Microbial Interactions: Prediction, Characterization, and Spatial Context

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Microbial communities are complex networks comprised of multiple species that are facilitating and inhibiting one another (as well as themselves). Currently, we lack an understanding of what mechanisms drive coexistence within these communities. We aimed to remedy this by studying the dynamics of coexisting communities, focusing on the complexity of their interaction networks, the impact of spatial dynamics, and the interplay of facilitating and inhibiting interactions. These limitations in our understanding prevent the furtherment of designing intentional communities for bioremediation, maintenance of healthy microbiota, and other functional communities. To better understand these microbial dynamics, we chose to address the problem from two fronts: computational modeling and exploring dynamics of cocultures. Through our 1-D model, spatial structure fostering more coexistence – especially when facilitation is present. For the coexistence assays, we determined that contact-dependent growth inhibition is a density dependent mechanism, and the use of a Tn-Seq mutant library to predict species interactions is possible, but needs further optimization to reconcile density dependent effects of interactions.

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Abbreviations

A.U.	Arbitrary units
CDI	Contact-dependent growth inhibition
CDiA	Contact-dependent growth inhibition toxin protein
CDiB	Contact-dependent growth inhibition transmembrane protein
CDiI	Contact-dependent growth inhibition immunity protein
dsRed	Red fluorescence protein
HGT	Horizontal gene transfer
LB	Luria broth
M9	Minimal defined media
MSA	Multiple Sequence Alignment
OD600	Optical density 600nm
RB Tn-Seq	Random barcode transposon insertion sequencing
RFU	Relative fluorescence units
Tn-Seq	Transposon insertion sequencing
UTI	Urinary tract infection
WM	Well-mixed
YFP	Yellow fluorescence protein

Prelude

Microbial communities affect our lives in many ways, from impacting human health to shaping the ecosystem around us.

There are many underexplored facets of how interactions among microbial species affect community composition and assembly. This is important for a better understanding of how communities are formed, how they are maintained, and potentially how we can modify and control them.

My work covers two particular aspects: In chapter 1 I investigate the impact of diffusible metabolites on community assembly and coexistence within a spatially structured environment. In chapter 2 I examine contact-dependent inhibition as an example of interactions that require physical contact between cells to explore its spread and ecological impact.

Since my work covers two rather distinct aspects, I have organized my thesis in two independent chapters, each with separate introduction, results, and discussions.

Chapter 1

Chemical mediators' impact on microbial community assembly and coexistence in well-mixed and spatial environments

Introduction

Microbes are ever-present from the deep ocean to the human digestive tract. However, it is estimated that as little as 5% of microbes have been discovered[1]. In some cases the community has been treated as a 'black box', focusing solely on the relation between an input (e.g. a carbon source) and a measurable output (e.g. a product or degradation of a chemical)[2,3]. In these instances, the microbial community composition is often not taken into consideration. On the other hand, often when microbes are more thoroughly studied, it is through *in vitro* experiments of single cultivable species or 16S ribosomal RNA gene sequencing of entire communities[4–8]. Both of these approaches help further the knowledge on microbes, but fail to address the interactions that microbes in a community have on one another. The spatial structure of the community is also lost through the process of collecting these samples leaving a gap in understanding of the organization of the microbes. Investigating the role interactions play in community dynamics and coexistence has become a major area of interest in microbial ecology.

By understanding community coexistence, in the future we can prevent or recover from dysfunctional microbiota compositions associated with disease [9]. Our

current methods of controlling pathogenic bacteria rely heavily on the use of antibiotics, which kills pathogenic and non-pathogenic bacteria alike. The drawbacks of antibiotic use are well known: antibiotic resistance and imbalance of the bacteria flora[9,10]. If the gut microbiota is viewed as a delicate ecosystem, then the treatment for diseases such as *Clostridium difficile* infection, obesity, and necrotizing enterocolitis could be to fix the imbalance in the ecosystem[9,11–13]. We must learn how the community assembles and coexists to better combat these prevalent diseases without damaging the heathy community as a whole.

Before communities can be intentionally manipulated for therapeutic means, we must first understand how a community achieves, maintains, and loses coexistence. We define coexistence as a state in which the species within the community remain present above a certain threshold though each of the species may undergo variations in abundance. To understand coexistence, the role microbial interactions play in both stabilizing and destabilizing a community must be better understood. Without lasting coexistence, microbial therapeutics would be only a temporary fix to a chronic problem.

Microbial communities are prevalent and impactful

Microbes are not found in isolation, but instead are found in diverse communities and environments. Within these communities, diverse microbial interactions create complex networks, of facilitation and inhibition through direct and indirect means. In natural communities, microbes can inhibit and facilitate each other through many mechanisms such as amino acid exchange (facilitation)[14,15] and

antibiotic production (inhibition)[16]. These interactions impact the ability of all species to maintain within the community by impacting growth[17], survival[18], and prevalence[19]. By understanding the network of microbial interactions, the community is broken down into the most fundamental parts that offer insight into the complex nature of the community's functionality.

Interactions can influence coexistence in microbial communities

Looking at a few species and determining the dynamics between them is insightful[20] , but it is unclear how this scales up to larger communities. It is also unclear if facilitation or competition drives diversity and stability of a community. A proposed reason for facilitation is the Black Queen Hypothesis [21] where some species lose gene diversity, forcing them to be reliant on others in the community to survive. The production of helpful resources can be attributed to leaky vital genes that allows for excess of a resource that is consumed by another member of the community [21].

For competition within communities stems from the limited resources present that are necessary for growth. There is the principle of competitive exclusion which asserts that species with too much niche overlap cannot coexist and it is a race to outcompete each other, forcing the other to be excluded from the environment [22]. This perspective on competition predicts that competition will lower species diversity.

