all are what made my time in the Bay Area the most fruitful. Especially the Black Queers; the gworls! I love you all so deeply. Y'all help me practice how to love and be loved in ways that are abundant and rich, and I am grateful for your care and presence in my life. I want to thank the old loves, new loves, and lost loves; all of whom contributed to my growth personally during my 20's, while pursuing my PhD.

With Love,

Gordon

Chapter 1: Introduction

1.1 Microbial Ecology and Microbial Interactions

Microbial communities exist across many environments, and are drivers of ecological processes such as carbon cycling [1]. Microbes participate in global biogeochemical cycling of macromolecules and are important contributors to human health and the development of multicellular organisms [2] . Over the last few decades, countless studies have documented the factors shaping microbial ecological processes, as well as the temporal and spatial distribution of microbes . This includes environmental factors (e.g pH, temperature, light, pressure, nutrient availability), as well as biological factors (i.e. the presence of other microbes, viral predation, grazing)[3, 4] . Microbial ecologists have become increasingly interested in studying microbes and their influence on local and global processes [5, 6]. Researchers do this by studying (1) microbial diversity in complex ecosystems through isolation techniques and omics approaches, and (2) *how microbes interact with each other and their environment.*

Microbes interact within and across species at the microbial community level, as well as across kingdoms to form bacteria-fungi, bacteria-plant and bacteria-animal interactions [7-11]. Interactions can range from positive to negative to neutral in their benefit to a microbe, and each of these interactions can have dramatic implications at the local, population and community level. Interactions can be classified further into categories such as mutualism, parasitism, and commensalism when the relationship between interacting partners is considered. For example, mutualism describes a partner pairing that is beneficial to both partners, while commensalism is used to describe interactions where one partner benefits and the other is unaffected. Interactions between dissimilar species are characterized as symbiotic relationships. The type of symbiosis depends on whether one or both of the partners benefit from the interaction. For example, a parasitic symbiotic relationship implies antagonism, in which one partner benefits without contributing to the relationship. A mutualistic symbiosis implies cooperation in which one partner provides a function or performs a behavior, and receives a benefit in return. Symbiotic relationships, and other types of interactions, can be obligate (i.e. at least one partner cannot survive independently) or facultative (i.e. at least one partner benefits, but is not necessary for survival).

Much of the work studying microbial ecology and microbial interactions relies on better understanding how these interactions contribute to more complex community-level processes, such as community stability, function and diversity.

1.2 Microbial Metabolism and Metabolic Products in the Extracellular Environment

Microbes survive by performing complex cellular metabolic functions. Microbes continually create and degrade proteins, peptides, lipids and metabolites based on their metabolic needs at a given moment. The environment microbes live in is continually in flux, as microbes consume and transform the energy and material resources surrounding them, creating proteins and other metabolites required for cell growth. Metabolites are small intermediate- and end- products of cellular metabolic processes, and have been regarded as “the ultimate response of biological

systems to genetic or environmental changes" [12]. Intermediate and end product metabolites are created through catabolic processes - the breakdown of complex molecules to simpler ones- and anabolic processes- the creation of complex molecules from simpler molecules. These products can be used by the microbe that produced them, or released into the environment, often as a "waste" product, to be used or sensed by other neighboring microbes. Different species of microbes have distinct metabolic processes and needs, and as a result, uniquely transform their environment based on these processes.

The metabolites required for growth, cell maintenance and reproduction vary depending on the needs of the organism, as it responds to biotic and abiotic factors. For example, temperature, light, oxygen and carbon sources are abiotic factors that can affect growth rates and the type of metabolisms a microbe performs. Biotic factors that can shape a microbe's metabolism include other bacteria that may either release usable products, inhibit growth via antibiotic production, or compete for a shared resource. Some microbes found in nutrient-deficient environments have adapted useful methods for acquiring the nutrients needed for growth. For example, many species that live in iron-deficient environments release siderophores, low molecular weight metal-chelating agents, to meet their iron requirements [13].

Collectively, the metabolites released by microbes make up the exometabolome. These metabolites can include amino acids, sugars, B vitamins and a host of other molecules that represent the products of diverse metabolisms [14]. Similar to how microbial metabolism affects the type of nutrients a microbe may require for growth, these same metabolisms can shape the type of metabolites released into the environment.

Release of metabolites into the extracellular environment can be mediated by transporter proteins, via active or facilitated transport, or diffusion across the lipid bilayer. The properties of the metabolite (i.e. hydrophobicity, size), and regulation of cellular homeostasis through taxa-specific metabolic regulatory networks, shape the rate and types of molecules released.

A broad range of metabolites have been found in exometabolomic studies, including those originating from central metabolism[15]. One explanation for this phenomenon is overflow metabolism, an evolutionarily conserved process where metabolic byproducts are excreted that could be used for catabolism or anabolism. Canonical examples of this include the acetate switch in *E. coli* [16], and the Crabtree effect in *Saccharomyces cerevisiae* [17]. Culture media from *E. coli*, *S. cerevisiae*, *Bacillus licheniformis*, and *Corynebacterium glutamicum* have been analyzed in batch culture. This analysis revealed that overflow metabolism is a general phenomenon that is not restricted to species-specific properties (i.e. Gram-negative vs. Gram-positive, prokaryote vs eukaryote)[18]. Paczia and colleagues also found correlations between the metabolic state of the cell and metabolite concentrations in the culture medium.

Additionally, overflow metabolism provides a mechanism for the active efflux of amino acids and other metabolic byproducts to prevent autotoxicity resulting from build-up of metabolites and to restore metabolic imbalances [19]. Efflux systems can reduce intracellular concentrations of amino acids or intermediate metabolites to prevent negative feedback inhibition. Examples of amino acids and metabolites that have known exporters involved in overflow metabolism are cysteine, glutamate, leucine, threonine, methionine, and aromatic amino acids, as well as energy-rich sources (i.e. mono and disaccharides, like maltose and lactose) and some cofactors (riboflavin)[20]. In the rhizosphere, microbial amino acid release contributes to plant root growth, microbial colonization, pathogenesis and symbiotic relationships [21]. Amino acids can also be released in response to stress; *Pseudomonas spp.* have been shown to release 22-26% of their intracellular amino acid pools in response to dilution stress [22]. *If bacteria are releasing*

a broad range of metabolites into the environment, what are the consequences at the population and community level?

1.3 Reciprocity, Metabolite Exchange and Cooperativity in Microbial Communities

The exchange of essential metabolites is an important process that governs microbial community growth and composition, and can lead to mutualistic and cooperative behaviors that stabilize microbial communities [23]. Metabolite exchange can involve the unidirectional, bidirectional or multidirectional sharing of a nutrient [24] . When metabolites are being exchanged bidirectionally, metabolisms of two species can become interdependent, critically linking microbes to each other through shared metabolite exchange. For example, some methanogenic communities contain many metabolic interactions, and contain strains with multiple auxotrophies that result in a network of interdependence [25].

Mutualisms can be categorized as a type of interdependent interaction, and range from facultative to obligate in character. Mutualisms broadly include interactions that involve two distinct species in which both benefit from the relationship. These types of interactions are widespread and ecologically relevant [26]. Early studies of mutualisms were dominated by theories of 'kin selection'[27] and 'reciprocal altruism'[28],that focused on the intrinsic fitness of individuals within a group. I prefer to use the conceptual framework developed by Connor (1995) that classifies mutualistic interactions based on the mechanisms of providing a benefit [29]. Connor (1995) describes three ways and organism can receive benefits from an interacting partner, through: (1) by-product benefits - where the byproducts of an organism incidentally benefit others, (2) investment - where organisms release costly products to increase the probability of receiving by-product benefits in return, and (3) purloined or stolen benefits that evolve from antagonistic interactions, such as parasitism [29].

Naturally evolved cooperative phenotypes have been shown to be important in many environments, including the rhizosphere and mammalian gut [30]. These phenotypes are thought to occur when microbes experience unusually stable environments that are nutrient-rich or constant, such as pathogens and symbionts [31, 32]. Pathogens and endosymbionts have some of the smallest genomes, with genome size correlating strongly to obligate association with host cells [31]. Other mutualisms in which the evolution is less clear are algal-bacteria symbioses. More than half of microalgal species are estimated to be auxotrophic for vitamin B_{12}[33]. For example, *Lobomonas rostrata*, a green alga vitamin B_{12} auxotroph, exchanges fixed carbon for vitamin B_{12} from a soil bacterium, *Mesorhizobium loti* [34]. Other examples of mutualisms in nature include the two obligate endosymbiotic bacteria, *Wigglesworthia glossinidia* and *Wolbachia*, found in the tsetse fly gut that metabolically complement each other and share the cost of producing thiamine [35], as well as *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron* in the human gut [36]. Each of these interactions contains an example of a microbe lacking the ability to produce all of the nutrients required for growth, and in turn, rely on reciprocal interactions to survive.

Studying mutualistic and cooperative interactions in natural samples can be difficult because of confounding interactions and metabolites in complex communities that could impact these observations. Synthetic co-culturing techniques have been used to study mutualistic behaviors in model systems. For example, *E. coli* leucine and lysine auxotrophs were co-cultured to study how the phenotypes of these strains changed during the course of adaptive evolution[37]. Auxotrophs of different species can also engage in reciprocal interactions, such as *E. coli* and

Salmonella enterica, and *E. coli* and *Acinetobacter baylyi* [38]. Auxotrophs are found to readily form cooperative interactions when co-cultured, with some even showing metabolic synergy [39]. These experiments show that reciprocity can be a directed act; and in addition to extended coevolutionary interactions, pre-existing conditions can provide positive feedback for the establishment of cooperativity.

Co-culturing work has shown that nutrient sharing can (1.) create stable mutualistic interactions that can switch between stabilities based on the nutrient availability in the environment [40], (2.) protect obligate two-way cooperative interactions from exploitation by non-cooperators [41], (3.) promote stronger cooperative interactions by sharing biosynthetically costly metabolites [42], (4.) favor cooperative interactions in spatially structured environments [43], and (5.) increase genotypic diversity in a community [44], to name a few. By disrupting synthetic systems with controlled perturbations, and comparing mutualism stability across strains with different genotypes, we can begin to clarify key elements that stabilize mutualisms and nutritional interactions in microbial communities.

1.4 Evolution and Maintenance of Cooperative Interactions

The two dominant areas of study surrounding cooperative behaviors are the maintenance and evolution of cooperation *within* a species and *between* species. Hamilton's theory of 'kin selection' laid the foundation for the study of intra-specific cooperation. This theory posits that genes involved in cooperative behaviors benefit gene copies in closely related species[27]. An example of this would include a bee stinging an invader to protect the hive and giving up its life in the process. A microbial example includes the cooperative behavior of siderophore production that is maintained in *Pseudomonas aeruginosa* populations when relatedness is high; and when relatedness is low, experiencing a population decrease[45]. Triver's model of 'reciprocal altruism' gave way to interspecies cooperation theory. This model explains relationships in which a cooperative benefit directed towards an individual is returned, resulting in a net positive benefit [28]. This model implies that a behavior is selected for because it tends to yield a positive fitness return. Additionally, 'by-product mutualism' was theorized as a type of cooperative interaction in which the benefit of producing a by-product automatically outweighs the cost of the product [29]. This would occur when the behavior of one individual incidentally benefits its partner, at no additional cost .

The evolution of mutualism remains difficult to discern for many theorists because it contradicts our understanding of natural selection as a force that drives individual fitness [46]. Multiple conditions have been agreed upon that are theorized to promote cooperation in both of these areas . Factors predicted to promote cooperation and mutualisms are low cost-benefit ratios for providing a benefit, partner fidelity feedback, partner choice and cooperative association [47]. Partner fidelity feedback can occur when the cooperative behavior of one species promotes a positive phenotypic effect, like increased abundance or survival, that provides a feedback benefit for the producing strain[48-50]. Partner choice occurs when the receiver of a benefit actively returns a benefit to the benefactor, and there is a conditional exchange between species that is reciprocated [48, 49]. Cooperative association occurs when individuals participating in the interaction receive benefits, and non-cooperating species are sanctioned, resulting in less benefits to non-cooperating species [51].

Most mutualisms are expected to evolve from by-product mutualism, where one organism selfishly benefits from the byproducts of another, with a low cost of production for the donor organism. Cooperative association is expected to be a rarer influence in the evolution of mutualism, because it relies on the cooperative behavior of the donor organism to increase the cooperativity in the receiving organism. Each of these traits can be adapted over time to improve mutualism.

1.5 Adaptive Evolution and Amino Acid Auxotrophy

Key to microbial survival is being able to adapt to diverse environments. As discussed above [See Section 1.2], a large breadth of metabolites can be found in the exometabolome. Some microbes have adapted to their environment and the presence of other organisms by losing the ability to synthesize one or more essential compounds for growth (i.e., becoming auxotrophic for one or more compounds). Genomic analyses provide evidence for the high prevalence of auxotrophy in nature and revealed natural bacterial isolates lacking complete biosynthetic pathways for producing one or more amino acid, cofactor or nucleobase[42, 52, 53]. These types of auxotrophies suggest that microbes must rely on receiving these compounds from a host or local prototrophic microbes. Some natural isolates cannot be cultured in the lab, and were only cultivatable in the presence of a beneficial or 'helper" microbe that released "growth stimulatory" molecules[54, 55]. For example, *Symbiobacterium thermophilum* was only able to be cultured in a laboratory setting when co-cultured with a *Bacillus* partner strain or its spent culture media[56]. Through this type of reductive gene loss, microbes can reduce metabolic costs by acquiring nutrients from other organisms.

The Black Queen Hypothesis (BQH) posits that adaptive gene loss occurs when a function is essential and sufficiently present in the environment to support the needs of the community [57]. Genes that serve little to no benefit are lost, decreasing the metabolic burden of the auxotroph because it no longer has to direct resources towards that particular gene product. It is suggested that the BQH is why researchers find examples of symbiotic organisms with truncated essential biosynthesis pathways [32]. Examples of this can be found through metagenomic and empirical surveys of bacterial isolates that appear to have undergone extensive genome reduction[58].
Functions lost as a result of genome reduction range from vitamin and amino acid biosynthesis [59, 60], to saturated fatty acid synthesis [58]. Auxotrophies for these types of molecules are particularly interesting because they are essential for all microorganisms, highly regulated and can be energetically costly.

Amino acids are important for microbes because they are essential building blocks of proteins and peptides, and can be used in a variety of other life sustaining processes. *E. coli* can use up to 11 of the 20 proteogenic amino acids as a nitrogen source [61], and some bacteria, such as *Klebsiella aerogenes*, can use amino acids as a carbon source [62]. Amino acid usage as nitrogen and carbon sources varies based on bacterial species and the environment; for example, tryptophan can only be used as a carbon source in non-catabolite repression conditions [61]. Some amino acids are also precursors to other essential metabolites , such as nucleotides and cofactors.

Amino acid biosynthesis pathways can be highly complex. Branch points for amino acid synthesis extend from different stages of the central carbon metabolism; this diverts intermediate resources and energy away from central metabolism and the creation of cellular biomass. Further, energy, in the form of ATP, is required for amino acid biosynthesis. The biosynthetic costs of

amino acid production has been calculated based on the resources required to (1) generate precursors for amino acid biosynthesis and (2) generate energy in the form of high-energy phosphate bonds and reducing power, such as NADH, NADPH and $FADH_2$ [63, 64]. Waschina and colleagues (2016) define and quantify the biosynthetic cost of amino acids based on the amount of exogenous carbon required to produce a mole of the given amino acid, relative to the amount of biomass that can be produced from the same amount of carbon [64]. An alternative way used by Akashi & Gojobori (2002) to calculate biosynthetic cost was to calculate the amount of ATP consumed in order to produce the amino acid [63]. Because amino acid biosynthesis pathways branch off from the central carbon metabolism, the estimated cost of producing an amino acid is also based on the carbon source used. The entry point of carbon into central metabolism and the intracellular pool of amino acids can significantly shift flux through metabolic pathways [65, 66]. This was empirically tested by Waschina and colleagues (2016), who found that the nutritional environment and the structure of the metabolic network are major determinants of metabolic production costs.

The cost of amino acid production, nutrient availability, the complexity of the biosynthetic pathway[67] and the efficiency of protein synthesis [63] represent metabolic constraints on microbial protein production. Microbes often use cost-minimization strategies for protein synthesis, in which more highly abundant proteins use less costly amino acids [68], and amino acid usage varies depending on the secondary structure of proteins [69]. For example, α-β mixed proteins contain a larger proportion of Val, Ile and Leu, while proteins with primary β pleated sheets contain a larger proportion of Trp, Tyr, Ser and Gln [69].

Predictive models suggest that the leakiness of amino acids from bacteria is linked to biosynthetic cost, with less energetically expensive amino acids contributing more to cooperative interactions via leakiness or other forms of metabolite exchange [70]. Additionally, the metabolic state of a microbial population growing in batch culture influenced the concentration of central carbon metabolites and amino acids released into the supernatant [18]. Literature on amino acid secretion, the detection of amino acids in media, and hypotheses about amino acid leakiness suggests great potential for amino acid release playing a significant role in nutritional interactions in nature.

Amino acid exchange has been shown to shape microbial community composition. Studies of amino acid transfer in a natural methanogenic population revealed that no single strain was able to produce all of its required amino acids, and the division of labor for amino acid production was not spread evenly [25]. This was suspected to lead to the intricate and diverse web of amino acid dependencies found within the methanogenic community. In this specific example, methanogens were able to refocus energy towards methanogenesis rather than biosynthesis, easing their metabolic burden. Additionally, the energy richness of the substrate diversified the composition of the microbial community to accommodate the transfer of electrons for redox reactions [25].

1.6 Cell Death and Viability

Microbial cell death remains an elusive and challenging process to study. In the earliest work on cell death, the dying process of a cell was often discussed as a consequence of ‘unbalanced growth’, and an event that occurred after all the more interesting biochemical and physiological activity had happened [71]. Since then, there has been a growing appreciation for bacterial death as a highly regulated process that greatly influences bacterial physiology and microbial community behaviors, such as biofilm formation [72, 73].

Despite the importance of being able to distinguish between living and dead cells, the difference is still difficult to disentangle using current microbiological techniques. *What makes a cell alive*? The definition of bacterial viability has changed during the lifespan of bacteriology from being characterized by the ability to produce progeny, to the ability to remain metabolically active. Viable but non-culturable (VBNC) bacteria are an example of bacteria that are unable to be cultured under lab conditions, but have been shown to be metabolically active and can maintain homeostasis [74].

Similar to our definition of viability, the definition of bacterial death is not straightforward. There is diversity even in death, when it comes to how bacteria lose their viability. For example, bacteria can (i.) lose cytoplasmic membrane integrity which prevents the ability to maintain homeostasis, or (ii.) there can be the inactivation of a narrow range of essential targets (i.e. ribosomes, protein biosynthetic enzymes, DNA, RNA) that causes rapid loss in viability but a slower degradation of cellular components and cytoplasmic membranes [75]. Some bacteria have been found to be non-viable, but structurally intact and impermeable to propidium iodide, a common fluorescent DNA stain used to count dead cells [76]. Additionally, extracellular DNA is found to persist in soils and can be resistant to degradation on mineral surfaces and aquatic particles [77]. This brings into question how reliable DNA-based measurements can be when determining microbial viability from environmental samples.

The best understood processes related to cell death are forms of regulated cell death pathways, such as bacterial programmed cell death (PCD), and accidental cell death in response to physical (e.g. temperature, pressure, osmotic forces), chemical (e.g. pH variation) or mechanical (e.g. shear) forces. Regulated cell death can be stress-driven and occur in response to intense perturbations intracellularly or extracellularly, or be a form of PCD that is purely rooted in developmental and physiological behaviors, such as biofilm formation or sporulation, and not in response to a bacterium's failure to adjust to a stressful condition or maintain homeostasis [73]. Bacterial PCD is highly regulated and serves important aspects of bacterial physiology and microbial ecology. One of the earliest accounts of studying bacterial death was autolysis, a self-destructive lysis process that was one of the first observable phenotypes related to bacterial death [71]. Autolysis was identified to be caused by autolysins which are a type of peptidoglycan hydrolase that cleave components of the peptidoglycan that are responsible for maintaining cell shape and providing resistance against osmotic shock.

How is it that these seemingly counterintuitive genetic modules, that result in self-destruction (i.e. bacteria lysing themselves), continue to be passed onto future generations? One way to better understand this is by considering bacterial multicellularity and phenotypic heterogeneity within bacterial populations. Specifically, phenotypic heterogeneity is used to describe isogenic populations with phenotypically distinct subpopulations that have unique genetic profiles [78]. This behavior allows for collective behaviors, like bet-hedging [79] and division of labor [80].