There are many perspectives trying to describe how coexistence arises in communities such as neutral theory – positing that these species are ecologically equivalent and able to coexist[23], a variety of relationships between microbes allows for diversity such as cross-feeding[24], rock-paper-scissors[25], and higher-order interactions not defined by pairwise interactions[26,27]. Alternative to neutral theory, niche theory speculates that coexistence arises due to niche overlap and fitness differences[28,29].

Most likely a combination of facilitation and inhibition leads to these complex diverse communities in our guts[30,31], soil[32,33], and nearly everyone other place on the planet. What parameters lead to coexistence of microbial communities? How do these parameters interplay with one another to affect a community's assembly and coexistence? What contributes to the communities to balance the growth rates and allow for coexistence?

Spatial context impacts community assembly and coexistence

In natural communities, microbes are present not just with each other, but dispersed in space throughout complex environments (plant phyllo sphere, human nasal passage, or waterways, as a few examples)[34–36]. Within these communities microbes interact via nutrients and inhibitors[37,38]. This is an important aspect to understanding community assembly and coexistence because diffusion of nutrients or inhibitors can shape interactions among members of a community. It is not always clear how the spatial structure of the environment will impact a community – especially one with a diverse set of interactions.

In lab settings, often well-mixed cocultures fail to produce coexistence, so exploring coexistence in a lab setting proves difficult. A spatial experiment can be performed, but understanding the dynamics and expanding these finding to larger more diverse communities is challenging. A better way to address questions about communities is using mathematical modeling. The introduction of a spatially segregated community allowed for a 3-species community to thrive together, when they outcompeted one another in a well-mixed environment [39]. Their takeaway was that "fences make good neighbors" for these microbial communities. This group created a microfluidic device to allow chemical mediators to pass between cells, but not the cells themselves to better understand how by changing the distance between the species the coexistence changed. They found without distance between them all 3 of their species could not survive, but by imposing spatial structure all 3 were able to stably coexist. In order to probe complex communities', models that incorporate the spatial context can be used to ask and address outstanding questions.

Mathematical Modeling of Microbial Communities Offers Valuable Insights

A mathematical model will offer insight into impact of the complex simultaneous interaction on the stability of the community. This will lead to predictions on how the community as a whole reaches coexistence and impacts members' growth. It offers predictive power and describes a system's response to a specific input or a system's response to a perturbation. For population models, within a community, each species is considered a population and described as a single entity. Thus, this type of model aims to evaluate community-level impacts

based on the overall impact experienced by each constituent population as a whole, not the individual members. This facilitates predictions pertaining to how populations will respond to changes in the environment and influence other populations in the community[40]. This model, however, does not account for heterogeneity within an individual populations[41]. Instead, it assumes a well-mixed environment, in which each individual is experiencing the same environmental factors and thus responds to changes in the environment similarly.

The implementation of a population model relies on data from the system (e.g. starting cell count, growth rate, and influence of species on one another) to predict changes in growth rate of each population in response to environmental perturbation. It is easily modified to model a variety of systems such as mutualism, competition, and predator-prey role reversal [40,42,43]. Once the parameters of the model are established, simulations can be performed which predict the impact of altering specific parameters on various factors on the overall community dynamics. This provides a consistent, modifiable system to study the population-level impacts a community experiences in response to individual, or simultaneous, environmental changes. In order to validate the simulations, *in vitro* and *in vivo* experiments are performed to compare the predicted outcome with the experimental outcome. This provides a metric for measuring the descriptive and predictive capacity of a mathematical model for the system. Mathematical models are continuously improved through the addition of newly obtained data pertinent to the system's dynamics. Through this iterative process, mathematical models become robust tools

for understanding the dynamics of a system and predicting the impact stressors have on the system.

Previous work on spatial modeling such as agent-based models have been used to model, bacterial communities experiencing different sub-environments such as gradients of moisture, nutrients, and temperature changes[44]. A previous model has examined how the moisture and roughness of the soil may influence microbial diversity within a soil sample[45]. In their model, they incorporate the parameters of different species interacting with their environment and the varying environments (soil roughness and moisture)[45]. By exploring abiotic impacts that affect individual cells within several populations, they were able to observe unique spatial patterns that formed for these communities.

The one-dimensional (1-D) model is meant to model microbes in a basic spatial environment. Often sequencing sets[46–48] from environmental and clinical samples find large microbial community diversity. However, lab settings are often limited to small, minimalistic versions of these communities. This arises from a multitude of challenges, from difficult to culture microbes to dominance of one strain within an artificial community. What allows for such a range of community diversity to be maintained in natural communities? By modeling these communities, I hope to gain insight into what parameters (or combination thereof) leads to larger stable microbial communities. By taking a 1D perspective we are considering space, but still keeping a simplified model of it. This approach could help us identify rules

and trends for coexistence that allows for understanding these complex, natural communities seen in environmental samples.

To understand microbial coexistence, we need to better understand two factors of their communities: types of interaction (facilitation and inhibition) and the environment (well-mixed and spatial). In the pursuit of studying these parameters' impact on community coexistence, we are focusing on community richness, which is a measure of the number of coexisting species present in a community at a given time. Natural communities display a great range of richness across time and space that is not fully understood. By modeling microbial communities based on the interactions of their substituent species, we gain a better understanding of the types of complex dynamics that lead to increased richness and coexistence. Similarly, by modeling these communities in both well-mixed and spatial environments we can detangle the impact of spatial dynamics on community interactions and niche formation that may be happening within these communities.

We use a chemical-mediator model for both the well-mixed and spatial condition because chemical compounds is a known interaction dynamic in microbes (cross-feeding[49,50] and antibiotic production[51]), pairwise interactions models over simplify indirect interactions[52,53], and the more recent identification and quantification of chemical mediators present within communities makes it more important than before to understand their facilitative and inhibitory impacts on members within those environments[54]. Others have explored modeling 2D microbial interactions with metabolic modeling of species and diffusible metabolites

and found that these metabolic processes are complex, but predictable[55]. By exploring how these chemical mediators' impact to coexistence, we hope to understand these trends within the interactions of communities.