The three model cases of PCD in bacteria that are extreme examples of division-of-labor are cannibalism, altruistic PCD, and fratricide. Cannibalism is observed in *Bacillus subtilis,* a Gram-positive soil dwelling bacterium that produces self-killing toxins [72]. Under nutrient limiting conditions, a subpopulation expresses a signaling molecule, Spo0A, that delays sporulation. These cells then express a killing factor that is secreted by a transporter protein , Skf, which lysis the subpopulation of B. subtilis not expressing *spo0A*. This killing of non-sporulating sister cells serves as a mechanism for providing nutrients to *B. subtilis* to delay endospore formation, which is an energetically costly process. *Myxococcus xanthus* are rod shaped Gram-

negative, spore forming bacteria that move using gliding motility. Under nutrient limiting conditions, *M. xanthus* use this motility to form aggregates that develop into fruiting bodies in preparation for myxospore formation [81]. In a highly coordinated fashion, 80% of the population undergoes autolysis that releases carbon and nutrients for fruiting-body development. *Streptococcus pneumoniae*, a commensal Gram-negative bacterium known for being the causative agent of pneumonia, triggers lysis as a mechanism of genetic recombination, termed fratricide [82]. During exponential growth, a subpopulation differentiates into competent *S. pneumoniae* in response to a peptide pheromone released through a quorum sensing mechanism. The non-competent cells undergo lysis and serve as DNA donors for the competent cells. It has been suggested that this process allows for the successful transfer of genetic material in pneumococcal biofilms. Each of these modes of lysis results from internal bacterial genetic programs, but some cell death genetic programs are governed by outside biological agents, as described in the next section.

1.7 Bacteriophage-Mediated Cell Lysis

Bacteriophages – viruses that infect bacteria - are the most abundant biological agent on earth and exhibit global and endemic biogeographical patterning [83]. Phages are obligate intracellular parasites that require a host to produce progeny. Increasingly, phage are being recognized for their role in microbial physiology, population dynamics and microbial evolution.

While phages are extremely diverse in structure, habitat and life cycle processes, there are a few steps common to all viruses: adsorption, expression of genes required for genome replication, and virion assembly, release and transmission. Bacteriophage life cycles can range from lytic, lysogenic, and pseudolysogenic to chronic infections [84, 85]. Lytic infections redirect host metabolism towards producing new phage progeny, which are released during lysis. Lysogenic infections occur when the genome of the temperate (or lysogenic) phage remains in the host in a dormant state. During the lysogenic cycle, phage DNA replicates along with the host until the lytic cycle is induced. Chronic infection occurs when phage particles are released by extrusion or budding, without cell lysis, and pseudolysogeny is when phage replication occurs in a fraction of the population. Each of these life cycles has different consequences for microbial population dynamics.

Similar to our understanding of autolysis mechanisms, loss of cell wall integrity characterizes some of the best studied and widespread examples of phage lysis. For many phages, virions are released following the transcription of phage lysis gene clusters that encode enzymes, such as endolysins and holins, that are necessary for the disruption of the bacterial cell membrane. Factors affecting the proportion of lytic and lysogenic infections is still unclear, but it is understood that these cycles play an important role in biogeochemical cycling and microbial population dynamics.

Viruses are major drivers of microbial mortality. In marine environments, they have been estimated to lyse 20-50% of the planktonic bacteria populations per day [86-88]. This large amount of microbial death has implications in microbial food webs, carbon turnover and global nutrient cycling. The prevailing concept that considers the impact of viruses on biogeochemical cycling in marine systems is the ‘viral shunt’. This concept highlights that lytic viral lysis returns the biomass of the lysed host cell back into dissolved matter, to be recycled for microbial growth, and is ‘shunted’ away from higher trophic levels [87, 88]. Macronutrients, such as carbon, nitrogen and

phosphorus, as well as micronutrients, iron and selenium, have been found to be released following viral lysis in aquatic environments [89-93]. Additionally, nutrient availability and dissolved organic carbon have been shown to fluctuate with seasonal changes in viral abundance [90, 92].

The dissolved organic matter (DOM), the organic matter able to pass through a 0.45 um filter, released from viral lysates is chemically and structurally distinct. Laboratory experiments reveal different molecule profiles of DOM released from virally lysed bacteria in comparison to mechanical lysis and exudate (i.e., supernatant) [94]. These three processes have different mechanisms of contributing to DOM release. DOM in exudates consists of molecules from overflow metabolism, or metabolites that are specifically exported for signaling or nutrient acquisition (See section 1.2). Mechanical lysis releases intracellular molecules from 'healthy' cells; an example of mechanical lysis in nature is 'sloppy feeding' by grazers [95]. And lastly, viral lysis releases molecules from cells that have experienced an extended infectious period under which there has often been considerable biochemical remodeling of the host's metabolism. Metabolomics of *Sulfitobacter* sp. 2047 shows elevated amino acid, nucleotide synthesis, and lipid synthesis when comparing the exudate of infected vs uninfected strains [96]. Contributions that have been shown to shape the composition and character of these metabolites are cell wall degradation and nucleotide synthesis [96, 97], exopolysaccharide degradation [98], lipid remodeling [99] and proteolysis of abundant protein complexes [94]. The fate of the released lysates depends on its bioavailability and how accessible the released nutrients are for neighboring cells. Laboratory experiments have revealed that viral lysis can relieve bacterial populations experiencing nutrient limitations, and have been shown to indirectly affect community structure [100]. Viral lysates were able to alleviate nutrient-limited cultures of *Aureococcus anophagefferens*, and phytoplankton inoculated into viral lysates had a faster growth rate and reached a higher biomass than phytoplankton inoculated in cell filtrate without lysis [89]. Similarly, significant levels of dissolved Fe have been reported from viral-mediated lysis and allowed for the sustainment of the local phytoplankton community [93]. Additionally, amino acid enrichments have been observed in viral lysates [94, 96], suggesting viral lysis could play a role in supporting amino acid auxotrophs.

Chapter 2: Emergence of Metabolite Provisioning as a By-Product of Evolved Biological Functions

Published: Gude, S., Pherribo, G. J., & Taga, M. E. (2020). Emergence of metabolite provisioning as a by-product of evolved biological functions. *Msystems*, *5*(3), e00259-20.

This chapter is work I co-authored and published during my Ph.D. that sets the theoretical foundation for Chapter 3.

Abstract

Microbes commonly use metabolites produced by other organisms to compete effectively with others in their environment. As a result, microbial communities are composed of networks of metabolically interdependent organisms. How these networks evolve and shape population diversity, stability, and community function is a subject of active research. But how did these metabolic interactions develop initially? In particular, how and why are metabolites such as amino acids, cofactors, and nucleobases released for the benefit of others when there apparently is no incentive to do so? Here, we discuss the hypothesis that metabolite provisioning is not itself adaptive, but rather can be a natural consequence of other evolved biological functions. We outline two examples of metabolite provisioning as a byproduct of other functions by considering cell lysis and regulated metabolite efflux outside of their canonical roles, and explore their potential to facilitate the emergence of interdependent metabolite sharing.

Introduction

Existence in the microbial world is contingent on the ability to compete in environments where even the tiniest advantage can mean the difference between survival and extinction. One way to gain an advantage over competitors is to use metabolites produced by others rather than performing all necessary metabolic functions independently. Indeed, most microbes depend on others for some metabolites, and at the same time, release metabolites into the environment [101, 102]. These interactions collectively form complex networks of interconnected metabolic pathways among numerous species (Fig. 1A). Of particular curiosity is the origin of these interactions: how does metabolic interdependence arise in a competitive world?

Dependence on others to fulfill core metabolic requirements starts with the loss of metabolic capabilities via loss-of-function mutations (Fig. 1 B,C). The emergence and maintenance of loss-of-function mutants can be explained by the Black Queen Hypothesis, which argues that the loss of a gene is favored by natural selection as long as the encoded function is sufficiently 'leaky' and hence continuously fulfilled by others [57]. Functions that change the extracellular environment, such as degradation of toxins, polysaccharide hydrolysis, and release of iron-scavenging siderophores, are inherently leaky, and therefore organisms that do not perform these functions can still reap their benefits (Fig. 2A) [103-105]. Another type of leakiness is the excretion of temporarily undesired, yet energetically valuable, metabolic byproducts such as acetate and lactate [16, 106] (overflow metabolism, Fig. 2B). Hence, secondary effects of biological processes, which evolved to benefit the organism performing it, can also provide benefits to unrelated organisms.

Unlike the examples above, metabolic end products such as amino acids, cofactors, and nucleobases are not considered inherently leaky because they are produced and used inside the cell. However, genomic analyses indicate that the majority of microbes are unable to produce one or more amino acids, cofactors or nucleobases (i.e., are auxotrophs) [42, 53, 107] suggesting that these intracellular metabolites are somehow leaky or that dedicated mechanisms of releasing them are prevalent. Auxotrophy and extensive genome reduction commonly occur in obligate endosymbionts, driven by genetic drift caused by genetic isolation and the bottlenecks that occur during transmission. Auxotrophy also commonly occurs in free-living microbes, though to a lesser extent, suggesting that auxotrophy can also arise via natural selection. In these relatively rare cases, auxotrophy may be supported by leakiness of other organisms in the environment [53].

In laboratory experiments, growth of some amino acid auxotrophs of *Escherichia coli* is supported by a partner in coculture, suggesting that actively proliferating microbial populations can be leaky for some intracellular metabolites . This suggests that nutrients can be provided within free-living microbial communities in the absence of a host. The mechanisms by which these metabolites are released in free-living microbial populations remain largely unexplored. How and why are these important and energetically valuable products released from cells? How can a seemingly non-beneficial behavior arise in free-living microbes? In other words, 'what's in it for the producer?'

Provisioning of resources to others can be beneficial to the producing population in reciprocal interactions: when one population provides a growth-enhancing metabolite to another, it may be rewarded with a larger amount of its required metabolite in return (Fig. 1D)[38]. Thus, once a single metabolic dependence has arisen, the subsequent evolutionary steps towards additional metabolic interdependences are thought to be adaptive [108]. Furthermore, for these interactions to be stable, effective mechanisms to exclude cheaters (individuals that benefit without contributing) must be present. Yet, still unclear is how leakiness for intracellular metabolites is initially established to allow for the emergence of metabolic dependence. For this initial evolutionary step to occur in free-living microbial populations, a route for metabolite release must be present. In addition, two criteria must be fulfilled. First, the mechanism of overproducing these desirable metabolites may not incur a fitness cost to the producer, since there is no reciprocation to compensate for it. Second, a sustained supply of the metabolite must be maintained in order for auxotrophs to become fixed in the population. Here, we outline how byproducts of evolved biological functions can lead to a sustained provisioning of intracellular metabolites, including energetically valuable amino acids, in free living, non-host associated microbial communities. We employ the term 'byproduct' to mean any secondary effect of a biological process [109], rather

than limiting its use to metabolic byproducts [108]. In the following sections we discuss two examples of metabolite provisioning mechanisms by considering lysis and regulated metabolite efflux outside their canonical roles (Fig. 2C,D).

Sustained metabolite provisioning as a byproduct of lysis

Lysis, a death process resulting from a loss of cell envelope integrity, is a fundamental part of microbial life. Microbial cells lyse when experiencing stress, and can be killed by competing microbes [110, 111]. Microbes also undergo lysis as part of developmental programs such as biofilm formation, fruiting-body development, and sporulation [81, 112, 113]. One of the most common mechanisms of lysis across diverse environments is mediated by viruses [86, 114]. Viral lysis is a key factor in modulating natural microbial ecosystems. In marine environments, viruses lyse an estimated 20-40% of microbial biomass each day, making them major drivers of phytoplankton mortality [86]. The importance of viruses in modulating microbial communities has also been demonstrated in controlled laboratory systems where viral predation was shown to alter competitive interactions between microbial species (Brockhurt fenton 2006).

Any lysis process inherently results in the release of intracellular material. Therefore, lysis may be considered a mechanism of 'leakiness' for metabolites that are normally contained within cells (Fig. 2C). The bioavailability of these cellular components may vary depending on the character of the molecule and how an organism accesses the nutrient. For example, the amino acids or metabolites sequestered within proteins can only be made available if proteases are present. Evidence for the release of bioavailable nutrients following cell lysis has been observed across various scales and systems. Release of iron, nitrogen, and carbon resulting from lysis has been shown to support microbial growth in aquatic environments, demonstrating that these cellular components are present in a bioavailable form (Fig. 3A) [92, 93]. Additional support for the availability of nutrients following lysis has been established in laboratory experiments. T7 phage lysis of *E. coli* was shown to support higher growth yields of *Salmonella typhimurium* in co-culture relative to conditions without phage, indicating the release of bioavailable nutrients, including a carbon source, via lysis [115]. Similarly, when nutrient-deprived, a subpopulation of *Bacillus subtilis* cells undergoes programmed lysis to provide nutrients to their kin in order to delay sporulation [112]. Together, these findings demonstrate that nutrients liberated via lysis can support the growth of microbes within and across species, and suggest that these nutrients can also contribute to the emergence of auxotrophs.

An individual lysis event results only in a transient release of nutrients, but for the emergence and maintenance of auxotrophic populations, a sustained nutrient supply is needed. One framework in which lysis can generate a sustained nutrient supply is the co-evolutionary arms race between viruses and their hosts. Red Queen dynamics predict the emergence of virus-resistant microbial hosts following a lysis-induced collapse of the host population [116]. This recovery lasts only until the host cells are again lysed by a newly evolved viral mutant, thus leading to repeated lysis events (Fig. 3B). Laboratory evolution studies indeed have observed such sustained co-evolutionary dynamics in phage-bacteria systems [45]. These boom-and-bust cycles created by an individual virus-host pair will thus produce a fluctuating supply of nutrients as a byproduct of lysis. However, virus-host co-evolution is not limited to a single virus-host pair, but occurs in the larger context of complex communities in which numerous viruses and hosts naturally exist. According to the kill-the-winner hypothesis, the most abundant microbial population within a community will be preyed upon most frequently, causing its population to collapse [117]. After this population collapse, a different microbial population will become most abundant and likewise

experience a higher susceptibility to viral predation and lysis. Population dynamics like these, coupled to co-evolutionary arms-race dynamics in the context of large microbial communities, are thus capable of creating overlapping and alternating boom-and-bust cycles (Fig. 3C). We propose that many uncoordinated, repeated lysis events can together provide a sustained supply of nutrients to enable the emergence and maintenance of auxotrophs. To date, limited experimental evidence for coupled oscillations within virus-microbe systems exists; *in situ* measurements of microbial and viral abundances with improved time-scale resolution could provide useful insight into these dynamics [118].

Co-evolutionary arms-race dynamics between viruses and their microbial hosts may halt for any particular virus-host pair, such as when microbes evolve full resistance [119] or when environmental factors modulate species interactions [40] . However, many other mechanisms of cell lysis exist and can also contribute to nutrient provisioning. For example, bacterial lysis is occasionally activated from within the host genome via prophage induction [120] , and lysis is also caused by non-viral mechanisms, such as autolysis induced by stress or as part of a developmental process, and killing due to microbial competition (Fig. 3 A,C) [81, 110, 111]. Autolysis has been shown to be important in supporting biofilm development by releasing DNA to form a structural component of biofilm extracellular matrices; this process could also support neighboring cells through released nutrients [113]. Thus, various combinations of these processes within large and diverse microbial communities can produce a sustained nutrient supply by creating many interspersed nutrient release events (Fig. 3A, C). Consequently, we posit that intracellular metabolites such as amino acids, cofactors, and nucleobases can perpetually be made available to support the emergence and maintenance of auxotrophs in microbial communities as a byproduct of evolved mechanisms of lysis.

Metabolite efflux as a byproduct of maintaining homeostasis

Efflux systems in microbes have long been appreciated as mechanisms to protect cells from the accumulation of toxins and metabolic waste products, excrete small molecules such as siderophores and flavins to fulfill a specific metabolic requirement, and export building blocks for the assembly of extracellular structures [121-124]. Recently, microbes have also been found to possess efflux systems for the excretion of amino acids and other intracellular metabolites [20] . These efflux systems have been speculated to act as ‘release valves’ that modulate intracellular metabolite levels to restore homeostasis following temporary metabolic imbalances (Fig. 2D) [20]. For example, cysteine exporters in *E. coli* are proposed to protect cells from toxic intracellular accumulation of cysteine, and a homoserine exporter has been suggested to play a role in maintaining homeostatic levels of threonine [20]. These processes thus perform a function akin to the release of temporarily undesirable metabolites during overflow metabolism (Fig. 2B), as both protect the cell from growth inhibition due to metabolite accumulation [16, 106].

We speculate that metabolite efflux systems can also provide benefits during unperturbed growth by releasing products accumulated as a result of continuous overproduction (Fig. 2D). Continuous overproduction, though seemingly in contrast to the principle of cellular economy, may be a consequence of the highly interconnected nature of cellular metabolism as well as an adaptive strategy to avoid the negative consequences of underproduction. When even one required metabolite becomes limiting, growth is reduced (Fig. 4, T_{under}). However, the highly interconnected nature of metabolic networks fundamentally limits the extent to which metabolite production levels can be optimized [125]. For example, aspartate is a precursor in alanine and asparagine biosynthesis, and serine is part of glycine, cysteine, and methionine synthesis pathways.

Consequently, the stoichiometry of certain sets of metabolites is hard-wired by the topology of the metabolic network. To satisfy the minimal requirements for all metabolites at any time, some metabolites need to be produced in excess [125]. One strategy for relieving metabolite accumulation is degradation. However, some organisms such as *E. coli* lack degradation pathways for certain metabolites, and therefore must remove excess metabolites by exporting them [125]. Continuous active export of these overproduced metabolites via designated efflux systems can avert their intracellular accumulation and partially diminish the negative fitness consequences associated with metabolite overproduction (Fig. 4, T_{over}, black arrows) [126].

Microbes may also have evolved to overproduce metabolites to buffer against inherent fluctuations in gene expression, enzyme activity, and regulatory systems (Fig. 4, blue arrows). One source of these fluctuations is the variation in gene copy number that occurs during genome replication, resulting in deterministic variations in gene expression levels [127]. Another source of fluctuations is the limited capacity of the cytoplasm, where proteins produced at low levels are particularly affected by stochastic effects [128]. For example, the average protein abundance in *Mycoplasma pneumoniae* is estimated to be 167 molecules per cell [129], and at least 10% of all proteins in *E. coli* are present at fewer than 10 copies per cell [130]. Therefore, if an organism would aim to produce the optimal level of all metabolites required for its growth (Fig. 4, T_{peak}), inherent fluctuations in protein levels (Fig. 4, blue arrows) would cause it to sometimes produce too much and at other times too little. Underproduction is always detrimental because it limits growth (Fig. 4, T_{under}). Overproduction can also decrease fitness when resources such as precursors and energy are wasted, or when metabolites accumulate to toxic levels (Fig. 4, T_{over}) [126]. The latter effect can be alleviated by actively exporting excess metabolites via designated efflux systems (Fig. 4, black arrows) [20].

Though seemingly wasteful, a strategy in which cells constantly overproduce to fulfill minimal production requirements, and export excess metabolites to avoid accumulation and toxicity, can be adaptive if overproduction is less harmful than underproduction (Fig. 4). Therefore, continuous export of some intracellular metabolites may be a fundamentally adaptive strategy that, as a byproduct, fortuitously provides nutrients to auxotrophs. Laboratory experiments have provided hints that some intracellular metabolites may indeed be exported. Growth of lysine, methionine, and phenylalanine auxotrophs of *E. coli* is supported by other *E. coli* mutants in co-culture, reinforcing the idea that some amino acids may be actively exported [42]. Furthermore, methionine and phenylalanine auxotrophs fared slightly better than other amino acid auxotrophs in pooled transposon sequencing (Tn-Seq) experiments, again suggesting that these amino acids can be made available by other cells [131].

Conclusion

The pervasiveness of auxotrophy throughout microbial genomes suggests that the release of intracellular metabolites is prevalent. Metabolite provisioning for the benefit of others can arise through various adaptive and non-adaptive mechanisms. For example, metabolites can be provided in the context of co-evolved partnerships, where evolved reciprocity or genome reduction leads to metabolic interdependence (Fig. 1 C,D). Here, rather than addressing partnerships that have co-evolved, such as endosymbionts and their hosts, we discuss how nutrient-sharing relationships can be initiated in free-living microbes (Fig. 1A). Lysis and metabolite efflux systems are, in principle, capable of creating a sustained supply of metabolites that can support the emergence and maintenance of auxotrophs, and therefore may contribute to the evolution of metabolic interdependence. Metabolite provisioning as byproducts of evolved functions may present a

complementary alternative to our current perception that specific interactions predominantly evolve between specialized partners (Fig. 1 B-D), by shifting the focus to non-specific metabolite provisioning that can benefit any organism (Fig. 1A). Thus, metabolite provisioning as a byproduct (Fig. 2) can lead to non-specific, many-to-many interactions in which multiple organisms provide and take up nutrients, as opposed to specific, one-to-one co-evolved partnerships. Such many-to-many sharing interactions may present a solution to the paradox of how the initial steps of nutrient sharing can evolve in free-living microbial communities in the absence of partner-specific, reciprocal interactions.

Acknowledgements
This work was funded by National Institutes of Health grant DP2AI117984 to MET and by the National Science Foundation Graduate Research Fellowship Grant DGE 1752814 to GJP. We thank Adam Deutschbauer and members of the Taga lab for helpful discussions. We also thank Kristopher Kennedy, Olga Sokolovskaya, Amanda Shelton, Kenny Mok, and Zachary Hallberg for critical reading of the manuscript.

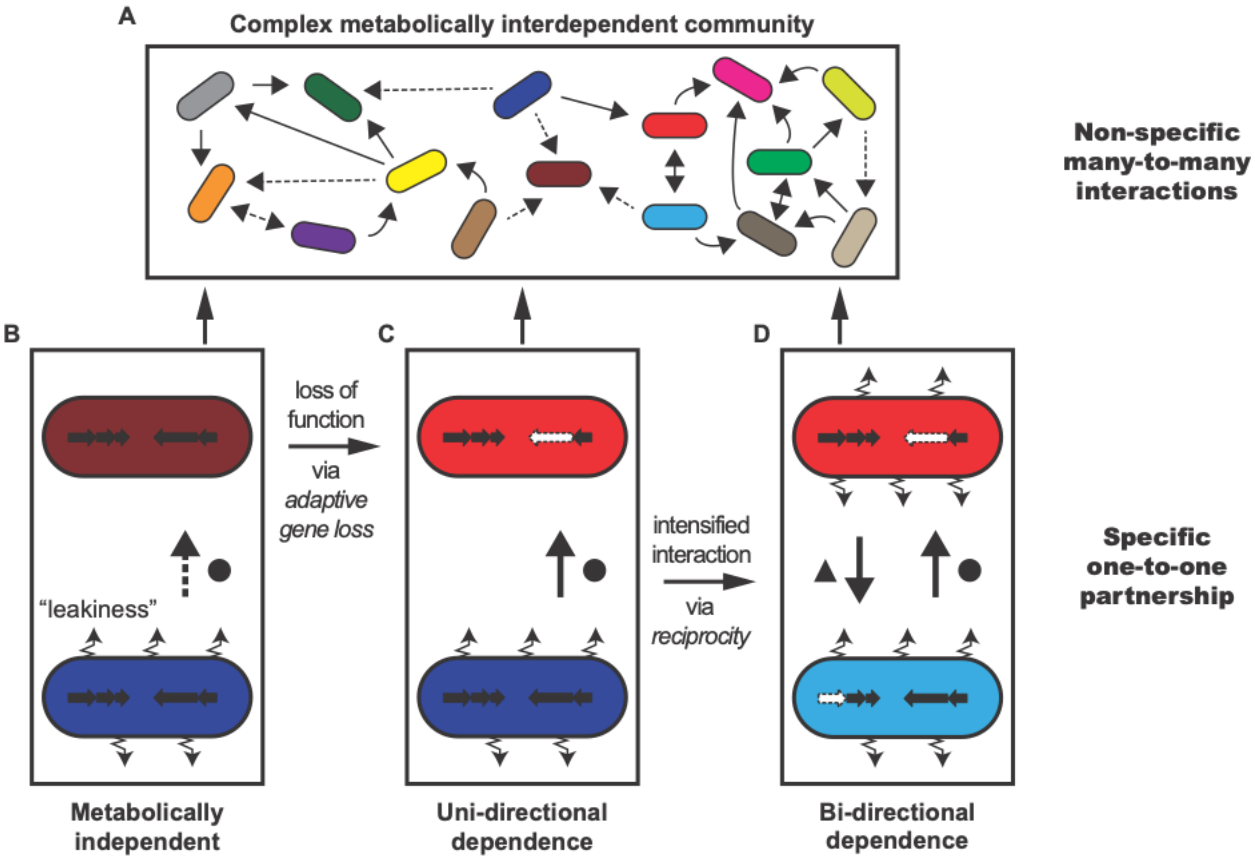

Figure 1. Structure and evolution of metabolically interconnected microbial communities. A. Microbes form complex networks of metabolic interactions in which many metabolites are shared among many species. **B-D.** Evolution of specific one-to-one partnerships. **B.** Generalists (dark red and dark blue) synthesize their own metabolites and thus are metabolically independent, but can engage in facultative interactions. **C.** A loss-of-function mutation via adaptive gene loss in one population (light red, auxotroph) is compensated by an obligate interaction with a 'leaky' producer

population (dark blue). **D.** Reciprocity facilitates intensified cooperation, resulting in a bi-directional obligate interaction. The original auxotroph (light red) supplies a growth-promoting metabolite to the original producer (light blue) which has evolved dependence on its partner. Facultative interactions are represented by dashed arrows and obligate interactions by solid arrows.

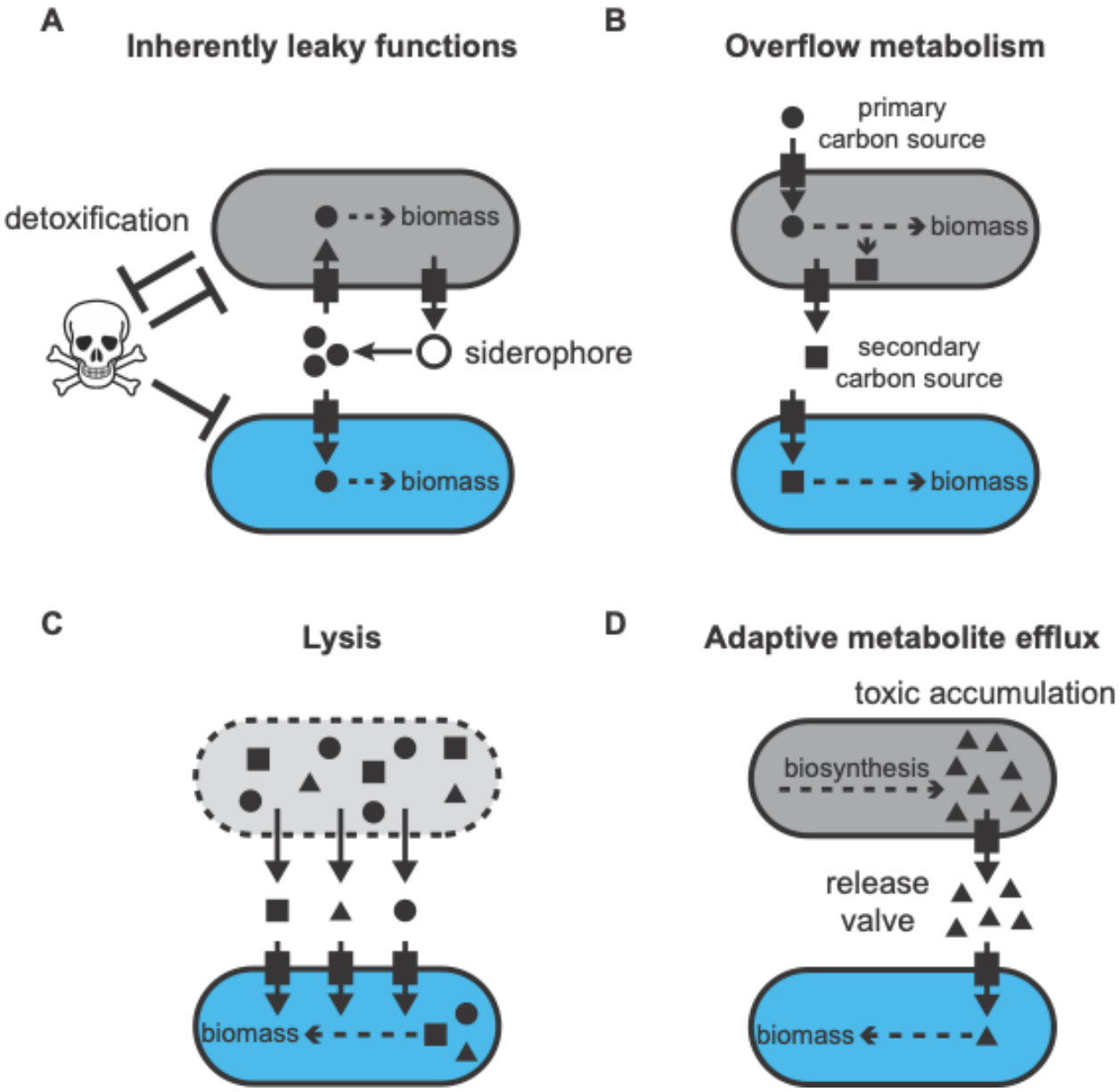

Figure 2. Mechanisms of metabolite provisioning via 'leakiness'. A. Some cellular functions are inherently leaky since they are performed outside of the cell. Examples include nutrient acquisition, such as siderophore secretion, and enzymatic detoxification of the environment, such as antibiotic degradation. B. Secondary carbon sources are released during overflow metabolism to maximize growth rate in the producer population (gray). C. Lysis, resulting from viral predation or other natural processes, releases intracellular contents into the environment. D. Accumulation of intracellular metabolites, which can inhibit growth, can be alleviated by specific metabolite efflux systems that function as a 'release valve'.

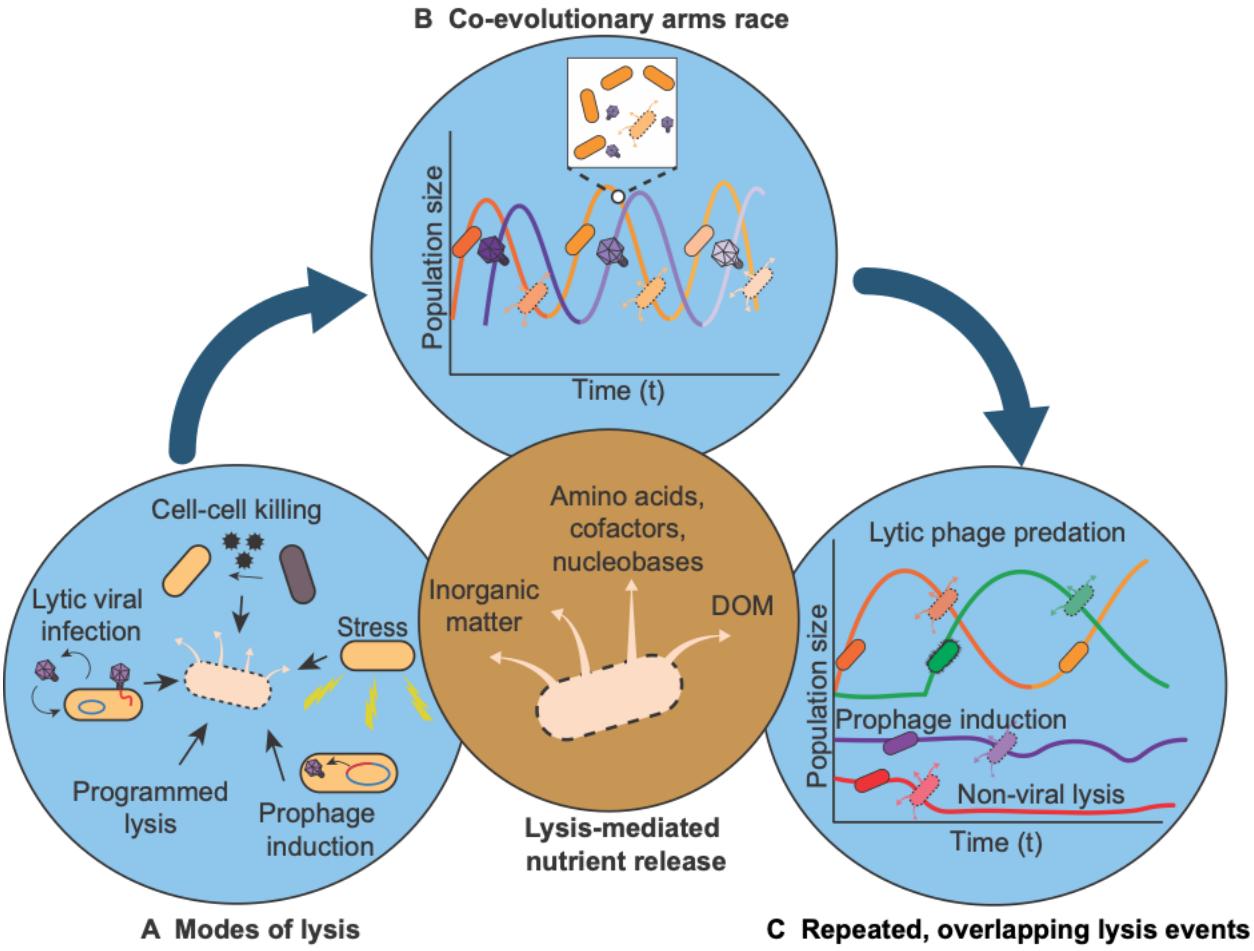

Figure 3. Lysis as a mechanism of nutrient provisioning. Lysis (center) releases inorganic matter (e.g., iron, phosphorus, and selenium), dissolved organic matter (DOM), and other intracellular metabolites synthesized in the cell (e.g., amino acids, cofactors, and nucleobases) into the environment. **A.** Different modes of lysis contribute to nutrient release. **B.** Viruses and their hosts co-evolve via Red Queen dynamics, resulting in population size and nutrient release fluctuations (boom-and-bust cycles). **C.** Overlapping lysis events across different populations can provide a sustained supply of metabolites.

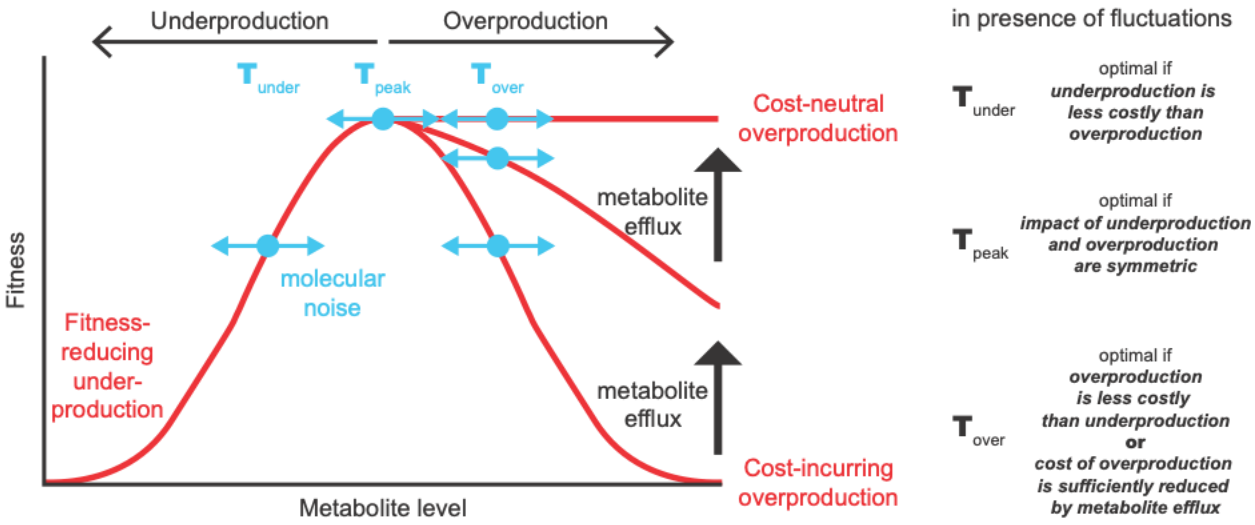

Figure 4. Regulated metabolite efflux can turn overproduction into an adaptive strategy. Fitness (red lines) is shown as a function of intracellular metabolite level. Underproduction or overproduction of a metabolite results in decreased fitness. Setting production targets (T, blue dots) is limited by inherent fluctuations (blue arrows) and the highly interconnected nature of metabolism. Regulated metabolite efflux can diminish the cost of overproduction by alleviating toxic effects of intracellular metabolite accumulation, shifting the fitness curve toward cost-neutral overproduction (vertical black arrows). Optimality of production targets (T_{under}, T_{peak}, or T_{over}) depends on the exact dependence of fitness on the intracellular metabolite level and the magnitude of fluctuations.

Chapter 3: Cell Lysis Releases Bioavailable Nutrients to Support Amino Acid Auxotroph Growth

Abstract

Auxotrophs - microbes unable to produce a required nutrient – have been identified in the majority of organisms across a range of taxa. Auxotrophy requires microbes to become nutritionally dependent on other cells in their community. The evolution of auxotrophy can be explained by the Black Queen Hypothesis, which posits that dependencies between microbes can evolve as a result of adaptive gene loss, where the loss of a gene becomes beneficial if enough of its product is present in the environment as a 'public good'. Interestingly, auxotrophies exist for essential nutrients, such as amino acids and cofactors, which are not thought to be inherently 'leaky'. Because amino acids and cofactors are produced and used intracellularly, it is unknown how or why such nutrients are released in significant enough quantities to support auxotrophic populations. Here I experimentally test the hypotheses presented in the previous chapter by exploring secretion and cell lysis as mechanisms of nutrient release that can support auxotrophic populations. I investigate this using *E. coli* amino acid auxotrophs to measure the amino acid bioavailability in supernatants, and cell lysates generated mechanically and by bacteriophage. I show that supernatants and mechanical lysates minimally support amino acid auxotrophy, while cell lysates from bacteriophage release a significant amount of bioavailable nutrients that support different amino acid auxotrophies. The amino acid type, mode of nutrient release and donor species are each factors shaping how well amino acid auxotrophs are supported. This work helps contribute to a mechanistic understanding of how auxotrophic community members may be supported in natural communities, and how auxotrophies may evolve for nutrients that are not inherently leaky.

Introduction

Nearly all microbes exist in complex communities, and collectively contribute to shaping community structure, composition and function by participating in interactions with other microbes. Nutritional interactions, such as competition for carbon sources, the transformation of one metabolite into another for a neighboring cell, as well as uni- and bi-directional forms of nutrient exchange, play important roles in maintaining microbial community diversity and stability.

Within communities, not all microbes are able to produce all of their required nutrients and, as a result, rely on neighboring microbes to meet their metabolic needs. Microbes that are unable to produce a required nutrient are called auxotrophs. Auxotrophs have been identified for a variety of nutrients, such as cofactors [33, 52, 132], nucleobases [133], and amino [25, 42, 60]

Amino acid auxotrophy has been studied in many systems, including endosymbionts of insects [31, 134], natural communities through metagenomics [25, 42], and synthetic co-cultures [37, 39, 135]. The true pervasiveness of auxotrophy needs to be tested because some predictions based on sequence may not always accurate [136], but metagenomic analyses suggest that auxotrophy may be common across many environments [42, 52, 58] [53].

Auxotrophies are thought to develop via mutations that result in gene loss from metabolic pathways. This type of gene loss can conserve energy when a microbe's metabolic needs can be met by neighboring microbes, rather than using energy to produce a gene product [44]. For example, in nutrient-rich environments, biosynthetic genes are found to be lost through selection [53]. This selective force has been described as The Black Queen Hypothesis (BQH), which

predicts adaptive gene loss when an essential function is sufficiently available in the environment, or 'leaky' enough, to support the needs of the community [57]. At first glance, the maintenance of auxotrophy in a microbial community seems counterintuitive, because increased dependence on other community members would make auxotrophs vulnerable to extinction. However, synthetic co-cultures and work from natural communities suggests that auxotrophs may be important for maintaining metabolic diversity [41], species heterogeneity [25], and overall community stability [137].

Amino acid auxotrophies are especially interesting because amino acids are essential for protein synthesis and some are precursors for nucleotides and cofactors. Natural isolates with amino acid auxotrophies have been found in a number of opportunistic and obligate pathogens[138], including *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients [60, 139]. Additionally, in a naturally occurring 5-member methanogenic community, no single species was able to synthesize all of its amino acids, revealing a division of labor for amino acid biosynthesis across the community [25]. What remains less clear about amino acid auxotrophs are the mechanisms of nutrient release that allow them to survive.

One mechanism of amino acid release is through secretion. Amino acid transporter proteins can mediate the secretion of amino acid through processes such as overflow metabolism or limited catabolism, both of which are responses to the amino acid accumulation inside the cell [66, 140, 141]. A similar mode of nutrient release is the formation of lipid-based nanotubes that are found to form between *E. coli* and *Acinetobacter baylyi* , but they only form under specific nutrient starvation conditions [142]. Another potential mechanism of nutrient release is through cell lysis. Some examples include 'sloppy feeding' in which a bacterial cell membrane is broken by grazing cells [95], programmed cell death in response to stress or developmental programs [143], and lysis resulting from interactions with microbial toxins or type VI secretion systems [144, 145].