In our model,[56] we determine the impact of chemical mediators on the overall community. This is a dilution model, modelled after environments such as a turbidostat where the necessary nutrients are continuously supplied. The impact of the chemical mediators will change the growth of the species, but all of the species have all necessary resources (such as a carbon source) supplied for them. We are trying to determine the impact of the interactions, not the richness of the environment itself. Our model can be used to test ecological ideas of the impact of spatial structure and microbial coexistence.

Methods

Simulation execution

All simulations were completed in MATLAB ® and run on the Sirius cluster at Boston College. Each set of parameters was simulated 500 instances, the default parameters can be found in Table 1. The files used are found in Table 2.

Chemical Mediator Model

A chemical mediator interaction model was used as previously described[56]. The mediators are produced and consumed by species within the well-mixed environment (Fig. 1). All functions and .m files can be found in Table 3. Table 2 indicates the default parameters for the variables in the simulation.

1D spatial model

Initially all species are in separate starting locations (species 1 next to species 2 and so on) (Fig. 4) with the same population size. The space does have edges (it is not a loop where species 10 is next to species 1). Similarly, to the well-mixed model, there is a range of assigned population reproduction rates (0.1 - 0.2/hr) that are randomly assigned to each population as well as the connectivity of the population – meaning which species are producing and consuming which mediators. This connection between each member of the community has a chance of being inhibitory or facilitative. This is controlled by the percent of initial interactions that will be inhibitory or facilitative. There is also a range for the rates of production and consumption of the mediators. Reflected in the well-mixed model

and the spatial model, after many rounds of dilution (approximately 100 generations), a subset of species coexist in the community and these species stably remain if the dilution scheme continued for 200 generations [56]. Each simulation was performed 500 instances and significance was determined via 95% BCa bootstrap confidence interval.

Results and Discussion

Comparison of Well-mixed and Spatial Environments

In natural communities, microbes are not present in a homogeneous environment, but dispersed in space which impacts the communities assembly and coexistence[39,57,58]. This structure is an important aspect to understanding community assembly and coexistence because diffusion of nutrients or inhibitors can shape interactions among members of a community. We know that space contributes to the structure of the interacting members of a community, often resulting in niches that limit contact between all members. Comparing a well-mixed model to a 1D spatial model will let us determine how much and what kind of an impact spatial structure has on community assembly and coexistence.

Our hypothesis for the introduction of space into a microbial community is that it will increase richness of coexisting communities because the interactions are weaker in a spatially structured environment. There is previous work that supports that space allows for neighbors to have both competition and coexistence compared to a homogenous environment[59,60]. By the species being able to disperse in space they can survive together because the movement into a new niche may not contain a superior competitor[59]. For our model we theorize that the weaker competition under certain condition would be due to gradients of chemical mediators which would be creating niches. These weaker interactions allow for increased species richness because the overall negative impact of inhibitory interactions is reduced while still allowing for moderate beneficial effects from facilitative interactions. In

this way, the presence of a single, strongly inhibitory chemical mediator will not impact the population of the consumer as significantly. I hypothesize that spatial distribution of interactions impacts the community assembly and encourages higher coexistence through the creation of niches compared to a well-mixed environment. I also predict inhibitory interactions will have less coexistence compared to facilitatory interaction networks, but the negative impact of inhibitory interactions on coexistence will be mitigated in spatial environments. I anticipate increasing both production and consumption rates to mimic the well-mixed conditions since this will be comparable to the homogeneous well-mixed environment. The goal is not to simulate a natural community, but to discover the patterns and trends that allow for coexistence within a 1D space.

1D microbial community model

The 1D spatial model works similarly way to the well-mixed model[56]; however instead of all of the chemical mediators being present for all microbes there is a 1D space (Fig. 1) across which the cells and mediators can diffuse. There is no competition for the space itself (more than one species can occupy the same location), but there is a difference in the populations and mediator present from location to location.

The 1D spatial model simulates mediator interactions between populations. One important parameter is the size of the space being simulated as well as the resolution of the data collected. For the simulations performed the size of the space simulated is 0.5 cm. With the size of the space the resolution of said space is

essential as well. To determine the necessary resolution, the absolute difference between the size of resolution (cm) and the highest resolution tested (0.002 cm). There is little difference in the values (Fig. 2). The chosen resolution is 0.005 cm in a 5cm space.

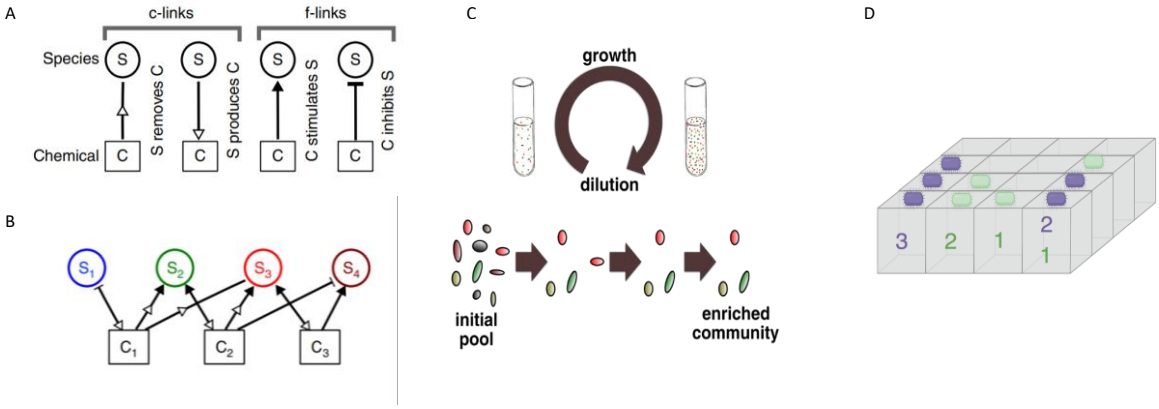

Figure 1 | Simulating a community of microbes engaged in chemical-mediated interactions in well-mixed and spatial environments. A) Species remove or produce chemical mediators, which stimulate or inhibit species. B) These communities can have complex interactions with one another. C) To simulate experimental set-up, the initial pool of 10 species goes through a series of growth and dilution steps. D) This is simulated in 1D space and a WM environment. Each experiment is simulated 500 times.