Cell lysis can also be initiated by bacteriophages (phages). The contribution of phage-mediated lysis to marine biogeochemistry and nutrient cycling has been well documented. Phage are significant contributors to bacterial mortality [86-88]; and phage lysates have been shown to contain a host of organic (i.e. C, P, N) and inorganic nutrients (i.e. Fe, Se), as well as dissolved free amino acids and dipeptides [93, 97, 146, 147]. Additionally, phages can reprogram the host's metabolism in ways that give infected hosts and uninfected cells compositionally distinct metabolite profiles [96, 148, 149]. Therefore, it seems plausible that viral lysis could contribute nutrients, such as amino acids, to support auxotrophs.

In this work, I explore secretion, mechanical cell lysis and phage-mediated lysis as mechanisms of nutrient release that are able to support auxotrophic communities. I show differences in amino acid bioavailability between the supernatants, mechanically generated lysates, and phage lysates of *Escherichia coli* and *Bacteroides thetaiotaomicron*, using a set of 11 *E. coli* amino acid auxotrophs. Based on the results presented below, I hypothesize that bacteriophage-mediated lysis is a dominant form of nutrient release in natural environments, especially for essential nutrients that do not appear to be inherently leaky.

Methods

Bacterial Culturing and Media

E. coli strains were streaked from freezer stocks onto LB agar plates. Single colonies were inoculated into 2 mL H1 minimal media supplemented with 0.2% glycerol (50 mM KPO_4, 67 mM

NaCl, 7.6 mM (NH4)SO4, 500 uM MgSO4, 1.25 uM Fe2(SO4)3, 0.2% [v:v] glycerol at pH 7.4) and grown with aeration at 200 rpm overnight at 37°C prior to all experiments. *E. coli* auxotrophs were supplemented with their required amino acid for all pre-culturing (methionine: 0.2 mg/mL, arginine: 0.2 mg/mL, proline: 0.5 mg/mL, glycine: 0.32 mg/mL, histidine: 0.625 mg/mL, phenylalanine: 0.2 mg/mL, lysine: 0.3 mg/mL, threonine: 0.2 mg/mL, leucine: 0.03 mg/mL, serine: 1.5 mg/mL, glycine: 0.625 mg/mL, tryptophan: 0.5 mg/mL).

B. thetaiotaomicron was routinely streaked onto Bacteroides Phage Recovery Media (BPRM) plates as described in [150, 151]; per liter of broth, 10 g casein peptone, 10 g meat peptone, 2 g yeast extract, 5 g NaCl, 0.5 g L-cysteine, 1.8g glucose, and 0.12g MgSO4 heptahydrate was added. After autoclaving, 10mL of 0.2um-filtered hemin solution (0.1%w/v in 0.02%NaOH), 1mL of sterile 0.05 g/mL $CaCl_2$ solution and 25mL of sterile 1 M Na_2CO_3 was added. For BPRM agar plates 15 g/L agar was added, and for BPRM top agar 7 g/L of agar was added. Prior to pouring BPRM agar and BPRM top agar, hemin and Na_2CO_3 were added. Strains were grown on BPRM media for 2 days, and inoculated into 5 mL BPRM or defined minimal media in an anaerobic chamber (Coy Labs) containing 10% H_2, 10% CO_2, and 80% N_2.

Defined minimal media for *B. thetaiotaomicron* was made as described; per 500 mL of broth, 50 mL Mineral 3B solution (per liter; 18 g KH_2PO_4, 18 g NaCl, 0.4 g $MgCl_2$ $6H_2O$, 0.52 g $CaCl_2$ 2H2O, 0.02 g $CoCl_2 \cdot H_2O$, 0.2 g $MnCl_2$, 10 g NH_4Cl), 5 g Na_2SO_4), 10 mL Hemin solution (100mg of hemin dissolved in 2 mL of 1 M NaOH and dH_2O brought up to 200 mL), 10uL of 1 mM vitamin B_{12}, 1.5 mL $FeSO_4$ solution (0.278 g $FeSO_4 \cdot H_2O$ per 100mL), 20 mL 20% sucrose, and adjusted to pH 7.1. After autoclaving, 20 mL of 10% $NaHCO_3$ and 10mL L-cysteine (100 g/L) was added to the broth.

Generating Mechanical Lysates for Auxotroph Assay

To generate *E. coli* mechanical lysates for the auxotroph assay, single colonies of *E. coli* MG1655 were inoculated into H1 minimal media and grown overnight to saturation. Cell cultures were then diluted 1:100 in fresh H1 minimal media. Once cells reached mid exponential growth ($O.D._{600}$ = 0.3-0.4), cells were centrifuged at 6000 rpm for 10 minutes and the supernatant was filtered using a 0.2 um filter and stored at -80 °C for further analysis. The cell pellet was washed 3 times with H1 minimal medium, resuspended in fresh medium and cells were lysed using a French Press (Thermo Fisher Scientific). Media alone was passaged through the french press and used as a control during the growth assays. The number of lysed cells was measured by removing aliquots of *E. coli* cultures before and after the french press. These aliquots were serially diluted 10-fold in 96 well plates, spot plated onto LB agar plates, and grown aerobically overnight at 37°C. The difference in CFU between the samples before and after lysis was calculated to be the number of cells lysed. This measurement was performed in triplicate.

To collect the mechanical lysate for the auxotroph assays, french pressed samples were centrifuged for 15 minutes at 6000rcp to pellet cell debris, and the supernatant was filtered using a 0.2um filter and stored at -80°C. Mechanical *B. thetaiotaomicron* lysates were generated similarly, except this strain was grown to mid exponential phase anaerobically at 37°C in defined minimal media supplemented with 0.4% sucrose. The number of cells lysed using the french press was measured by taking aliquots of *B. thetaiotaomicron* cultures before and after the french press, serially diluting 10-fold in 96 well plates, spot plating onto BPRM agar plates, and growing the plates anaerobically at 37°C for 2 days. Each lysate was generated in triplicate.

One-Step Phage Growth Curve

The one-step phage growth curve was used to determine the average phage burst size and the amount of time required to generate phage lysates for the auxotroph assay [152] . One step growth curves were performed in triplicate as in [152]. Briefly, *B. thetaiotaomicron* was grown in Bacteroides Minimal Media supplemented with 0.4 % sucrose at 37 °C in the anaerobic chamber (10% H_2, 10% CO_2, and 80% N_2) to a cell density ~1 x 10^7 CFU/mL. The ϕSJC12 phage lysate was added to obtain a multiplicity of infection (MOI) of 0.01 (1x10^5 PFU/mL). Bacteria-phage mix was incubated for 10 min at room temperature (RT) to allow for phage adsorption. Afterwards, cultures were placed at 37 °C and 0.4 mL aliquots were taken for each timepoint. Half of the sample was added to a few drops of chloroform, vortexed for 10 seconds, and placed at room temperature until the chloroform settled. The remaining 200 ul were stored on ice and processed during the experiment. These samples were diluted 10-fold up to 10^{-8}, and spot plated onto BPRM top agar overlays (0.35 % w/v) to measure infective centers and free phage, and plated onto BPRM agar (1.5 % w/v) to measure cell survival following phage addition. Three biological replicates were measured for each growth curve. At the end of the experiment, chloroformed samples were diluted 10-fold up to 10^{-8}, spot plated onto BPRM top agar overlays (0.35 % w/v), and grown overnight in an anaerobic chamber. One step growth curves for T4rI and λvir were performed similarly, except that cultures were grown aerobically in H1 minimal media supplemented 0.2% glycerol at 37°C. For PFU counts, samples were spot plated on LB top agar overlays (0.7% w/v) and CFU measurements were spot plated on LB agar (1.5% w/v).

Generating Phage Lysates for Auxotroph Assay

To generate *E. coli* phage lysates for the auxotroph assay, overnight cultures of *E. coli* MG1655 were grown to saturation and diluted 1:100 in fresh H1 minimal media. Once cells reached mid exponential growth ($O.D._{600}$ ~ 0.3), T4*rI* phage was added at an MOI of 3 for 100 minutes. Each phage lysate had a mock treatment that served as a control. The mock treatment was created by filtering phage stocks twice using 100 kDa centrifugal filters (Amicon). After 100 minutes, the phage treated culture and the mock treatment were collected by centrifuging the cell cultures at 6000 rpm for 10 minutes, and filtering out the bacteria using a 0.2 um filter. Aliquots of the phage treated culture were taken at the beginning and end of the incubation period and

titered. The difference between these two aliquots was used to calculate the number of new phage particles generated during incubation. This value was divided by the burst sizes calculated from the one-step growth curves, to estimate the number of cells lysed by phage. The samples were further processed using a 100 kDa centrifugal filter to remove phage particles. Samples were stored at -80 °C. Phage lysates for λvir was performed similarly except the incubation time was 150 minutes. Phage lysates from *B. thetaiotaomicron* were similarly processed, except the strain was grown anaerobically at 37°C in defined minimal media supplemented with 0.4% sucrose. *B. thetaiotaomicron* was incubated with φSJC12 for 4 hours. All lysates were generated in triplicate.

***E. coli* Auxotroph Assay**

Amino acid auxotrophs were pre-cultured in biological triplicates aerobically in H1 minimal medium supplemented with 0.2% glycerol at 37°C with their required amino acid for 18-24 hrs until saturation, and then washed 3 times in H1 minimal media to remove amino acids supplemented during the pre-culturing step. Following the wash step, the cultures were diluted to an $O.D._{600}$ = 0.02 in H1 minimal media supplemented with 0.4% glycerol and 100ul of culture was dispensed into 96-well plates (Corning ®). One hundred microliters of each lysate treatment was

then added to each well, bringing the final $O.D._{600}$ to 0.01, and the final glycerol concentration to 0.2%. The plates were shaken at 1200rpm for 24 hours at 37°C in a Southwest Science Heated plate shaker. Absorbance measurements were measured using a multi-well plate reader (Tecan Spark). Colony forming units (CFU) were calculated after 24 hours of growth by spot platting 10-fold serial dilutions onto LB agar plates.

Phage Lysate Preparation and Titering

E. coli MG1655 was used as the host for the propagation of T4*rI*, which was received from the Carolina Biological Supply Company. High titer stocks of *E. coli* phage were generated by growing cultures at 37 °C with aeration in either LB or H1 minimal medium to $O.D._{600}$ = 0.2-0.3, and adding phage to an MOI of 0.01. After about 7 hours, chloroform was added to lyse remaining cells. After the chloroform settled, the sample was centrifuged at 7000 rpm and the lysate was filtered using a 0.2um filter. Phage stocks were titered by spot plating onto soft agar overlays. High titers for λvir were generated similarly.

B. theta was used as a host for the propagation of φSJC12 phage. φSJC12 was received from Andrew Hryckowian and Bryan Merrill, and was isolated from the San Jose-Santa Clara Regional Wastewater Facility. To generate high titer stocks of φSJC12, *B. thetaiotaomicron* was generated similar to Hyrckowian et al, 2020 [151]. In brief, a soft agar overlay method was used where 0.5mL of saturated *B. thetaiotaomicron* culture was combined with 1-10 uL of high titer φSJC12 stock for 20 minutes to allow for phage adsorption. Then 4.5mL of molten BPRM top agar (0.35%) was poured onto BPRM agar (1.5 %) and incubated anaerobically overnight at 37°C. Top agar overlays that showed a "lacey" pattern (confluent lysis) were flooded with sterile phage buffer (an autoclaved solution of 5 mL of 1 M Tris pH 7.5, 2 g NaCl, 5 mL of 1 M MgSO4, in 500 mL of ddH2O) and incubated at room temperature for at least 2 hours to suspend the phage. The phage buffer was removed, passed through a 0.2 um filter and stored at 4 °C for future experiments. For phage lysates used to generate lysate treatments for the auxotroph assays, minimal media top agar was used instead of BPRM top agar.

Calculating Amino Acid Auxotrophs per Lysed Cell

To calculate the number of auxotrophs supported per lysed cell, the difference in CFU between the treatment (i.e. phage, French press, or supernatant) and its control (i.e. mock phage, French press control, no addition control) was divided by the estimated number of lysed cells. See above sections for estimation of cell lysis for mechanically generated lysates and phage generated lysates.

Calculating Amino Acid Concentration per Cell

A standard curve was used to determine the concentration of amino acid required to reach its maximum growth yield (A_{max}) (See Bacterial Culturing and Media for amino acid concentrations). I calculated the difference in CFU (C_{diff}) between auxotrophs grown on the amino acid concentration determined from the standard curve and auxotrophs grown without amino acids supplemented. I then used the concentration of amino acid used in the culture (A_{max}) to calculate the total molecules of amino acid per mL of culture (M_{max}). From here, I calculated how many CFU (T) were supported per molecule of amino acid (C_{diff} divided by M_{max}), and then dividing by the difference in CFU between treatments (i.e. phage treatment – phage mock treatment), to calculate the molecules of amino acid per mL (B). To calculate ug/mL of amino acid, I multiplied B by the molecular weight of the amino acid, and divided by Avogadro's number.

Results

Supernatants support minimal growth of amino acid auxotroph populations

Synthetic co-culture experiments have shown that amino acid sharing is possible when two strains with different auxotrophies are cultured together [42, 135]. In some cases, co-cultures are not productive without further engineering or evolving one of the strains to excrete or produce more of a given metabolite [38]. These examples suggest amino acids can be secreted from producers, and sometimes, at levels high enough to support an auxotrophic strain. To explore this hypothesis systematically, I tested whether *E. coli* and *B. thetaiotaomicron* naturally excreted amino acids at levels sufficient to support auxotrophs by measuring the growth of amino acid auxotroph strains cultured with supernatants from wild type bacteria. This is distinct from published co-culture experiments because I am specifically measuring the amount of bioavailable amino acids from the supernatant collected from a growing culture.

I collected cell-free spent media from wild-type *E. coli* and *B. thetaiotaomicron*, each cultured in minimal media. I then cultured a set of eleven amino acid auxotrophs on these supernatants without amino acid supplementation and measured how well each auxotroph grew based on viable cell counts (colony-forming units, CFU) and optical density ($O.D._{600}$). I calculated the number of auxotroph cells supported per donor cell by comparing the auxotroph viable cell count to the number of wild-type cells measured in the original culture. I found that supernatants from both *E. coli* and *B. thetaiotaomicron* minimally supported most of the 11 amino acid auxotrophs used in this study, with each wild type donor cell supporting the growth of less than 0.1 auxotroph cells in all except one case (Fig. 1A and 1B). The exception was the Trp auxotroph, in which each donor cell was estimated to support about one Trp auxotroph. Together, these results suggest that secretion may not be a dominant form of amino acid provisioning.

Mechanically lysed cells support more auxotrophic growth than supernatants

Bacteria tightly regulate intracellular pools of amino acids and other metabolites [153], and the concentration of these intracellular pools can shape flux through metabolic reactions and dictate reaction rates based on whether enzymes sites are predominately filled or empty [154]. It is possible that lysing bacteria would release concentrated nutrient pools inside the cell, and provide more bioavailable nutrients than what is typically secreted by bacteria. To test this, I mechanically lysed *E. coli* and *B. thetaiotaomicron* and grew the auxotrophic strains on these lysates. I compared the growth of amino acid auxotrophs on the mechanical lysate to the number of lysed cells to calculate the number of auxotroph cells supported per mechanically lysed cell.

The results show that mechanically lysed *E. coli* contains more bioavailable amino acids than *E. coli* supernatants, though less than one auxotroph cell was supported by each lysed donor cell (Fig 1C). Lysates generated from mechanically lysed *B. thetaiotaomicron* also contain more bioavailable amino acids than what was present in the supernatant collected from *B. thetaiotaomicron* (Fig 1D). Additionally, comparing across mechanical lysates from *B. thetaiotaomicron* and *E. coli* suggests that these bacteria contain different amounts of several amino acids (Fig 1C and 1D). This is not surprising given that different species of bacteria have distinct metabolic needs and processes. Interestingly, for *B. thetaiotaomicron*, the majority of the amino acid auxotrophs that were most supported by mechanical lysates trended towards more biosynthetically costly amino acids. The overall conclusion of these results is that mechanical

lysates provisioned more amino acids than supernatants, but still support less than one auxotroph per donor cell.

Differences in Amino Acid Bioavailability from Cells Lysed in Stationary versus Exponential Phase

Microbes can exist in dense communities, and are expected to fluctuate between exponential and stationary phase growth depending on nutrient availability. Metabolism shifts as bacteria enter different growth phases, and when bacteria enter long periods of nutrient starvation they can undergo dramatic physiological changes [155].

The experiments presented above measure amino acid bioavailability in bacterial cells lysed during exponential growth. I next tested how different growth phases could affect the level of amino acids released in supernatants and mechanically lysed cells. In this study, I lysed *E. coli* cells in stationary phase and exponential phase by sonication, and measured the percent growth yield differences across different supernatant and mechanical lysate treatments using 7 of the 11 amino acid auxotrophs.

I observed distinct differences in the ability of lysates and supernatants of exponential versus stationary phase donor cultures to support the growth of several of the auxotrophs, as measured by the maximal $O.D._{600}$ achieved in comparison to cultures containing excess of the required amino acid (Fig 2). The majority of tested amino acid auxotrophs were more supported by supernatants from stationary phase than exponential phase. For mechanically lysed cells, Met, Arg, and Thr were more bioavailable in stationary phase lysates than exponential phase lysates, while Leu, Gly and Trp were more bioavailable from exponentially growing cells. This suggests that some amino acids are sequestered during exponential phase and released during stationary phase, while for others, the concentration of amino acids in lysates and supernatants increase or decrease together from exponential to stationary phase.

These findings align with work from other studies that directly measured metabolites from the exometabolome of *E. coli* grown in batch culture. Carbon intermediates and a small number of amino acids were measured in the supernatant of *E. coli* grown over time, and differences were observed between the concentration of metabolites during the early growth stage when compared to later stages [15, 18]. In summary, the level of bioavailable amino acids in supernatants and mechanical lysates is distinct between stationary and exponential phase cultures, and these differences could significantly affect the level of nutrient provisioning required to support auxotrophs.

Phage lysates strongly support auxotrophs of biosynthetically costly amino acids

Phages are highly abundant and ubiquitous in natural environments [83]. Phage-infected host cells have vastly different metabolisms from uninfected cells, resulting in different extracellular metabolite profiles [149]. This suggests that following phage infection, changes in flux through the host's metabolism could also influence the concentrations and types of metabolites that become available to neighboring microbes following lysis.

To investigate how phage infection and lysis shapes amino acid bioavailability, I generated phage lysates from wild-type *E. coli* and *B. thetaiotaomicron* using lytic phages. For *E. coli*, I used T4*rI* phage, a 'rapid lysis' T4 mutant that is unable to undergo lysis inhibition (LIN), and measured the growth of amino acid auxotrophs on *T4rI* phage lysates following filtration to remove phage particles. *T4rI* phage lysates strongly support auxotroph growth, and depending on the amino acids, one donor cell can provision enough nutrients to support anywhere between 11 and 47

auxotrophs (Fig 3A) . For each of the amino acid auxotrophs tested, T4*rI* phage lysate supported significantly more auxotrophs per lysed cell than the mechanically generated *E. coli* lysate.

Given that T4*rI* phage lysate is able to provide more bioavailable nutrients than mechanically lysed cells, I next tested whether the type of phage affects the concentration and types of metabolites released following lysis. To study this, I generated *E. coli* lysates using λvir, a lytic version of wild-type λ that is unable to lysogenize its host. Similar to T4*rI* phage lysate, λvir phage lysates provision high concentrations of amino acids that are able to support as many as 4 to 27 auxotrophs per donor cell (Fig 3B). Lysates generated by T4*rI* phage supported close to double the number of Phe and His auxotrophs as λvir lysate, and nearly 6 times more Met and Lys auxotrophs.

Microbial communities contain many different species of bacteria , which may all be lysing at similar times. To explore how the lysis of different bacterial species can affect the types of amino acids that are bioavailable, I infected *B. thetaiotaomicron* with the lytic phage φSJC12 [151]. Similar to *E. coli* phage lysates, φSJC12 phage lysate supports auxotrophy to a higher degree than *B. thetaiotaomicron* mechanical lysates, with, in some cases, 33 to 38 auxotrophs being supported per donor cell (Fig 3C). Additionally, these results suggest that different bacteria have the potential to provision higher concentrations of some amino acids in comparison to others. For example, one φSJC12 lysed *B. thetaiotaomicron* cell is able to support around 34 Lys auxotrophs, in comparison to 12 and 2 auxotrophs for T4*rI* and λvir, respectively. And *E. coli* phage lysates are able to better provision Leu, with T4*rI* lysates supporting 26 Leu auxotrophs and λvir lysates supporting 20 Leu auxotrophs per lysed cell, while one φSJC12-lysed donor cell supports only one Leu auxotroph. This suggests that the phage lysis of different bacteria may be important in provisioning different types of nutrients for auxotrophs in the community.

Concentration of Bioavailable Amino Acids in Mechanical and Phage Lysates

Absolute concentrations of intracellular metabolites, including some amino acids, have been measured in *E. coli* grown on multiple carbon sources [154]. These measurements are useful for understanding how intracellular metabolite concentrations affect free energy and the rate of metabolic reactions inside cells. Chemical procedures for measuring absolute concentration do not tell us the relationship between intracellular metabolite concentration, and the concentration required for cell growth. Using amino acid auxotrophs to measure amino acid bioavailability, I can learn how the concentration of an amino acid inside a cell correlates with the concentration that is bioavailable, or accessible to neighboring cells, when a cell is lysed.

To estimate the concentration of bioavailable amino acids per lysed cell, I supplemented each amino acid auxotroph with a range of concentrations of the required amino acid to determine the minimum concentration required to reach maximal growth. I then used this value to calculate the number of cells supported for a given concentration of amino acid added. This was used to determine the total concentration of amino acid required to support the growth of one auxotrophic cell. I used this value to estimate the concentration of amino acid in the mechanical and phage lysate treatments (Table 3).

The concentration of Phe and Trp measured in the mechanical lysate from *E. coli* are comparable to previously reported LC-MS/MS data in *E. coli* [154]. The amino acid concentrations measured using the *E. coli* auxotroph strains for most amino acids was less than those measured using LC-MS/MS. It is possible that my *E. coli* mechanical lysate measurements are lower because amino acids are somewhat inaccessible when incorporated into proteins.

When comparing *E. coli* phage lysates to the absolute concentrations measured for each amino acid in Bennet et al, 2009, many of the amino acids were measured to be higher in the phage lysates. Viral lysates have more diverse and compositionally distinct organic matter than mechanical lysates and supernatants (Ma et al, 2018), and it would be interesting to explore whether amino acid bioavailability is affected by these differences.

Discussion

Nutrient provisioning is a key element involved in maintaining microbial community diversity and stability. Auxotrophs that have lost the ability to synthesize essential nutrients rely on nutrient provisioning to fulfill their metabolic needs. Amino acid auxotrophy is predicted to be common across bacteria [42, 108], and co-culture experiments have explored nutrient exchange, cooperativity and community stability between auxotrophs and have been seminal to refining the rules for how metabolite interactions shape microbial communities [37, 39, 42]. Yet, the mechanism for how nutrients are released in significant enough quantities to support amino acid auxotrophs has not been explored in detail.

When investigating whether donor cells could secrete enough amino acid to support amino acid auxotrophs, very little amino acid provisioning was observed. In *E. coli*, most amino acid auxotrophs, except the Trp auxotroph, required a range of 10-100 donor cells to support a single auxotroph (Fig 1A). The Trp auxotroph may grow better on supernatant than other amino acid auxotrophs because when *E. coli* is grown with glycerol as a carbon source, Trp is converted into indole, ammonia and pyruvate at high rates as a result of catabolite repression [156]. The supernatant collected from wild-type *E. coli* may contain indole precursors, and indole can be converted into Trp by the Trp auxotroph. This highlights that one limitation of this study is that most auxotrophs were generated by a single gene mutation, so it is possible that some strains could be using precursor molecules that are further downstream of the deleted gene. Trp is also one of the most lipid permeable amino acids because of its hydrophobicity [157], which could result in more Trp being released into the supernatant than other amino acids.

When studying whether lysis could be a significant source of bioavailable amino acids, it became clear that, for most amino acids, mechanical lysates supported more auxotroph growth than supernatants in *E. coli*. This result was similar when comparing mechanical lysates and supernatants from *B. thetaiotaomicron* . This difference was expected because bacteria regulate the size of intracellular amino acid pools based on metabolic requirements [153], and I would expect these pools to serve as reservoirs of nutrients following lysis. Based on my results, mechanically lysed cells could be a source of amino acids for amino acid auxotrophs, and potentially other types of auxotrophy, even though grazing and other forms of shear forces that can disrupt microbial membranes may not be as common as phage-mediated lysis.

Phages are a significant source of naturally occurring lysis, and have been shown to be major drivers of microbial turnover and nutrient cycling in marine environments and soils [86, 100]. Additionally, in laboratory experiments where *S. typhimurium* is co-cultured with *E. coli*, *S. typhimurium* grows to higher levels when *E. coli* T7 phage is present, relative to conditions without phage [115]. This lends additional evidence for the ability of phage lysis to provide bioavailable nutrients to support the growth of neighboring microbes.

Phage-mediated lysis, across host and viral strains, released large concentrations of amino acids in comparison to both supernatant and mechanical lysate treatments. This suggests that phage lysed cells can be large reservoirs of amino acids for auxotrophs in nature. Additionally, T4*rI* and

λvir phage lysates have distinct bioavailability profiles, where one phage is better able to support a specific auxotroph than the other, revealing how lysis by different phages that infect the same host may have different capacities for supporting amino acid auxotrophs. Relatedly, phage lysates from *E. coli* showed a different set of bioavailable amino acids than the phage lysate from *B. thetaiotaomicron*. Together, this provides evidence that the phage and species of bacteria lysed can affect the types of nutrients available for auxotrophs.

The energy required to synthesize an amino acid varies considerably across amino acid types [63, 67], and the metabolic cost can differ based on the carbon source available [64]. Interestingly, strains auxotrophic for more biosynthetically costly amino acids were better supported by phage lysates and B. *thetaiotaomicron*'s mechanical lysate. For *E. coli* phage lysates, Phe, His, Met, and Leu auxotrophs were best supported, and for *B. thetaiotaomicron* phage lysates, Phe, His, and Lys auxotrophs were best supported in comparison to less energetically costly amino acids (Fig 3). Bacteria tend to use less costly amino acids for highly expressed proteins, revealing the relevance of major codon usage bias during protein synthesis [63]. The ability of lysates to better support auxotrophs for costly amino acids could be a reflection of codon usage biases.

In an analysis of more than 6,000 bacterial genome sequences, over 80% were predicted to be auxotrophic for Phe and His, and over 70% for Leu and Lys [42]. The results presented here show that the phage lysates I tested can support these auxotrophs, whose required nutrients are expensive. Given that different hosts are better at provisioning specific amino acids than others (Fig 3), the less costly amino acids may be acquired from other bacteria that were not tested in this study. Together, these data support that viral-mediated lysis can be a mechanism of nutrient release that supports amino acid auxotrophs, especially auxotrophs for nutrients that may be costly to produce *de novo*.

Cell lysis is a challenging process to study because there are a lack of methods for detecting and measuring cell death. This work and other research on phage lysis and cell death continue to show the importance of better understanding lysis, especially as it relates to nutrient provisioning. Microbes are contributing to extracellular nutrient pools when their membranes are ruptured due to environmental, chemical, and biological processes, and phages are likely to play a key role in this contribution.

Bacteria	Strain	Auxotrophy	Genotype	Reference
E. coli	JW2786-1	Arginine	*ΔargA::kan*	(Baba et al, 2006)[158]
E. coli	MG1655	Methionine	*ΔmetA, ΔmetB, ΔmetE, ΔmetH, ΔmetC::kan*	This study.
E. coli	JW2535-1	Glycine	*ΔglyA::kan*	(Baba et al, 2006)
E. coli	JW2004-1	Histidine	*ΔhisB::kan*	(Baba et al, 2006)
E. coli	JW5807-2	Leucine	*ΔleuB::kan*	(Baba et al, 2006)
E. coli	JW2806-2	Lysine	*ΔlysA::kan*	(Baba et al, 2006)
E. coli	JW2580-1	Phenylalanine	*ΔpheA::kan*	(Baba et al, 2006)
E. coli	JW0233-2	Proline	*ΔproA::kan*	(Baba et al, 2006)
E. coli	JW2880-1	Serine	*ΔserA::kan*	(Baba et al, 2006)
E. coli	JW0003-2	Threonine	*ΔthrC::kan*	(Baba et al, 2006)
E. coli	JW1254-2	Tryptophan	*ΔtrpC::kan*	(Baba et al, 2006)
E. coli	MG1655	Wild-type		This study
B. thetaiotaomicron	parental	Wild-type		This study

Table 1: List of bacterial strains used in this study.

Strain	Host	Reference
T4rI	*E. coli*	Carolina Biological Supply Company
λvir	*E. coli*	This study.
φSJC12	*B. thetaiotaomicron*	(Hryckowian et al, 2020) [151]

Table 2: List of viral strains used in this study.

		Calculated ug/mL Amino Acid				
	ug/mL Amino Acid LCMS (Bennett et al, 2009)	Ec French Press	λvir	T4*rI*	Bt French Press	ϕSJC12
Lys	111.4	0.19 ± 0.23	3.99 ± 1.27	16 ± 5.2	1.51 ± 0.16	14.75 ± 3.08
Pro	108.38	8.18 ± 7.54	0.30 ± 0.11	0.11 ± 0.07	2.16 ± 0.37	2.03 ± 0.89
Iso	57.45	-	-	-	-	-
Leu		0.35 ± 0.16	4.85 ± 2.16	3.41 ± 1.02	0.1 ± 0.03	0.05 ± 0.03
His	27.15	10.72 ± 3.11	81.18 ± 32.49	29.50 ± 43.41	8.13 ± 0.62	43.77 ± 5.65
Met	19.25	1.50 ± 0.23	6.58 ± 1.64	29.43 ± 8.01	4.77 ± 0.75	0.28 ± 0.24
Phe	6.95	3.46 ± 0.68	40.85 ± 14.26	42.47 ± 11	2.76 ± 0.70	12.67 ± 0.75
Trp	4.82	7.99 ± 2.91	0.01 ± 0.002	0.22 ± 0.17	15.97 ± 10.50	0.24 ± 0.08
Ser	1.58	12.73 ± 4.07	0.003 ± 0.001	0.15 ± 0.07	-	-
Thr	21.32	1.47 ± 0.22	0.01 ± 0.006	0.014 ± 0.003	0.19 ± 0.012	0.26 ± 0.17
Arg	99.12	3.88 ± 1.88	0.20 ± 0.1	0.17 ± 0.07	0.64 ± 0.13	0.12 ± 0.11
Gly	not measured	96.26 ± 40.49	0.03 ± 0.005	0.03 ± 0.02	-	-

Table 3: Calculated concentrations of bioavailable amino acids for mechanical and phage lysates. Amino acids are organized from highest concentration to lowest concentration (except for threonine and arginine) based on the Bennet et al, 2009 analysis[154].

A. The absolute concentrations of amino acids grown on M9 minimal media supplemented with 0.2% glycerol as measured from Bennet et al, 2009. The absolute concentration for threonine and arginine were only measured on glucose in Bennet et al, 2009. All other amino acids were measured on glycerol in M9 media. Glycine was not measured in their analysis.

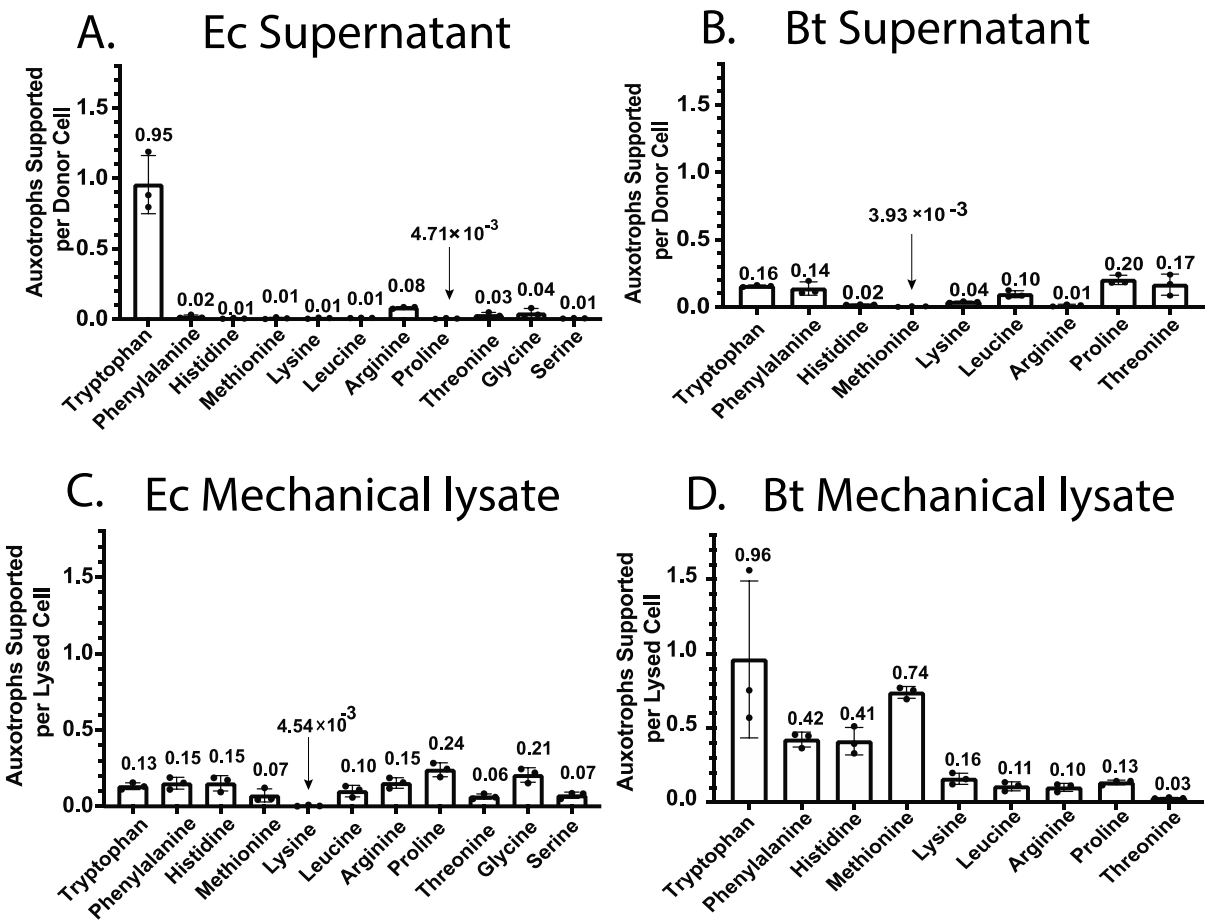

Figure 1: The number of auxotrophs supported per donor cell was measured for (A.) *E. coli* and (B.) *B. thetaiotaomicron* supernatant treatments and for each of the mechanical lysate treatments: (C.) *E. coli* French pressed lysate, (D.) *B. thetaiotaomicron* French pressed lysate. The amino acids are organized in order from left to right based on the estimated biosynthetic cost of producing one molecule of the amino acid[42].

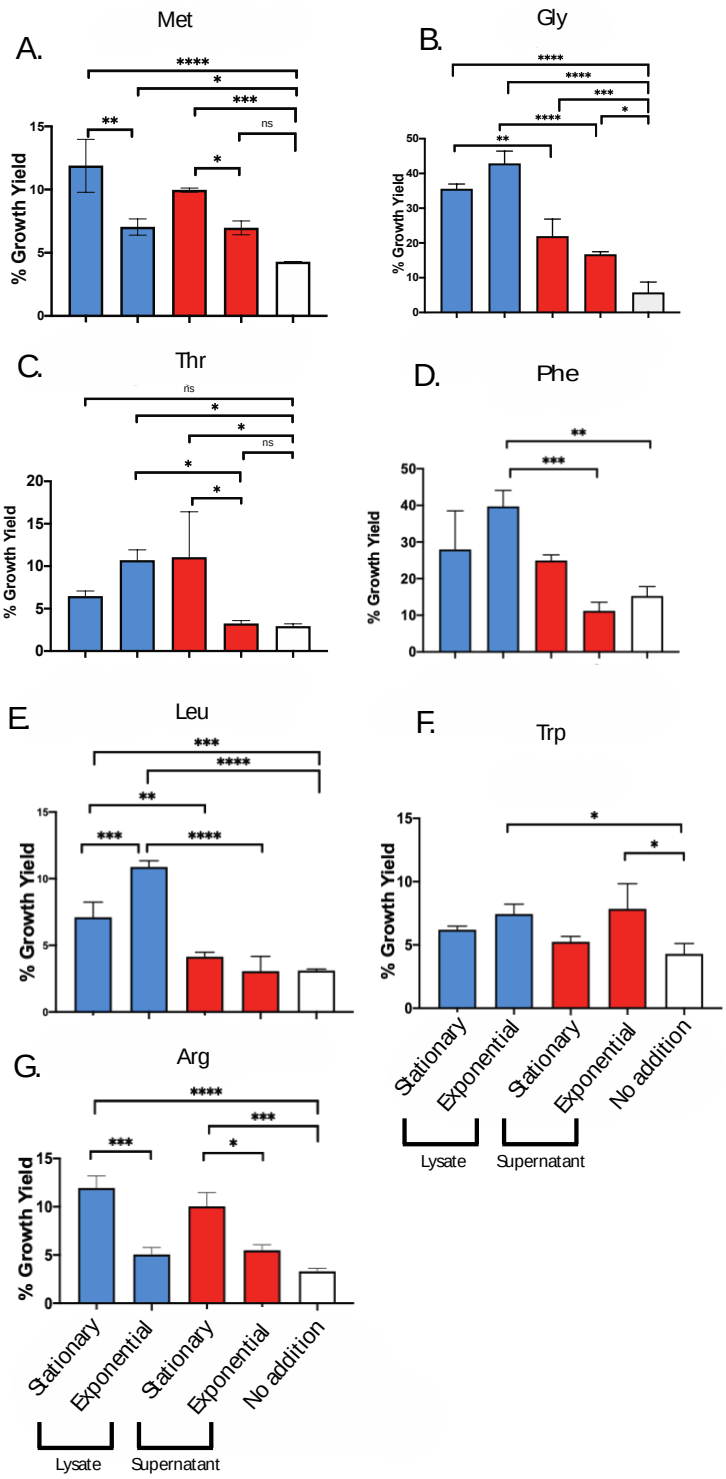

Figure 2: The growth yield of each amino acid auxotroph was measured after treatment with lysates and supernatants from either stationary or exponential phase *E. coli* cultures. (A.) Trp auxotroph, (B.) Phe auxotroph, (C.) Met auxotroph, (D.) Leu auxotroph, (E.) Arg auxotroph, (F.) Thr auxotroph, (G.) Gly auxotroph ($p<0.0001$ = ****; $p<0.0002$= ***, $p<0.0021$=**, $p<0.0332$= *)

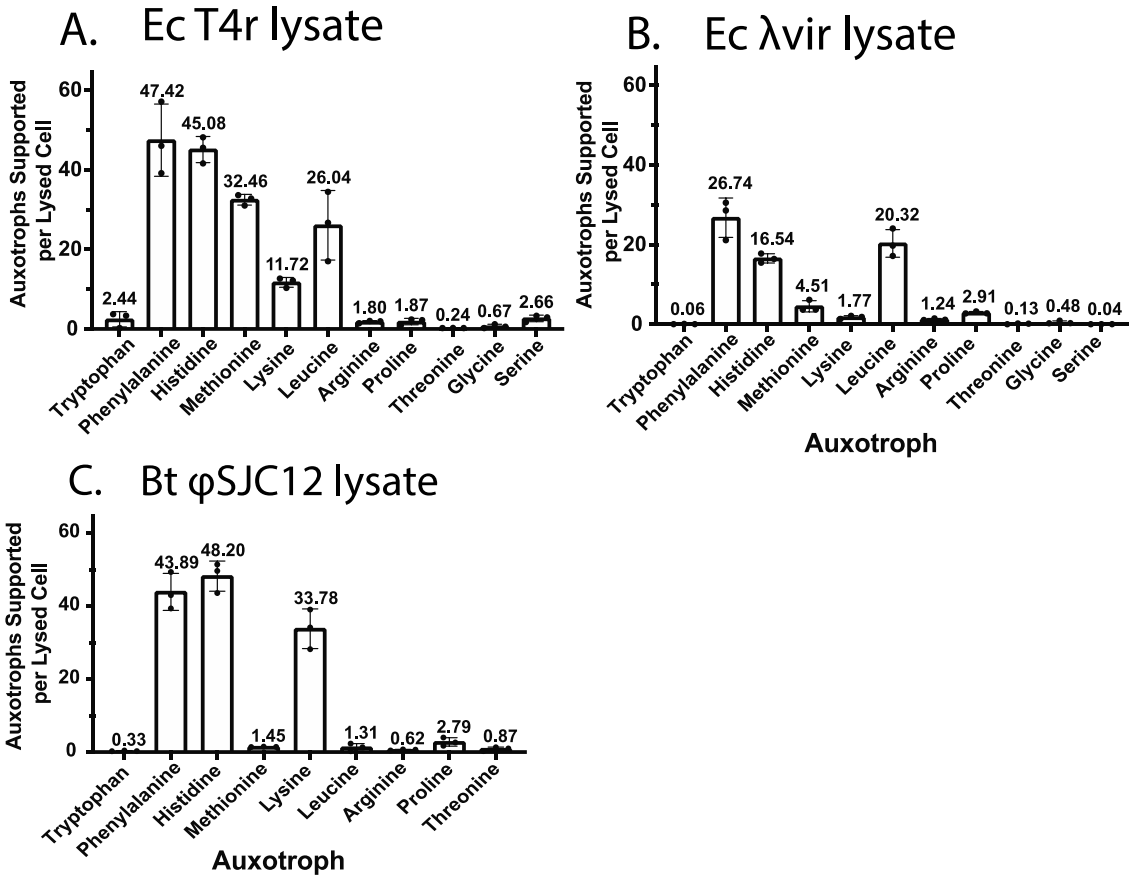

Figure 3: The number of auxotrophs supported per phage lysed cell was measured for (A.) *E. coli* T4ri phage, (B.) *E. coli* λvir phage and (C.) *B. thetaiotaomicron* ϕSJC12 phage. The amino acids are organized in order from left to right based on the estimated biosynthetic cost of producing one molecule of the amino acid [42].