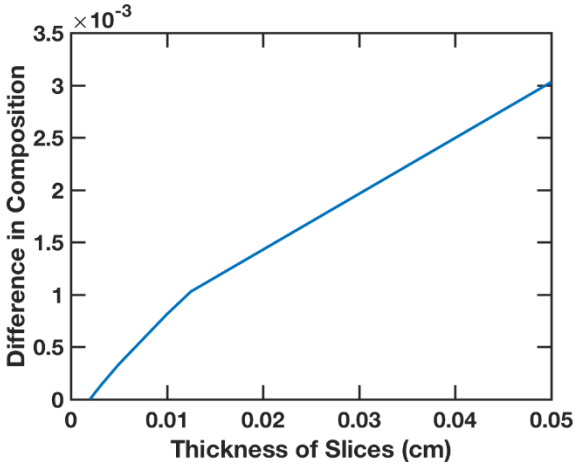

Figure 2 | Determining necessary resolution of 1D space. Comparison of changing the resolution of the 1D space to determine the impact on community composition. To determine the difference in composition, the average richness of all the final communities was compared to the average for a given resolution.

Similar to the well-mixed model, for each simulation we simulate three different initial interaction networks. For the work done in this study, "Positive" indicates an initial interaction network that is 90% facilitative interactions, "Even" indicates 50% facilitative, and "Negative" indicates 10% facilitative. We hypothesize that facilitative interactions will increase mean richness because facilitation has been shown to drive biodiversity in natural communities in a multitude of ways such as stress amelioration, increasing access to resources, and increasing habitat complexity[61]. We intend to continue to explore inhibitory interaction (rather than simulating completely facilitative communities) since there is evidence of a prevalence interactions between microbes being inhibitory though this may be a bias of the analysis done to understand these communities [62].

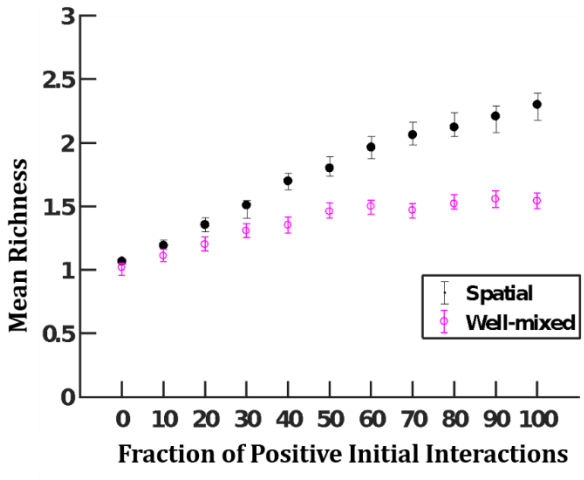

Figure 3 | Mean richness is defined as the median number of coexisting species (samples = 500). We chose the initial number of species types *Nc* = 10 and the number of mediators *Nm* = 5. The same initial community networks were compared with differing ratio of positive interactions. Error bars show bootstrap estimates of 95% confidence intervals for the mean values.

By looking at a range of initial interaction networks (Fig. 3), we find that facilitation in a spatial environment leads to more coexistence. Especially within a spatial environment. This is due to the weakened impact of interactions in a spatial environment. By introducing a gradient of chemical mediators, the species being inhibited are no longer as strongly impacted. This also remains true for the facilitative interactions, but facilitation maintains larger communities by facilitation between species maintains balance of a community. We find that self-facilitation is self-serving reducing coexistence (Fig. 8). As the ratio of positive interactions increases, there is a higher mean richness observed in both spatial and well-mixed communities. Spatial has higher mean richness compared to well-mixed, increasing as positive interactions increase (Fig. 3). Spatial environments allow for niches where slower growers can use chemical mediators to remain in the community

without competing with fast growers, leading to this increased richness. Conversely previous modeling work has demonstrated how competition can lead to higher coexistence in a well-mixed environment[63]. This difference in outcome stems from their model having primary metabolites being model so a well-mixed model favors necessary resources being present.

We find in both well-mixed and spatial environments we often have few species in the final enriched community (Fig. 3). This is partially due to the number of starting species (10) because there will also be a reduction in surviving species through the growth and dilution steps (Fig. 1). This is also due to the competition of growth rates within the community. None of the species are competing for necessary resources, such as a carbon source, so it is often a competition of randomized growth rates. The species with the highest growth rates will remain and those with slower will be outcompeted. The goal is not necessarily to create the largest community, but rather find trends of coexistence with the manipulation of parameters. If the goal were to be having higher coexistence within this simulation then reducing the range of randomized growth rates would allow for more coexistence since otherwise it is a growth rate race.

Since this is a spatial model, the dispersal rate of the species was also important to determine. A faster dispersal rate mimics a faster cell motility, and conversely the opposite is true for the slower dispersal rates indicating slower or even no motility. By changing the dispersal constants of cells, we can determine the impact of motility on mean richness in a community. For our parameters we

compared species in a well-mixed environment (infinity), intermediate speeds, as well as non-motile (0) (Fig. 4). We predicted that a reduction of dispersal would increase mean richness through the creation of niches, which are not found in the well-mixed condition. We find that coexistence increases with reduced cellular migration with less coexistence present in the well-mixed condition compared to the non-motile species (Fig. 4). In addition to the difference in outcome based on motility, we also find a difference with the type of initial interaction network. For the Positive initial interaction network, the mean richness was the highest, while the Negative condition remained largely unchanged throughout each dispersal constant (Fig. 4). For the remainder of the study we used the dispersal rate 5e-8cm^2/hr, since this dispersal rate led to a notable difference between initial interaction networks, but it still allowed for some movement of the species within the 1D space (Fig. 4).

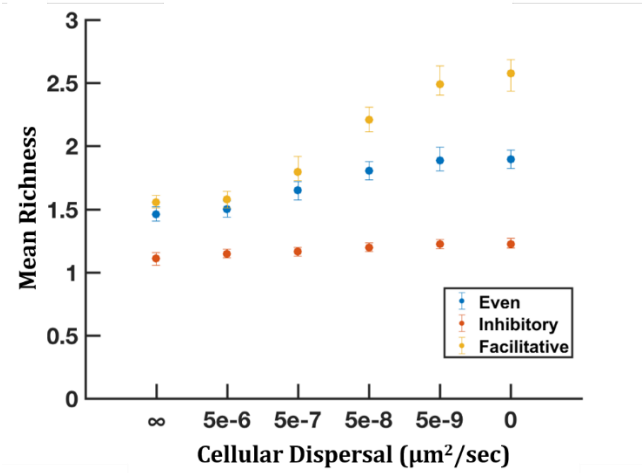

Figure 4 | Impact of the reduction of motility and ratios of interactions on community mean richness. Mean richness is defined as the median number of coexisting species (samples = 500). We chose the initial number of species types *Nc* = 10 and the number of mediators *Nm* = 5. The mean richness was compared for fast motility (homogenous) ranging to no motility. The interaction ratio was also changed from mostly stimulating (yellow), even

(blue), and mostly inhibitory (red). Error bars show bootstrap estimates of 95% confidence intervals for the mean values.

An example of a community is visualized in Fig. 5. The initial interaction network is an even network. The thickness of the arrows indicates the strength of the interaction. The top half of the arrow is for production and the bottom half is for consumption. In this example community, species 3, 7, and 10 survive and coexist in the final community, and there are both facilitative and inhibitory interaction in the final community. There is also a non-interacting species present, species 10. There is also a self-facilitating and self-inhibiting species (#7). This representative community demonstrates how complex even a 3 species coexisting community can become.

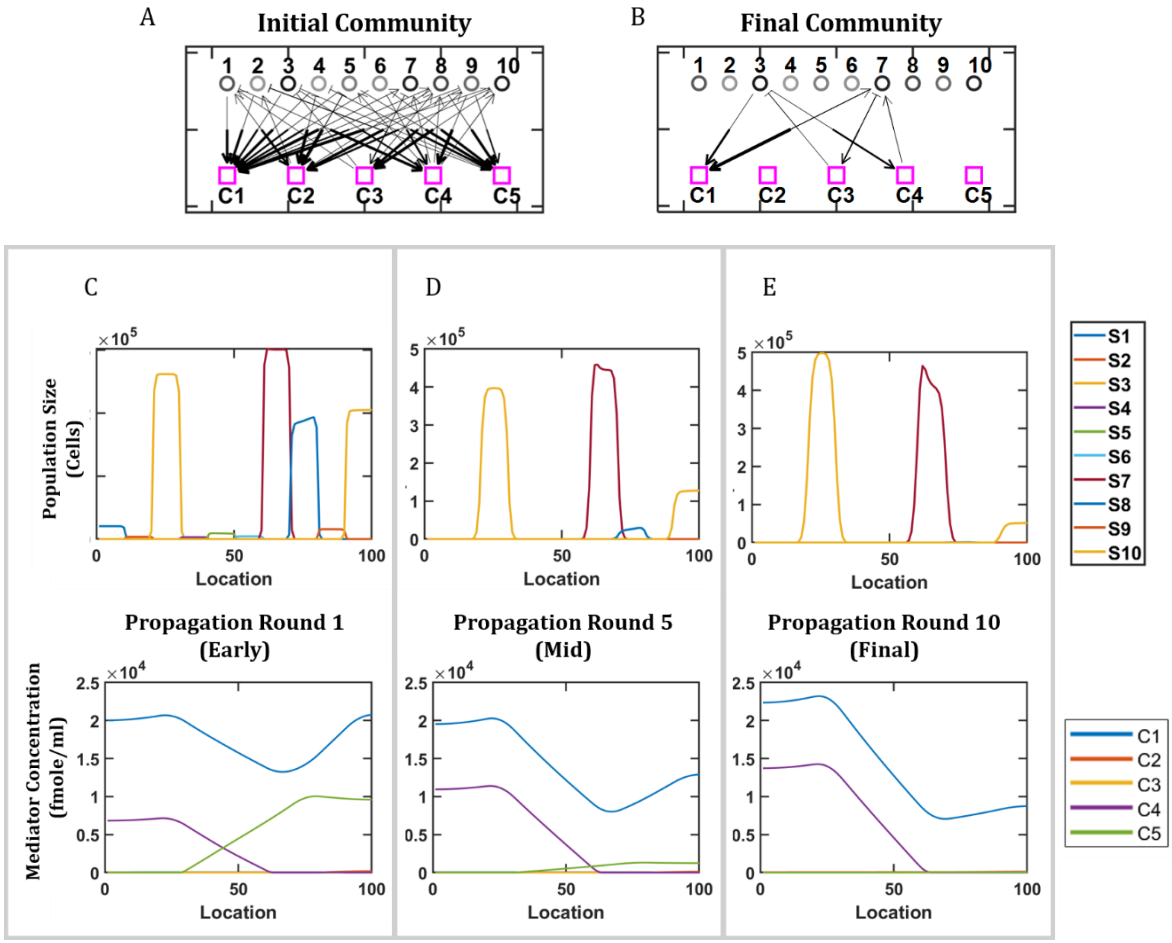

Figure 5 | Example community network. An example of an even interaction network with default parameters. The initial (A) and final (B) community are shown with arrows indicate facilitation/production and blunt ends indicate inhibition. This same community is show at early (C), mid (D), and final (E) community location(top) and chemical mediator location(bottom).