Chapter 4 : Nutrient Overproduction as a Byproduct of Auxotrophic Mutations

Introduction

Microbes exist in multispecies communities that are composed of complex and layered nutritional interactions. Nutritional interactions are foundational to the stability and function of microbial communities [159], and can have vast implications for human health and global microbial processes, such as carbon cycling. Nutrient interactions between microbes can range from competition for a shared nutrient source [111], to mutualisms where nutrients are exchanged in ways that benefit both partners [29].

Auxotrophs – organisms unable to produce all of their required nutrients for growth – rely on interactions where the nutrient they need is provided by other microbes. These forms of interactions can be unidirectional, where a metabolite is transferred from one bacteria to another, or bidirectional, where metabolites are exchanged reciprocally between organisms. Auxotrophies exist for a variety of metabolites, and surprisingly, some auxotrophies exist for metabolites that are essential for cell growth and costly to produce, such as amino acids and vitamins. While evidence suggests that amino acid [42, 108] and vitamin [160] auxotrophy may be common, it is not obvious why bacteria release or secrete metabolites that are expensive to produce or essential for cell growth.

Synthetic co-cultures have become invaluable tools, offering experimentally tractable ways of studying nutritional interactions and auxotrophy [39, 135]. For some co-cultures, strains were engineered [135] or evolved [38] to establish a nutrient interaction, and in other cases, adjusting environmental conditions was enough to establish an interaction, such as a co-culture between *Chlamydomonas reinhardtii* and a CO_2-producing yeast [161]. *Mee et al, 2014* conducted an in depth study that used co-cultures to understand properties associated with amino acid exchange in microbial communities, and revealed that some amino acid auxotrophs lend themselves to be more cooperative in co-culture than others [42]. This work also revealed that some amino acids are released in significant enough quantities to support auxotrophic populations. Additionally, some evidence suggests that auxotrophic mutations can alter flux through other biosynthesis pathways [162, 163]. This suggests that some auxotrophs may inherently overproduce specific metabolites, and thus, impact the types of nutritional interactions in which auxotrophs participate .

Here I developed an obligate mutualistic co-culture system, to further explore amino acid nutrient secretion, and I hypothesize that nutrient overproduction as a result of auxotrophy can be an important mechanism shaping microbial communities (See Chapter 2 Hypothesis)

Methods

Bacterial Culturing and Media

E. coli co-culture strains strains were streaked from freezer stocks onto LB agar plates. Single colonies were inoculated into 2 mL H1 minimal media supplemented with 0.2% glycerol (50 mM KPO_4, 67 mM NaCl, 7.6 mM $(NH_4)SO_4$, 500 uM $MgSO_4$, 1.25 uM $Fe_2(SO_4)_3$, 0.2% [v:v] glycerol at pH 7.4) , 0.2 ug/ml L-methionine and grown with aeration at 200 rpm overnight at 37°C prior to all experiments. M_{Lys} and M_{Thr} pre-cultures were additionally supplemented with 100 mM L-lysine and 300 mM L-threonine, respectively.

Co-culture Experiments

Co-culture strains were pre-cultured in biological triplicates aerobically in H1 minimal medium supplemented with 0.2% glycerol and 0.2 mg/mL L-methionine at 37°C for 18-24 hrs until saturation, and then washed 3 times in H1 minimal media to excess methionine supplemented during the pre-culturing step. Following the wash step, each co-culture pair was inoculated into 96-well plates (Corning ®) at a 1:1 ratio to a final $O.D._{600}$ = 0.02 in H1 minimal media supplemented with either 0.2 mg/mL L-methionine, 10 nM Cbi and 50 nM DMB, or Cbi and DMB alone. For experiments containing strain M_{Lys} and M_{Thr} , 100mM L-lysine and 300mM L-threonine were added to all culture conditions, including the original M strain. Plates were incubated at 37 °C with continuous shaking in a multi-well plate reader (Tecan Spark) for 24 hours. Absorbance at 600nm and fluorescence (mCherry: excitation 530/25 nm, emission 590/35 nm; GFP: excitation 485/20 nm, emission 528/20 nm) were recorded every 10 minutes.

Construction of Co-culture strains and plasmids

To construct strain M, *E. coli* MG1655 Δ*cobTSU* Δ*cobC* strain was received from [164]. Subsequently, Δ*btuR*::Kan^R, Δ*metE*::Kan^R, Δ*thrB*::Kan^R, Δ*lysA*::Kan^R were transduced into this strain via P1 transduction from E. coli JW-1262, JW-3805, JW-0002, JW-2806, respectively, which were obtained from the Keio collection [158]. To construct strain B, a similar process was performed, but included the transduction of Δ*metH*::Kan^R into *E. coli* MG1655 from *E. coli* JW-3979 that was obtained from the Keio collection. After each transduction, the Kan marker was subsequently removed by recombination of the flanking FLP recombination target (FRT) sites as previously described [165].

Fluorescent reporter plasmids are based on pUC19[166], and sfGFP and mCherry inserts were assembled using isothermal cloning [167]. Fluorophores were tested in each strain to confirm no fitness differences between the mCherry and sfGFP inserts. All bacterial strains and plasmids are listed in Table 1 and Table 2, respectively.

Results

Developing an Obligate Mutualistic Co-culture System

The synthetic co-culture system I developed contains two engineered *E. coli* strains that share methionine and vitamin B_{12} (Figure 1). Strain B (orange), the B_{12} producer, is a methionine auxotroph that lacks both the B_{12}-dependent and B_{12}-independent methionine synthases, *metH* and *metE*, respectively. When vitamin B_{12} precursor molecules, 5,6-dimethylbenzimidazole (DMB) and cobinamide (Cbi), are added to the media, strain B is able to synthesize vitamin B_{12} using CobT and other B_{12} biosynthesis enzymes, and releases B_{12} into the environment. Strain M (green), the methionine producer, requires exogenous B_{12} or methionine in order to grow. In this co-culture, strain M received B_{12} from Strain B. Strain M has *metE* and *cobT* genes deleted, meaning it is unable to produce methionine unless B_{12} is present, and it is not able to synthesize vitamin B_{12} from B_{12} precursor molecules. In summary, strain B produced vitamin B_{12} from B_{12} precursor molecules, and releases B_{12} into the environment. Strain M uses vitamin B_{12} to produce methionine for itself and for strain B using the B_{12}-dependent enzyme MetH.

To test whether strains M and B are able to support each other's growth in an obligate mutualism, both strains were cultured together in the presence and absence of B_{12} precursor molecules, Cbi and DMB. When the co-culture was grown in the presence of Cbi, DMB and

methionine, both strains were able to grow to similar optical densities because both strains can use methionine to fulfill their metabolic needs (Fig 2A.). When the co-culture was only supplemented with Cbi and DMB, each strain grew, but to different optical densities (Fig 2B.). Strain M was able to grow to a higher maximal O.D.600 than strain B. This suggests that strain M is receiving more of its required nutrient from strain B, than strain B is receiving from strain M. The growth of each individual strain in co-culture is limited by the level of nutrient released from its respective partner.

Auxotrophic mutations can increase nutrient overproduction to improve co-culture growth

Auxotrophs rely on outside sources to provide their required nutrient (cite). This means that a nutrient, such as an amino acid, needs to be secreted or released in a large enough quantity to support the growth of the auxotroph. Chapter 3 provides some evidence that some supernatants in exponential and stationary phase are able to support the growth of amino acid auxotrophs to a small extent. For example, Chapter 3 suggests about 0.01 Met auxotrophs are supported per donor cell based on growth on supernatants (Ch. 3, Fig 1A), so Met secretion is expected to be limited in the co-culture between strain M and B, as we observe in Fig 2.

Nutrient overproduction can also be a mode of nutrient release that is able to support growth of other microbes. One example of this is overflow metabolism, when a nutrient accumulates inside the cell to levels that require their efflux out of the cell [106, 168]. I hypothesize that another potential mechanism that could contribute to nutrient release is nutrient overproduction as a byproduct of an auxotrophic mutation. Microbial metabolic pathways are highly interconnected [125], and because of this, when an auxotrophy forms through mutation, it can redirect metabolic flux through other metabolic pathways. For example, flux through the Met biosynthesis pathway has been shown to be increased when disrupting either the Lys or Thr biosynthesis pathways because these three pathways share a common precursor molecule [162, 163]. What remains less clear is how these altered fluxes created through auxotrophic mutations might shape nutrient provisioning in a mutualistic co-culture.

To test whether an auxotrophic mutation could redirect flux through another biosynthesis pathway to improve co-culture growth, I introduced ΔlysA and ΔthrB mutations separately into strain M, which I will refer to now as M_{Lys} and M_{Thr}, respectively, and co-cultured each strain with strain B. When strain M_{Lys} and M_{Thr} were each grown in co-culture with strain B in the presence of Cbi, DMB, Lys and Thr, both co-cultures grew faster and with a shorter lag time than the original co-culture without ΔlysA and ΔthrB mutations (Fig 3). Thus, by disrupting either the Lys or Thr pathways, I was able to improve the overall growth of the co-culture through an auxotrophic mutation. This validates that ΔlysA and ΔthrB mutations do alter flux through Met biosynthesis, and that disrupting Lys and Thr biosynthesis can increase Met provisioning in co-culture.

Discussion

Synthetic co-cultures have been useful in elucidating how nutrient exchange dynamics affect cooperativity and community stability (cite). Nutrient exchange interactions that have been documented in co-cultures include amino acid – amino acid exchange [37, 39, 135], vitamin – sugar exchange [169], carbon – nitrogen exchange [161, 170], carbon – carbon exchange [171](Summers et al, 2010), and vitamin – vitamin precursor exchange [172] (Sathe et al, 2022).

Here I have developed an obligate mutualistic co-culture sustained by amino acid- vitamin exchange. The vitamin B_{12} producing strain (strain B), provisions a sufficient amount of B_{12} for strain M to grow, but the methionine producer (strain M) is limited in its ability to support strain

B. The difference in maximal growth could be due to more vitamin B_{12} being released than Met, or a result of the different physiochemical properties and biological roles of these two nutrients. Vitamin B_{12} is a co-factor that can be reused over 100 times before it becomes inactivated via oxidation [173] ,while methionine is consumed by being incorporated into proteins. This could explain why strain M is better supported by the secretion of B_{12} from strain B, and why strain B's growth is limited by the release of methionine from strain M. The growth of this co-culture is reliant on supplementing the co-culture media with vitamin B12 precursor molecules, Cbi and DMB, which is an additional element that can be manipulated in future experiments to alter the level of B12 production. The distinction in growth capacities between strain M and B (Fig 2B) can be used as a barometer to further explore nutrient provisioning (i.e. the release of nutrients) and nutrient exchange (i.e. the sharing of nutrients between pairs), and how phage lysis and nutrient overproduction may support auxotrophic populations.

Co-culture strains can be evolved [38] or engineered [41] to release more nutrients, but in these cases, the engineered or evolved strain in the co-culture experiments was not a nutrient provider as a result of auxotrophy. Here I show how two different auxotrophic mutations can redirect flux through methionine biosynthesis to improve co-culture growth. Having a nutrient provider as a result of an auxotrophic mutation can allow us to explore how auxotrophies that redirect flux through other biosynthesis pathways, both contribute to and rely on nutrient provisioning in communities, and have implications for understanding the evolution of mutualisms that may result from a single mutation; both creating an auxotroph and, at the same time, creating a more cooperative nutrient provider.

Bacterial Strain	Strain Name	Genotype	Reference
E. coli MG1655	B	△ metE△metH	This study.
E. coli MG1655	M	△cobTSU, △cobC, △metE, △btuR	This study.
E. coli MG1655	M_{Thr}	△ thrB, △cobTSU, △cobC, △metE, △btuR	This study.
E. coli MG1655	M_{Lys}	△ lysA △cobTSU △cobC △ metE △ btuR	This study.

Table 1: Strains used in this study.

Plasmid Name	Plasmid Backbone	Insert	Source
pGP161	pUC19 – Amp^R	mCherry	This study.
pGP162	pUC19 – Amp^R	sfGFP	This study.

Table 2 : Plasmids used in this study.

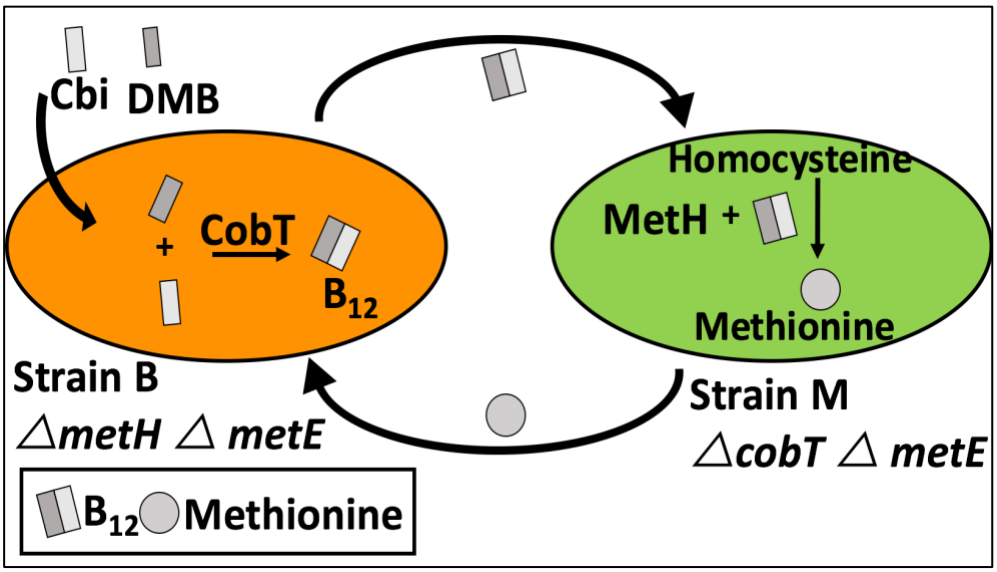

Figure 1: Nutrient flow of methionine and vitamin B_{12} in a synthetic *E. coli* co-culture. Strain B (orange) is a methionine auxotroph that lacks both the B_{12}-dependent (Δ*metH*) and B12-independent methionine synthase (Δ*metE*). Strain B is able convert vitamin B12 precursor molecules, 5,6-dimethylbenzimidazole (DMB) and cobinamide (Cbi), into vitamin B12 using CobT, a vitamin B_{12} biosynthesis enzyme. Vitamin B_{12} is released from strain B and imported by strain M (green). Strain M requires vitamin B_{12} to produce methionine for itself and strain B. Strain M produces methionine in a B_{12}-dependent manner by using MetH.

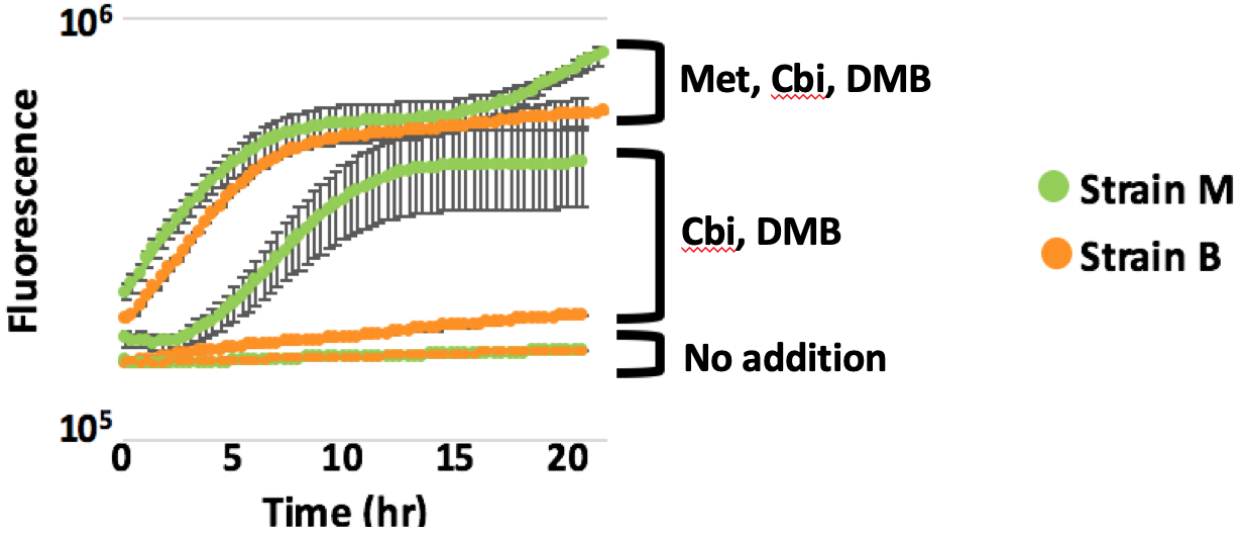

Figure 2 : Growth of fluorescently labeled strains in co-culture. Strain M was labeled with GFP and strain B was labeled with mCherry. Co-cultures of both strain M and B were supplemented with either methionine, cobinamide and DMB, cobinamide and DMB, or no addition.

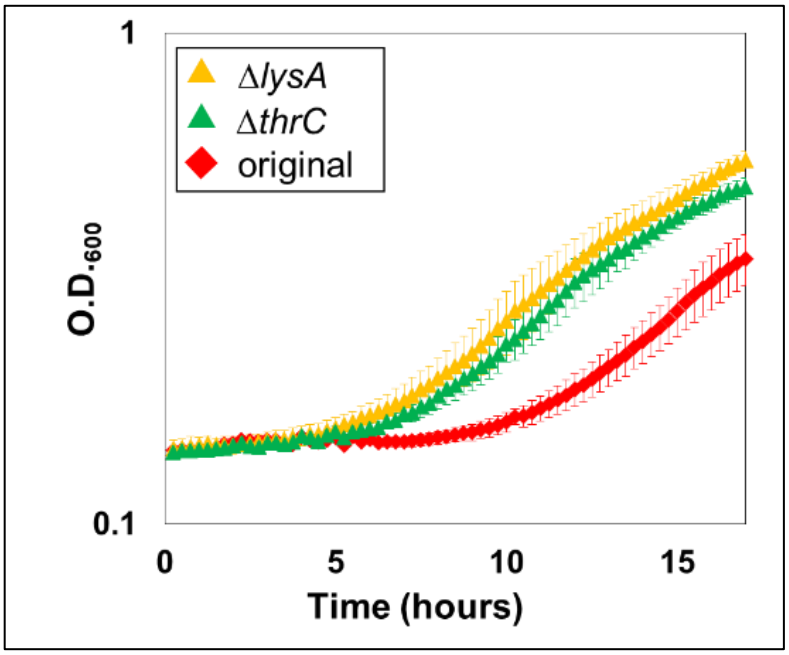

Figure 3: Redirection of metabolic flux in auxotrophs. M_{Lys} (Lys auxotroph), M_{Thr} (Thr auxotroph), or the original M strain were cocultured with strain B. Lys or Thr was added to the cultures containing the M_{Lys} and M_{Thr} strains, respectively. Total growth of the coculture is shown.