In our model, we find that a species' low dispersal rate limits movement, but chemical mediators readily diffuse through space (Fig. 5). We also find there is still a chemical mediator gradient even with the cellular dispersal being limited. The populations are also varied between each species with 3 and 7 being the largest (and the interacting species) and 10 maintaining low population levels within the community.

Spatial structure supports more coexistence, especially when facilitation is prevalent

Another important consideration is the impact of the prevalence of facilitation versus inhibition. Similar to Niehaus, 2019, we find that there is a steady increase in coexistence when facilitation becomes more prevalent[56]. Notably, the spatial environment supports coexistence even more when facilitation is prevalent.

For example, a 2-species community with density-dependent interactions will have differing outcomes depending on if the interaction is inhibitory or facilitative[64]. If species A is inhibiting species B, then it will lead to the exclusion of species B from the community. If species A is able to inhibit species B enough that the growth rate of species B cannot overcome the inhibition (especially with the growing population of species A), then species B will no longer exist in the community. Conversely, if species C is facilitating species D this will increase the growth rate of species D. If the population rapidly increases, then species C will decrease, reducing the population of species D because facilitation is limited. If species C goes extinct then the size of species' D population will also be reduced because the facilitation link is also removed. The way that interactions can lead to coexistence is by facilitating the growth of slower growers and inhibiting the growth rate of faster growing populations.

The well-mixed and spatial environment follow the same trends as the average mediator production/consumption rates vary

We find that in addition to spatial structure fostering coexistence of chemically interacting microbes, the communities exhibiting more facilitation also support more coexistence. I predict that increasing both production and consumption rates to mimic the well-mixed conditions since this will be comparable to the homogeneous well-mixed environment. The spatial environment thus far as increased mean richness, so we anticipate the same for the production and consumption rates because of the space allowing for niches and gradients of chemical mediators (Fig. 5). We find the same trend (similar results in each parameter as well as initial interaction network) for production and consumption rate, but the impact mean richness more strongly in the spatial environments (Fig. 6). We posit this is because space allows for a reduction of the inhibitory affect by creating the chemical mediator gradient. The microbes that are in closer proximity to the inhibitor are more strongly receiving the inhibition while the space allows other species which are further away to be less drastically impacted.

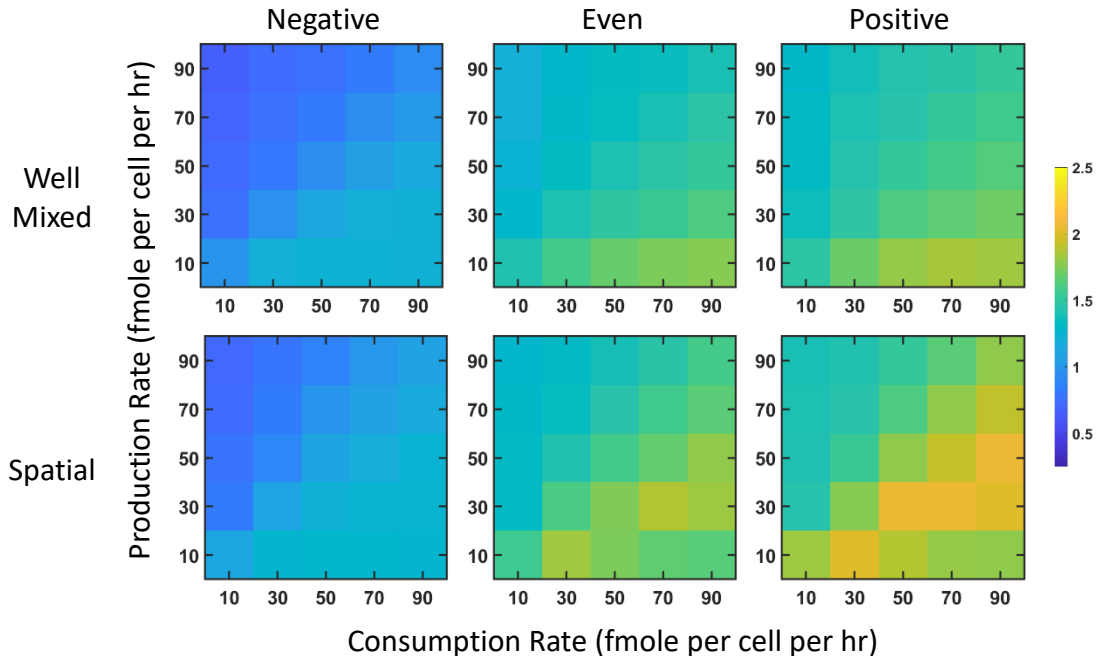

Figure 6 | Optimal production and consumption rates in spatial and well-mixed environments. We ran simulations in communities starting with 90% inhibitory interactions (left), 50% inhibitory interactions (middle), and 90% facilitative interactions (right). Each simulation was performed in well-mixed (top) and spatial(bottom) environments. Darker colors indicate lower mean richness and lighter colors indicate higher mean richness.

Maximum mean richness is observed at intermediate levels of prevalence of producers and consumers in both spatial ad well-mixed cases

We hypothesize that many producers and intermediate number consumers would increase mean richness because there will still be niches present since not every member of the community is consuming and producing all chemical mediators. If each member was producing and consuming all available resources this would reduce the survival of the members to a growth rate race where the fastest growers who are starting faster as well as receiving the most facilitation will

persevere. In our model for coexistence, interaction should make a difference, otherwise it is a growth rate race.

We find that producers and consumers affect coexistence differently in spatial and well-mixed communities. I find that restraint of consumption favors higher coexistence within a community. A community with most of the species consuming most of the chemical mediators (even in Positive, where nearly every species is facilitating) defaults to the fastest growers will prevail. This situation becomes solely a comparison of growth rate, whichever species are faster will survive, because the impact of the chemical mediators is mitigated since all the species consume all available mediators. There must be a balance struck for the production and consumption of the mediators within a community. We find that it is beneficial to have many producers when the interactions are facilitative (Fig. 7), and the modulation of the number of consumers is what changes the coexistence of the community. Once all the chemical mediators are available such as a saturated environment, then the importance comes from which species are being impacted by the mediators and what type of mediators they are. If the interactions are inhibitory then saturation is negatively impacting coexistence, but if the interactions are facilitative then they will benefit the richness of the community. If most of the microbes are producing most of the chemical mediators in the Even and Positive interaction networks, this could be beneficial to the community because of the increased chance for facilitative mediators.

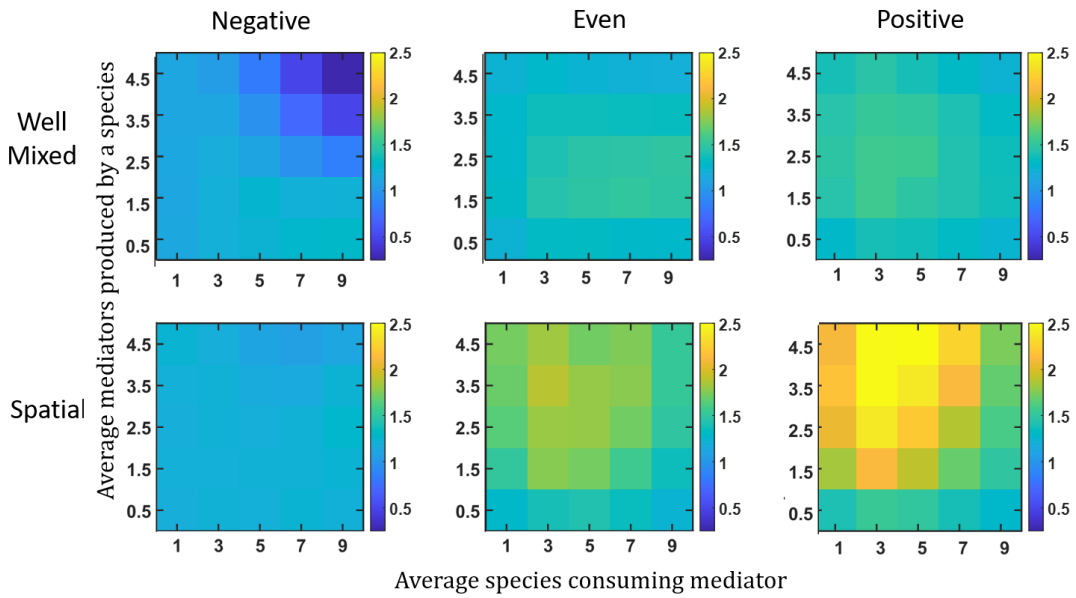

Figure 7 | Optimal producers and consumers in spatial and well-mixed environments. We ran simulations in communities starting with 90% inhibitory interactions (left), 50% inhibitory interactions (middle), and 90% facilitative interactions (right). Each simulation was performed in well-mixed (top) and spatial(bottom) environments. Darker colors indicate lower mean richness and lighter colors indicate higher mean richness.

We conclude that the interplay between producers and consumers in a community is truly about maintaining balance. By being able to modulate parameters in our model, we find that less can be more. By limiting the consumption of a community, it allows species to be dependent on one other and linked together rather than individual microbes existing together until one fast growing species overtakes the community. This is one answer to how species with a variety of growth rates are able to coexist in a community.

One aspect of the community that would reduce coexistence is self-facilitation because it allows for that species to dominate and outcompete other species (Fig. 8) Facilitation between species maintains balance of a community, but self-facilitation is self-serving reducing coexistence. Our data suggests that when

there was only one species left in the final layout the majority of the time the only link present was an sf link, suggesting it decreases coexistence (Fig. 8). When there were two or more species left in the final layout, the data suggests there were other links present than just sf links (Fig. 8).

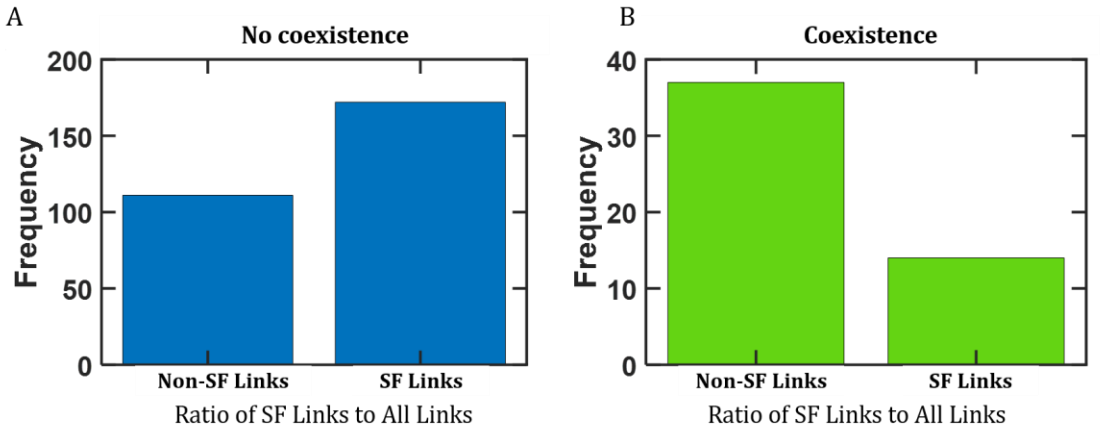

Figure 8 | The ratio of self-facilitating (sf) links to non-self-facilitating links present at the end of the simulation. A) Communities with a single species in the final community and B) communities with two or more species in the final community. Work performed by Alexander Lobanov.