Chapter 5: Socialization of Doctoral Students in the Biological Science Laboratories

Abstract

STEM graduate education and doctoral student training programs are responsible for producing educators and researchers that lay the ground work for new products, the advancement of scientific and technological knowledge and paradigm shifting discoveries. However, there remain ethnic, racial and gender-based disparities in Ph.D. - requiring STEM fields and faculty positions. The current interventions (i.e. increasing enrollment of marginalized groups in doctoral programs) ignore other intermediary elements, such as doctoral student socialization, that can also contribute to the high attrition rate of marginalize communities in STEM doctoral programs. Here I use relevant theories of socialization and identity construction to explore how students are socialized in biological science doctoral programs. By conducting interviews with 32 doctoral students across two biological sciences departments, I identify key elements of socialization that shape doctoral students experiences and learn how day-to-day informal interactions affect access to scientific help and expertise.

Introduction

4.1 What is STEM Education?

Over the last few decades there has been a push for Science, Technology, Engineering and Math (STEM) education that stems from concerns related to having a large enough skilled workforce to fill STEM jobs and careers, and maintaining the economic and educational competitiveness of the U.S. on the global stage [174]. Those who believe in the values of STEM education feel STEM education can increase problem-solving skills, analytical thinking, and critical thinking skills [175]. But what makes up a STEM education?

STEM education has been defined as a meta discipline where STEM teachers use integrated approaches to teaching and learning where different STEM disciplines are treated as "one dynamic, fluid study" [176]. The National Science Foundation includes economics, sociology, political science and psychology in their definition of STEM[177] . While describing content is one part of defining STEM education, an additional element involved in defining STEM education is how it is implemented [176]. Some question whether it is enough to take a course in each of the four STEM areas, or whether the four areas of STEM need to be integrated into one or more courses for a student's education to be identified as a STEM based education. The foundation of most K-12 and higher education STEM education in the U.S. choose to emphasize the relationships and conceptual connections between STEM fields through curricular and pedagogical coherence [178].

Expectations and definitions for graduate STEM education have been less defined. The Committee on Revitalizing Graduate STEM Education for the 21st Century, a committee through the National Academies of Sciences, describes that in an ideal graduate STEM system students should encounter " a variety of points of view about the nature, scope and substance of the scientific enterprise and about the relationships between science, engineering and society and they would be encouraged to understand and grapple with the differences of opinion, experiences, and ideas as part of their graduate education and training"[179]. This committee also advises that

graduate STEM education should challenge students to consider the ethical and cultural issues related to their work, as well as the broader needs of society [179].

4.2 Graduate STEM Education and Universalism

Graduate education and the training of doctoral students are essential in helping the U.S. maintain its leading force in the world's economy and solve some of the world's most pressing problems. Additionally, graduate education is responsible for (1) producing the educators and researchers of the future that will lay the groundwork for new products and paradigm shifting discoveries , and (2) directly contribute to the national goals of cultural, economic and technological development [179]. We have become highly reliant on technically trained graduates to advance scientific and technological knowledge, develop new technology, and make new discoveries. This heightens the importance of understanding how to continue to make graduate education adaptable to the changing economic landscape and the diverse populations it serves.

After students are trained in the sciences, they become members of the national labor force and are prepared to participate in technology innovation and the knowledge production economy. From 1850 to 2010, the share of jobs requiring high level STEM knowledge increased from 9.5% to 18.8% [180]. Science, because it is a high-status occupation, often rewards its members with high personal income and social prestige [181]. The relationship between a science occupation and these rewards is suggested to be based on sciences subscription to *universalism* [178], a core sociocultural norm that Merton (1996) uses to describe the scientific community [182, 183]. Universalism is a philosophical concept that some ideas have more universal context than others, and that knowledge is free of bias and subjective elements, including the race, ethnicity, gender or other personal attributes of the scientists [184]. In the context of education, universalism assumes a high level of objectivity when evaluating STEM students, which is why science education is often seen as an opportunity for socially disadvantaged groups climb the social ladder [185]. Merton (1957) describes this by stating "to restrict the scientific careers on grounds other than lack of competence is to prejudice the furtherance of knowledge" [186]. While universalism is an ideology that governs our perceived norms and expectations of science, we continue to observe disparities in engagement and participation in science [187], leading us to believe that access to scientific careers is not based on competence, but other social factors.

Graduate STEM education is positioned within the broader educational landscape, and because of this it is also subjected to the general dynamics of the general education system in which educational attainment is highly dependent on social characteristics such as family socioeconomic status, race and ethnicity, family structure, gender, school and neighborhood [178].

4.3 Theory of Socialization

Doctoral students in STEM, and their movement into the STEM labor force is dependent on the successful socialization of students into the culture of science and technology related fields. Socialization theory was first used to study graduate students through the work of Merton (1957). By pulling from work on the sociology of medical education and reference group theory, Merton described socialization as *a process* in which a person develops a sense of their professional self, with characteristic skills, values, knowledge and attitudes that guide behavior during particular professional situations [186]. Wiedman and Stein (2003) develop this definition by further by discussing socialization as "the process through which individuals gain the knowledge, skills and values necessary for successful entry into a professional career requiring an advanced level of specialized knowledge" [188]. The process of learning within doctoral programs would not be

possible without socialization as a process of spreading shared knowledge, values and attitudes to its members.

Gardner (2007) performed empirical work that revealed variability in the way doctoral students from different disciplines and social groups are socialized [189]. This informs us that socialization is dependent on *who is being* socialized, *who is doing* the socialization, and under what *environmental context*. For a doctoral student in the biological sciences, the identities held by the student, their relationship with their research advisor, committee members and lab mates, as well as the type of institution (i.e.; public, private, R1) could all influence how a student is socialized.

While socialization happens at the institutional level , disciplinary culture is argued to be the primary source of faculty identification and socialization [190](Johnson et al, ; Kuh and Whitt,1988; Tierney and Rhoads, 1993). And because many biological science doctoral students are being primarily mentored by faculty members, this suggests that disciplinary culture may also be a salient factor in doctoral student socialization. Learning a discipline's commonly shared assumptions, values, and epistemologies form the groundwork for successful integration into a particular professional community. Departments can also establish organizational structures that facilitate certain forms of socialization processes. This can occur by fostering explicit socialization (e.g. developing core course requirements, setting expectations for faculty-graduate student relationships) and implicit socialization (e.g. helping foster informal interactions such as peer mentoring organizations and social hours between graduate students and faculty members). The departmental and disciplinary culture provides the experiences necessary for doctoral students to learn the attitudes, norms, values and skills of their fields.

The Tiery and Rhoads (1993) theory of faculty socialization investigates socialization through faculty culture, where culture is said to shape and be shaped by social interactions. In this theory, they refer to graduate education as an anticipatory socialization step required of students before becoming a faculty member. This anticipatory stage is where graduate students learn the values, norms and expectations that come with being a researcher. This process is important for doctoral students as they begin to integrate into their discipline, and undergo role commitment as a member of a group [190].

Socialization is not a uni-directional process in which students are solely the receivers of organizational culture, norms and values. The newly enculturated members of a group also participate in the "resocializing" of organizations as they change traditional notions and require the organization to respond to different needs (Schein, 1968). Van Maanen and Schein (1979) outline five (5) dimensions of organizational socialization that describe how individuals experiences in an organization are structured both by the individual and by others in the organization. The five dimensions include : collective vs individual (i.e. whether entering members are sharing a similar experience as a collective); formal vs informal (i.e. whether structured activities are completed as a "rite of passage" or learned through trial and error); sequential versus random (i.e. whether the route towards an organizational position is clearly defined, or if there is ambiguity and uncertainty); fixed vs variable (i.e. whether the time required to move through a particular social role is known or is it variable and less clear); serial vs disjunctive (i.e. whether there is a transfer of knowledge and expectations from a mentor or is there a lack of role models) and investiture vs divestiture (i.e. whether an organization is more affirming and welcoming of members anticipatory socialization or if there is the stripping of individual personality and characteristics that do not align with organizational culture). Navigating the unique combination of these dimensions at a given university are important for doctoral student

matriculation into STEM fields, and inadequate socialization within this complex, dynamic and interactive process is theorized to lead to doctoral student attrition (Gardner, 2007; Golde, 1998).

Golde and Dore (2001) performed a national study of doctoral student experiences in the arts and sciences that highlighted: (1.) a lack in understanding what doctoral studies entail, and (2.) mismatch between the purpose of doctoral study, student's aspirations and the realities of careers available following doctoral study. These reflections from doctoral students experiences aligned with the Consensus Study Report of The National Academies of Sciences, Engineering and Medicine (NASEM) that there needs to be a cultural shift that goes from being research enterprise and higher education-centered to student-centered, with an emphasis on the diverse needs and challenges of STEM graduate students [179] (NASEM, 2018). Scholarly work has been published about students experiences in doctoral programs [191-193], but what remains unclear are the elements of socializations in biological science doctoral training programs, and the effect of these elements on students experiences.

4.4 Participation in Science and Identity Development

Interests in the different patterns of participation and engagement in science and engineering has led educational researchers to study the role identity plays in how an individual perceives accessible and desirable career goals [187, 194]. Individuals are involved in constructing their identity based on race, gender, family socioeconomic status, who they want to become, and how they are positioned and defined by the people around them [195]. There is a growing interest in building more theory on professional identity construction, especially under the conditions of stigmatized cultural identities [196], which becomes necessary when we consider organizations interest in diversity and equity. Career success is often associated with successful professional identity construction [196, 197]. This is especially important when we consider the increased interest in increasing diversity in the professoriate and PhD-level STEM careers.

An individual's perception of available and desirable identities are grounded in their understanding of how these identities are positioned within a particular social context (Holland et al, 1998), which includes the profession and organization they aspire towards. Particular characteristics are assigned to specific identities, such as a "good scientist", "future faculty member", "promising student" and "sloppy scientist". These identities become goals to aspire to or avoid. Each of these identities can be characterized as a *model of identity,* which can be used to understand one's current identity, imagined future identity and the identities of people around them [195, 198]. Individuals that enter a new institutional space are influenced by the models of identity that are most recognized and valued [199]. Additionally, the extent to which students feel their identity is recognized and valued in science education influences their engagement with science and whether they see themselves pursuing science after college or university [187, 200]. When marginalized groups identify a conflict about who they are and who they think their doctoral program needs them to, there becomes a negotiation between their scientific identity and cultural identity in order to achieve academic success become [195, 201] . Students studying science often feel the need to adapt to white, middle class culture of science [202].

Claude Steele (1997) developed a concept called "stereotype threat" which has been used to understand why underrepresented groups (i.e. race, gender, ethnicity, etc.) withdrawal and dis-identify with academic identities [203, 204] . Stereotype threat can be described as a marginalized group's fear and anxiety of fulfilling negative stereotypes that corresponds to an identity they have. Supporting a positive self-image as a scientist is important in self identifying as such. If the performance of an individual conflicts with their positive self-image, Osborne and Walker (2006)

describe three outcomes that can occur: (a) de-identification from the domain that conflicts with an individual's positive self-image, (b) the seeking of support to improve one's performance or (c) escapist tactics such as absenteeism or withdrawal [205].

Carlone and Johnson (2007) developed a model of scientific identity using qualitative data collected from women of color in science [191]. This model illustrates three components important for the strong formation of a scientific identity : competence in one's knowledge and understanding of scientific content, the ability to perform relevant scientific practices, and the ability to receive recognition by oneself and others in the scientific community as a science person. Multiple groups have discussed the role of social factors and socialization in scientific career attainment [191];Lewis 2003). Entering a doctoral program and becoming a researcher requires doctoral students to reconceptualize their personal and professional selves and negotiate already held identities (Austin & McDaniels, 2006). Part of this reconceptualization includes how doctoral students participate in and make sense of themselves in scientific discourse. Gee (1996) describes Discourse as:

> A socially accepted association among ways of using language, of thinking, feeling, believing, valuing, and of acting that can be used to identify oneself as a member of a socially meaningful group or "social network' or to signal (that one is playing) a socially meaningful role. (pp 42-43)

The language that is used within the scientific community is associated with ways of acting and epistemologies that have shaped sciences values. For example, the continued use of militaristic vocabulary (i.e. "arsenal", "defense systems") reveals sciences deep connection with military research during World War II (examples Richardson, 2017; Li et al, 2017).

Mentoring also plays a critical role in doctoral students scientific identity development. Through these relationships one learns the values, attitudes, skills and knowledge of their particular discipline. The critical role of mentors in the biological sciences lends itself to the social structure of science, and the way tacit knowledge is passed down through observation and example [186](Merton, 1957). New students must learn the location and proper usage of laboratory equipment, what experimental controls are necessary to make a clear and persuasive argument, experimental design and a variety of other skill sets that will vary by lab environment and discipline. A significant component of doctoral training in the biological sciences is provided through tacit instruction, much more than in the humanities. While this knowledge is primarily passed down through one-on-one mentoring, this practice has been criticized for failing to address the needs of students from diverse backgrounds [195]. Faculty can unconsciously privilege students whom they deem "motivated" or "capable" scientists, resulting in the biased extension of professional opportunities to their mentees such as involvement in reviewing research papers, writing grants, and network connections. Mentorships are seen as more successful when doctoral students take-on the characteristics and values of their advisor and what it means to be a scientists, but Hall and Burns (2009) mention that this raises questions about power relations, equity, and the agency of doctoral students [195].

Guiding Research Questions

The research questions guiding this study are : How are doctoral students in the biological sciences socialized as they navigate their doctoral programs, and how does this socialization shape their experiences as doctoral students and scientists. I hypothesize that doctoral students make sense of themselves in relation to their daily interactions with labmates (i.e. graduate students, postdocs, lab staff) and faculty mentors, and that informal interactions in lab shape doctoral students access to scientific help. To investigate these hypotheses, I interviewed 32 biological science students about their mentorship experiences, daily interactions in lab, and other social aspects of navigating a lab environment.

Methods

This study was reviewed and determined to be exempt by the University of California, Berkeley Institutional Review Board (FWA#00006252).

Participants

This study seeks to address gaps in doctoral education literature by exploring how doctoral students are socialized while navigating biological science doctoral programs. Western University (pseudonym) is a public research university on the west coast with a high research activity, with an enrollment of approximately 40,000 undergraduate and graduate students.

Based on the assumption that doctoral students are socialized most by their discipline and departments [206], I selected two biological sciences departments, Life Sciences A (LSA) and Life Sciences B (LSB) that often collaborate frequently in faculty hires and training grants. Multiple academics have joint faculty appointments between LSA and LSB, and are asked to be committee members for qualifying exam committees and dissertation committees from doctoral students from both departments. Because of the institutional structures that have helped foster the relationship between these two departments, I will assumed they operate using a similar set of institutional logics, which includes practices and behaviors, with respect to doctoral student training.

The selection criteria for being a participant in this study was to be a 2nd, 3rd or 4th year doctoral student in either LSA or LSB (Table 1). These years are the focus of this study because students in LSA and LSB take their qualifying exam during the second year of their program. During year 1 of a PhD , students are enrolled in classes and rotating in 3-4 labs for 10 weeks each. This serves as a trial run to decide which lab students would be interested in joining for their dissertation. While year one is also an important socialization stage for newly accepted doctoral students, I am most interested in the phase of graduate school when doctoral students are no longer taking classes and are becoming fully committed to their dissertation research projects. Year 2 serves as a benchmark for when students begin transitioning from student to independent researcher. All, except two of the 2nd year doctoral students interviewed had completed their qualifying exam by the time of the interview.

It is also important to acknowledge the variation of experiences across LSA and LSB based on the unique lab environment each doctoral student joins. Across LSA and LSB, there are 138 labs. A total of 14 labs are accounted for in this study, and 5 of the labs are run by women principle investigators (PI's). Each laboratory has its own culture that is created by the PI, graduate students, postdoctoral scholars, laboratory staff and undergraduate students. Because of this, there is the opportunity for great variability in the experiences of doctoral students as they continue to develop as scientists. Of these 14 labs, three of the labs have 2-3 graduate students represented in the

sample. These three cases serve as important data to examine how different individuals experience the same lab environment.

I used a purposeful sampling strategy (Merriam, 1998) to recruit a diverse group of participants with respect to social identity (e.g. gender, race/ethnicity). Doctoral students were recruited by emailing departmental listservs and asking administrators and graduate coordinators from LSA and LSB to distribute study invitations to doctoral students within their department. Participants were also asked to share the study information with peers who they believed met the study requirements (i.e. snowball sampling). Because of my interest in race and gender with regard to scientific identity construction and the small sample population of women and racialized people within the departments I was studying, it was important to maximize range for these individuals. This was completed by directly contacting individuals that I had met through my personal network and by asking interviewees if they knew of anyone that would fit the criteria for this study.

Data Collection

I collected data using in-person semi-structured interviews. Each interview lasted 60-90 minutes and was audio recorded and transcribed verbatim using Temi, a speech to text transcription service, and checked for accuracy. I conducted all of the interviews for consistency purposes. I designed the interview questions to gain an in depth understanding of the mentorship relationships between doctoral students and their advisors and peers, as well as their relationship to their scientific identities. All identifying information described in the interviews (i.e. names of individuals, techniques, names of model organisms) were removed and replaced with pseudonymns to maintain participants privacy. Specifically, questions probed doctoral students about their perspective on what it means to be a scientist, the role of social networks in scientific professional development, the process of selecting and integrating into a new laboratory, identifying departmental and laboratory expectations, and exploring interpersonal interactions with lab members, faculty advisors and other salient mentors in science. I employed phenomenological research methodology to identify firsthand perspectives on the ways doctoral students made sense of their experiences navigating their doctoral programs. As a doctoral student in a biological sciences program applying phenomenology, I am not detached from my own presumption, a perspective that Zahavi (2019) has addressed [207].

Researcher Characteristics

My experiences and perspectives as a researcher are also relevant to interpreting these results. I am a doctoral student in the biological sciences department at UC Berkeley and have training in the life sciences. I spent a year being mentored by Dr. Heather Haveman, a faculty member in the UC Berkeley Sociology Department, who served as one of my principle guides as I designed my research questions , developed interview surveys, and processed data. I have participated in mentored research experiences in the biological sciences for over 9 years, and have been an active participant in mentoring undergraduate and doctoral students.

Results

Lab Meetings as Important Sites of Socialization

Lab meetings are one of the essential elements of a doctoral degree in the biological sciences. While the structuring of these meetings can vary depending on the needs and interests of

the lab, most lab meetings contain similar elements. These elements include, hearing about the research of other labmates, brainstorming alternative approaches to experimental and project challenges, and receiving feedback on research from their labmates and PI. Many labs also incorporate a journal club into their lab meetings, where they discuss, critique and engage with literature relevant to their labs research interests.

Lab meetings represent one of the many sites of socialization into the scientific profession, where hierarchical systems in science are maintained and reinforced. Prior to entering a doctoral program, interviewees have often attended lab meetings as either an undergraduate student, lab tech or post baccalaureate student. Because all doctoral students have experience working in a laboratory prior to entering a doctoral program, they have been socialized, to different degrees, prior to graduate school to understand the hierarchy within a labspace. Multiple students discussed seniority playing a role in lab meetings. Seniority most often referred to individuals who were "more senior" in the lab, and had "put more time in". One student described in detail his typical encounters with senior scientists during a lab meeting.

> I still feel sometimes - this might just be the character of some of the people in the lab, but people do speak a lot and they have also spoken over me. I don't tend to interrupt or speak over people that's not my character to do that. I don't think it's polite, but I do get a sense that some people - sometimes people feel like they turn to me and explain to me when actually I do understand what I'm talking about. So that is still a case in the lab. I definitely feel it at lab meetings, especially with the most senior people in the lab. " (Interviewee A; Asian Man, International Student)

Senior scientists can be perceived to have more authority because of the number of years of research experience, and/or their length of time in the lab. This authority can give senior scientists insights and critiques higher value, and sometimes, more legitimacy in lab meetings. Additionally, during this interview, Interviewee A mentioned that senior scientists did not speak over other graduate students as much as they spoke over him. He wondered whether this could be due to the disciplinary background of the other graduate students, and how other graduate students training was more aligned with the training of the senior scientists. This reveals potential disciplinary distinctions made by lab members, that can shape interactions with members with different research backgrounds. An additional component that should be considered is the racial background of Interviewee A and how differences in cultural values between the U.S. and Asian countries may affect interactions during lab meetings. Asian students share collective racialized experiences as they navigate postsecondary institutions; regularly facing slights and insults similar to other non-White groups [208]. Evidence suggests that Asian students in classes often feel more pressure to conform to western norms of "talking more" in order to receive participation points in class [209]. Interviewee A later discusses how he has needed to become more assertive and find ways to interject when a senior scientists is speaking over him.

> As I have grown, you know in terms of seniority in this lab, I've become more assertive about what I say. So they might interrupt me, but I might talk back now and say "hey, well, actually this is what I was thinking. You didn't understand what I was saying". (Interviewee A ; Asian, International Student)

When another doctoral student was asked about her experience during lab meetings, she responded "…if you ask me who talks a lot in [lab] meetings, it's all the men." (Interviewee B ; White Woman). This was a common comment among labs, especially labs that were not predominantly women. Another doctoral student discussed his observation of how he saw himself developing as a more vocal scientist in comparison to a woman who entered the lab during the same year.

> I've become more comfortable voicing- asking questions and saying things in lab meeting than she has and I'm sure that is partly because I'm a man. (Interviewee C ; White Man).

Interviewee C discusses his comfort level with participating during lab meetings, and expresses his awareness that his level of comfort is not shared among his labmates. Statements from both Interviewee B and C are examples of gendered imbalances that exist during lab meetings. These imbalances show trends towards men taking up more sonic space than women during lab meetings. This aligns with evidence from college classrooms where men were observed to speak more frequently, speak without raising their hand, and interrupt speakers [210].

Lab meetings serve as an essential socializing element for doctoral students as they are learning how to communicate research findings in their discipline, troubleshoot experiments, and practice presenting experimental data to an audience of colleagues. Student's that get less speaking time are undercut in their ability to engage, learn and receive scientific feedback during lab meeting. Disparities in speaking time can occur as a result of scientific seniority, as we see with Interviewee A, or gender, as we see with Interviewee B and C. Classrooms require equitable participation to be built into the framework of the class in order to eliminate disparities in participation [211], and lab meetings could benefit from a similar framework.

Informal interactions among labmates shape access to scientific guidance

The transfer of academic cultural and scientific knowledge from senior to junior scientists is an essential element in the socialization of doctoral students into their discipline (cite). Traditionally, doctoral education has been modeled using the 'apprenticeship model', in which the dominant form of mentorship a student receives is from one-on-one interactions with their faculty advisor (Walker et al, 2009). While this could be the case in some instances, most students discussed receiving mentorship from multiple individuals in the lab during their doctoral training.

Mentorship can take many forms. More experienced graduate students and postdoctoral scholars were said to provide advice to younger students on how to write a successful grant or fellowship application, and would critique PowerPoint and poster presentations. These mentors also shared material resources with doctoral students, such as reagents, protocols and bacterial strains; and taught younger doctoral students how to use equipment and perform new experimental techniques. Each of these resources provided by mentors helps doctoral students gain technical expertise in their field, learn how to communicate effectively to scientific audiences, and develop habits of mind for thinking through the scientific method.

Much of this transferring of knowledge occurs through informal interactions - frequent, unplanned and brief encounters - throughout day-to-day lab activities. For example, some students learn the norms of how to present and synthesize their findings into an engaging story over lunches with labmates. One student described how having positive interactions with his labmates outside of lab shaped his future interactions with these same individuals, within the lab.

> I spent basically the entire day with them outside of lab which I - hadn't happened before and so that might have been- maybe it may be kind of turning point in terms of my - my comfort with them in a more informal way... I felt that I was defending maybe my project more [before]. And now it's more discussing. (Interviewee D ; Asian Man)

The change in getting to know his labmates on a more personal level through activities outside of the lab changed how he perceived his relationship to his labmates. Rather than protecting the image of himself and his ability to do good work, becoming more comfortable with his labmates allowed him to be more willing to "discuss" his project and allow for reciprocal dialogue, rather than "defend" his project against critique. This sentiment is echoed in the following quote.

> The people that I'm closest with in lab are the ones that I'm most likely to share about, like an experiment that I don't understand, just because I know that they're not going to make fun of me if it's, like a dumb mistake that I didn't realize that I made. (Interviewee E ; White Latinx Woman)

This suggests that students are more likely to seek outside scientific help when they have the psychological safety of being able to fail and make mistakes. Psychologically safe environments are environments where students feels a sense of safe to take risks, such as proposing new ideas, or asking questions [212]. The benefits of psychological safe environments have been observed to improve students focus on learning in medical education environments [213] . Given a large part of learning in a research lab is assessing and re-assessing null or failed experiments, it is essential for students to learn in a constructive environment where they feel comfortable talking about their failures with more experienced researchers. This will allow doctoral students to learn how to improve and avoid similar challenges in the future. The creation of friendships in lab make the space comfortable for receiving advice from those you consider friends. One student mentioned:

"I kind of wind up being friends with [my benchmates]. And as a result you also kind of wind up getting advice from them on the science." (Interviewee F , Asian Woman) .

Many doctoral students discuss the development of lifelong friendships, as well as professional relationships in their labs.

Informal conversations in and out of the lab with labmates can serve as a mechanism of social cohesion necessary for doctoral students to make progress on their research project, and can shape the real and perceived challenges students feel, as it relates to research-related tasks. The consistency and strength of these informal interactions could also influence scientific confidence, the amount of support students feel they are receiving, and prevent lengthy graduate school careers. A student below discusses in-depth his relationship with his primary postdoctoral mentor.

> [We meet] about every two weeks, kind of one-on-one, in a way. It can be pretty informal. He just might walk over to my bench and we'll chat for forty minutes or so. Mmm, I email with him regularly. Yeah, he might send papers my way. I might send papers his way. He might ask for my help on certain, like things that he might be doing and, and that's kind of how that developed. And last year - about a year ago now, almost a year ago now. He came to me with another project. He said "Hey, you know, I'm kind of- I've got a lot of things to

> do. I have a lot of things to write up. This is a project - is another project which is unrelated to yours right now, but I think it's going to be really good and frankly I need someone to do it. Would you consider doing it?", and this time last year my project situation was not looking that promising so I thought maybe it would be good to diversify a little bit. And so I took on that project and ... that's the project I'm writing up first even though I spent only a year on it. (Interviewee G; White Man)

The network tie between the doctoral student and postdoctoral mentor, which was strengthened through consistent and frequent informal interaction, resulted in a publishable research project and, as was discussed later in the interview by Interviewee G, an opportunity to build their confidence as a scientist by knowing they can successfully collect positive results.

Constructive informal interactions vary across identities and affect access to expertise

While the number of hours students spend in lab varies from 60 to 80 hour work weeks, a large portion of their working hours are interacting with labmates. This creates opportunities for friendships to develop, as well as less positive relationships.

> [I have a labmate] that would make a lot of like- jokes like that [about not being American]. And I think that affects our professional relationship too. Because - because I start, I start disliking him as a person. I would also not want to collaborate with him, [or] ask him for advice… [my PI] like, suggests a collaboration. Like 'You can do these experiments together' and I'm just like, "Ugh, We can," and I would if we need to, but I'd rather not." (Interviewee H ; White Woman)

This international doctoral student discussed how jokes about her not being a U.S. citizen shaped her comfort level in collaborating with a labmate. International students work to overcome communication and cultural differences, and can also experience prejudice and stereotyping that can lead to negative psychological outcomes that affect students educational experiences [214]. Additionally, international students have diverse needs in how they navigate cross-cultural boundaries to advance in their field, and research shows that these students can experience difficulty engaging in a variety of social situations within the U.S. university system [215]. The labmate's jokes made Interviewee H uncomfortable to the point of wanting to avoid future interactions, which shapes the type of scientific expertise she is able to receive as she continues through her graduate training.

Lab personnel are often crafted based on the interests of the lab. Postdoctoral scholars and graduate students are attracted to a given lab because of what they hope to gain technically, but also for the expertise they bring from their previous research experiences. Not having access to a labmates expertise because of their race, gender or nationality precludes students from valuable scientific capital - science-related forms of social and cultural capital. Science-related social capital includes scientific social networks, and science-related cultural capital includes education, knowledge and qualifications [187]. A student from a lab with over 20 lab members, expressed that not being willing to ask a particular labmate for help was an "inconvenience". This phenomena could be more stark in smaller labs with less than 10 members, or in a lab where it is necessary for

the doctoral student to interact with a given labmate because of the labmates unique expertise in the lab.

During the development of informal interactions, doctoral students discuss how shared hobbies, such as sailing, mushroom hunting, rock climbing and sports, helped them develop close connections with their labmates. But when students are not able to develop these interactions because of dissimilar interests, students can feel like an outsider.

> Um, there are times where I do feel like an outsider when I hang out with, uh, my lab as a group, um, because of just different hobbies and interests and, you know, it's just not me … Sometimes, it's just some-sometimes I just, I just want to get away from it because I just don't feel like I fit in...when I, you know, when I do get out of my comfort zone, I'm hoping, I'm hoping that it'll increase my desire to just interact with people in a social setting, which hasn't been the case within, like the past year or so." (Interviewee I; Black Latinx Man)

Not feeling comfortable engaging with labmates during social events and informal conversations means not being able to develop strong network ties that can lead to gaining more scientific capital. Interviewee I's statements about "not fitting in" with other labmates aligns with findings from other studies that show Black students, and students from other marginalized communities, are less likely to feel they belong in STEM [216, 217]. Sense of belonging refers to a student's feeling connected and value by other members within an organization or group. Students' sense of belonging are impacted by peer interactions and interpersonal relationships [216], and can shape interest and pursuit of science careers for students from marginalized communities [218].

Work related conversations and brainstorming also happens during these social gatherings, which means individuals not present are not able to contribute to or benefit from these interactions. This is exemplified further in the following quote by another doctoral student.

> I guess if I don't really necessarily talk to them too much, there's no sort of like trust necessarily built in there....It makes it sort of hard for me to like build a rapport that I need with other people in my lab to really, I guess, uh, connect with them and have, you know, conversations with them about my work. (Interviewee J; White Latinx Man)

Social events, such as beer hours, lab lunches, and weekend trips with labmates are opportunities to develop trust and rapport with other members of the lab. Students in labs where they feel integrated into the social circle describe the benefits of having close friendships and relationships with their labmates.

> Seeing all of these really wonderful people around me who are great in all of their own unique ways, but at the same time also aren't perfect and you know, acknowledged that. And are totally open about the things that they don't know. I think it's made me feel more comfortable admitting what I don't know. Whereas in the past, I- I didn't feel that way. I was like, if I admit what I don't know, then I'm admitting that I'm not deserving of, you know, being here. (Interviewee K; White Woman)

And another student discusses what makes her lab a good lab environment.

> ..for me, like a good lab environment was a place that people were willing to share their ideas and their time with each other. (Interviewee L; White woman)

The sharing of time with each other seems to be one of the key components of establishing strong relationships in lab, and in turn, this can lead to the generation of scientific capital.

Relationships with PI shape access to different forms of scientific capital

During the first year of graduate school in LSA and LSB, students complete their core curriculum modules and participate in rotations. Rotations are 10 week training periods where students have the opportunity to work in a lab of interest on a small research project. This opportunity allows students to immerse themselves in the lab culture by interacting with lab members and the PI. Some doctoral students looked for very specific values when deciding which lab to join for their dissertation. Some recurring themes outside of lab research topics included: a collegial and collaborative work environment, peers that are hardworking and friendly, a lab that challenged students to critically think and analyze data, the prestige and reputation of the lab, as well as the history of lab funding. Selecting a lab to join can be stressful for many students because the lab they choose often determines the PI they will work with for the remainder of their graduate careers. PI's shape the culture of their lab, and are often one of the main sources of input on research project direction, and can be considered the main individual responsible for socializing their students into the discipline. How PI's mentor graduate students and cultivate their lab culture can be seen as a reflection of how they were trained to be a scientist, and what they feel is necessary in order to be a successful scientist.

PI's can serve as bridges between collaborating research groups, write recommendation letters, and be advocates for their students on the job market. One student discusses their feelings about their relationship with their advisor.

> My relationship is that I want to impress my adviser to con- to show that I'm doing well and that I'm making, you know, finding cool stuff out . And that I think about things. So it's definitely - I want to impress. (Interviewee M; Mestizo Man)

The need to impress may come from experiential knowledge passed down from previous graduate students about the benefits of having positive relationships with your PI. Not only are PI's responsible for writing recommendation letters for their students, but through PI's, doctoral students have access to scientific social networks.

> So [my current PI] and my former Undergrad PI actually know each other really well. So I was kind of lucky in that, um - my undergrad PI who told me like, "she's a great PI, you would love the stuff they do". Um, so science wise, I think I got a very cherry picked, like perfectly primed position to join [my current] lab. So [my current PI] was my first rotation. (Interviewee F; Asian woman)

Reputation and recognition in the sciences has been discussed in depth by Merton (1988) in *The Matthew Effect in Science, II* [219] . The Matthew Effect is a phenomenon in which eminent scientists disproportionately get more credit than non-eminent scientists even if they both provide

similar levels of contribution [219]. More famous scientists are more likely to receive grant funding, fellowships and access to resources. Funding is attractive to doctoral students because it allows for more experimental flexibility and research exploration because one can purchase expensive technical equipment and worry less about reagent scarcity that is a concern in smaller labs with less funding. Doctoral students from smaller labs often had to be more aware of the types of experiments and research trajectories they pursued. Interestingly, the Matthew Effect seems to also benefit doctoral students who are legacy children of well-known scientists. Here, a student describes two other members of her lab, and how their status shapes the PI's relationship with them.

> And one thing about the lab that is, like very intimidating is that, two of the graduate students are like science legacy children. So, like one of them is like the son of the president of my alma mater, who also was like also this very important like, you know, scientist or whatever and then another one is like a son of like a very important geneticist that like, you know, so like the more you learn about the people who are there it's like ivy league or science royalty and it feels very like that's why you're [the PI's] favorite. (Interviewee N; White Latinx Woman)

This type of favoritism can lead to disparities in resource allocation (e.g. time spent discussing research projects) towards graduate students. This student then goes on to explain how this favoritism affects her development as a scientist in comparison to her peers.

> He's not going to come to my desk to shoot the shit with me. He's gonna go to one of the three [men who are] friends to go shoot the shit with them and then that's when they start going and having cool ideas and stuff like that. He doesn't do that with me. He goes on sailing trips with some people and not other people...He did this bullshit thing last year where he was letting some people go to more prestigious conferences and not others based on where their data were, but it kind of didn't make sense why he wasn't sending – like, I asked to go to this conference because - because I hadn't gone to a lot of conferences . (Interviewee N ; White Latinx Woman)

While this is one of the more extreme cases of different treatment amongst graduate students by the PI, another student from a different lab shared similar sentiments about his tense relationship with his PI.

> One grad student gets invited out to dinner or goes and has, uh, you know, one-on-one coffee discussions, uh, you know, once or twice a week or, um, you know, like has one and a half hour meetings versus the typical 15 minute meetings for everyone else or, uh, has their project basically focused on, uh, like 70% of the time, whereas everyone else sort of makes up, you know, a minor of it. (Interviewee J; Mestizo Latinx Man)

Both of these quotes highlight how the time allocated towards doctoral students varies based on PI's preferences. And this can have significant consequences for the professional development of students not receiving this treatment. Students that receive the necessary support from their advisors receive advice and suggestions on how to overcome challenges with data analysis.

> Like I was being like, oh, “I have all this analysis to do”. And she's like, “Is there anyway to like automate this, do you need an Undergrad?” and I was like, “actually I wanted to figure out, like how to write like a macro code for it”. It's kind of a complicated macro. And the more I thought about it, I was like, crap, I don't know if I have the skills for this. Um, she was like, “oh, [recent PhD acquaintance] did a lot of that in her PhD. Like, why don't you ask her?”. (Interviewee O; Asian Woman)

This conversation reveals (1.) how a simple statement can lead to a process of brainstorming more efficient ways for a student to complete a task, (2.) the benefits of self reflective dialogue between doctoral students and their PI to identify areas for technical improvement, and (3.) the ways in which a PI can use their social network to connect doctoral students in their lab to resources that may not have immediately been accessible or clearly defined. The student below discussed his interest in applying for postdoctoral positions.

> And more recently he's had, he's invited speakers or whatever for, for our lab. Um, and he's told me explicitly "this person that's coming to visit may be a good fit for you" for whatever reason ... He invited a guest speaker for one of the, one of his classes to do like a, um, a guest lecture and he's like, ah, “why don't you take him from the seminar to lab. And then you guys can go and grab a coffee or something and hang out, talk or whatever. Um, you know, just moving from here to here and you guys can hang out and get to know each other or whatever”. And so he did that. Um, and he told me explicitly like, he might be someone that you might be interested in. (Interviewee P; Mestizo Latinx Man)

This is an example of a PI using his social capital to provide his graduate student with an opportunity to network with other scientists in the same discipline.

Conclusion

Doctoral programs in the sciences train future scholars and field experts contribute to research productivity and the creation of new knowledge. While most work on disparities in STEM education and high student attrition rates in STEM have been conducted on undergraduates [220], doctoral students working in biological science labs are a unique and understudied population in that they have made firmer commitments to a field and have had more time to reflect about their interests through coursework, research experience and extracurricular activities. Studies have been conducted on doctoral student persistence in the mathematical sciences [192], the role of support networks to graduate students of color [221], factors contributing to graduate students sense of belonging [217], and science identity formation with respect to advisor relationships among women of color PhD graduates [191]. Less work has specifically focused on doctoral students in the biological sciences, and the elements of socialization that are important for their development as scientists.

Graduate student success in STEM and their movement into the STEM labor force is based on the successful socialization of students to the organizational culture of science and technology related fields. Students more well integrated into their lab environment have easier access to learning shared disciplinary knowledge, values and attitudes held by other members of their lab, and the scientific community. Students lacking these strong connections may struggle to

successfully socialize into the academic science community, and thus, may find alternative career paths that align better with their interests and values. Navigating the unique combination of these dimensions at a given university is important for doctoral student matriculation into STEM fields, and inadequate socialization within this complex, dynamic and interactive process is theorized to lead to doctoral student attrition [189, 222].

When students enter a research lab, they are entering into an environment with resilient and taken-for-granted norms that guide member's behavior. Merton (1957) describes various institutional imperatives that comprise the culture of science, one of them being universalism. This ideology demands that science be open to all talents, with the goal of science and science education being the furtherance of knowledge. Despite this ideology, there remains structural barriers (e.g. access to science resources, hostile lab environments) to participation in science. Sites of scientific research (e.g. national labs, universities, and industry), are not neutral bureaucratic entities [223]. This reveals itself in practice through the varied levels of participation and (dis)engagement of women and people of color in science. My interviews with doctoral students in LSA and LSB reveal that women and students of color experience barriers to participation in biological science labs due to weaker informal relationships with labmates and PI's. This work reveals that informal interactions are important and effective in the transferring different forms of scientific capital to doctoral students.

Carlone and Johnson (2007) developed a model of scientific identity using qualitative data collected from women of color in science [191]. This model illustrates three components important for the strong formation of a scientific identity : competence in one's knowledge and understanding of scientific content, ability to perform relevant scientific practices, and achievement of recognition by oneself and others in the scientific community as a science person. Multiple groups have discussed the role of social factors and socialization in scientific career attainment [191, 218]. Interviews with doctoral students show knowledge and deeper understandings of technical and theoretical scientific concepts are shared through informal interactions, and can influence how well an individual feels a sense of belonging to the scientific community. This work expands on our understanding of key sites where doctoral students are socialized into their profession, and the role of informal interactions in the establishment of scientific capital.

Informal conversations are important in developing social cohesion among lab members. While there are no clear agreed upon terms of what constitutes formal language in conversation, but what is clear is the unique nature and value of informal communication [224]. Prior observational studies of informal conversations reveal that informal conversation functions to transmit office culture, coordinate group activity, execute work-related tasks and establish healthy team dynamics [225]. These interactions also help members of the same organization learn about each other and their work and facilitate collaborations. Physical and organizational proximity has been shown to support frequent opportunistic conversations, and plays a role in scientific research collaboration [226]. This work reveals that informal interactions affect access to different forms of science capital.

While the stereotypical image of a laboratory scientist is an individual that works in isolation; working in a laboratory is fundamentally a social process. My work suggests that seniority, race and ethnicity, and gender shape the training experiences of doctoral students in unique ways that affect their access to different forms of scientific capital. Socialization of doctoral students in biological science doctoral programs occurs through day-to day interactions with labmates and PI's, and can differ based on the race and gender of the doctoral student. Continuing to study doctoral student socialization and identifying underlying mechanisms for differences in

informal interactions in the STEM doctoral programs is necessary if we are to create scientific spaces that welcome and affirm the lived experiences, backgrounds and cultures of diverse communities.

Descriptor		n	(%)
Gender			
	Men	12	38%
	Women	18	56%
	Genderfluid/Nonbinary	2	6%
Race			
	Black	3	9%
	White	23	72%
	Asian	6	19%
	Mestizo/Mixed Race	2	6%
Ethnicity			
	Latinx	6	19%
	Indian	3	9%
	Japanese	1	3%
	Vietnamese	1	3%
	Puerto Rican	1	3%
	Filipino	1	3%
Year of Degree			
	2	5	16%
	3	12	38%
	4	15	47%

Table 1: Study Sample Demographics (n=32)

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