Current Results and Future Steps

In order to more completely understand the community dynamics that lead to coexistence, further examination into the location of microbes in space needs to be done. This can be executed by taking a known existing community and mixing the initial location to determine the strength of the neighbor's impact on the other members. This would allow us to address more questions about the strength of gradients within our community and the impact of close and far neighbors. By comparing this to known microbial mat communities we could relate how strength of interactions and how neighbors change diversity of the community.

In this study, we performed simulations with a single dispersal rate, which mostly kept the species in the same location they began the simulation (Fig. 5), by comparing these results with a faster dispersal rate we can more clearly understand the interface between spatial division and well-mixed. By gradually increasing the dispersal rate until it is essentially well-mixed we can explore patterns of coexistence and assembly in a more diverse range of communities. By simulating a diverse range of dispersal rates, we can better understand what may lead to high diversity within 16S sequencing of natural microbial communities and in the future be able to predict their community organization.

Chapter 2

Exploration of bacterial contact-dependent growth inhibition community impact and prediction of interactions

Introduction

Contact-Dependent growth Inhibition

A dichotomy of interactions often exists within a microbial community. In natural communities, microbes can inhibit and facilitate each other through many mechanisms. One such example of inhibition is contact-dependent growth inhibition (CDI), which is a negative cell-to-cell interaction that can strongly impact the dynamics of a community. The CDI mechanism is a naturally occurring inhibition system discovered in a strain of *E. coli* isolated from rat intestines.[65] The mechanism has since been determined to be present in α-, β- and γ-proteobacteria[66]. As a Type Vb secretion system, CDI is a two-partner system consisting of three genes, CDiB, CDiA, and CDiI[67]. CDiB is a transmembrane protein that allows for the presentation of CDiA, which is a hemagglutinin-repeat protein[6867]. CDiA carries the toxic inhibitory effect of the system and when the effector (CDI) cell comes into contact with a target cell, the C-terminal end of CDiA detaches and enters through the target's transmembrane protein, BamA[68,69]. When encountering an *E. coli* cell that does not have the CDI mechanism, there will be inhibition. If a CDI cell comes in contact with a related CDI cell, the CDiI immunity protein will neutralize the CDiA toxin. This prevents CDI cells from inhibiting their

kin within the community. When the C-terminal end has entered the target cell, it degrades DNA or RNA, which inhibits target cell growth[70].

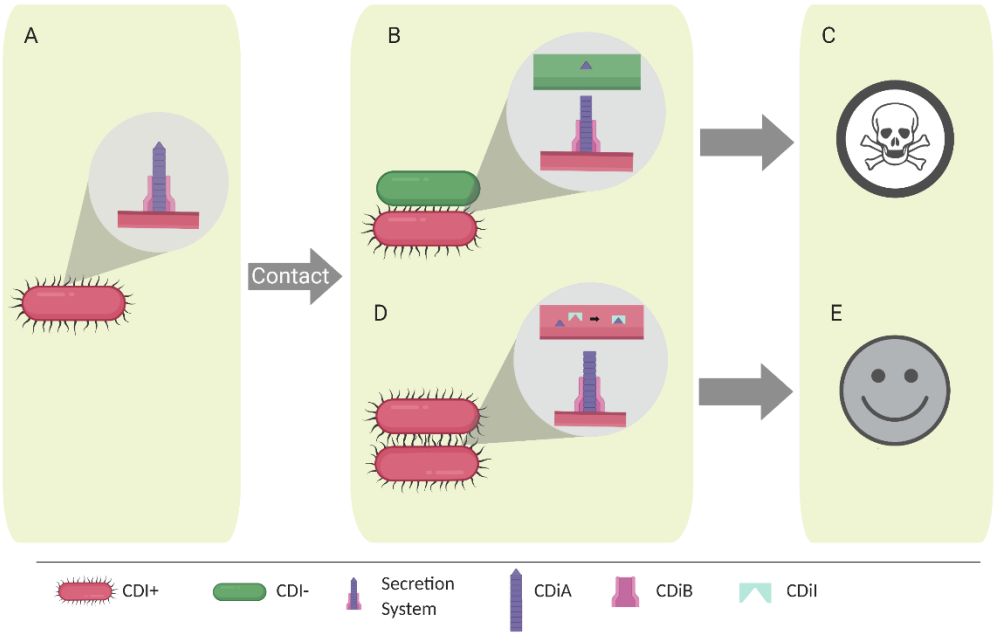

Figure 9 | Mechanism of contact-dependent inhibition. A) a CDI+ effector cell comes into B) contact with a target cell (green) causing C) inhibition of the target cell. D) Alternatively, the CDI+ effector cell comes into contact with another CDI+ effector cell where the CDiI immunity protein neutralizes the CDiA C-terminal toxic end resulting in E) no change to the CDI+ target cell (red).

The CDI mechanisms have an important ecological impact, and offers insight into how pathogenic bacteria overtake a niche and invade a community. By specifically inhibiting other *E. coli* that lack that CDI machinery, CDI positive effector cells have a unique effect on a community. Not only is the context specific to spatial communities (contact is required for inhibition), but the target is genetically very similar to the attacker. Even though the molecular machinery of CDI has been studied in detail, the impact on population dynamics has not been well-described. The CDI interaction is found in uropathogenic *E. coli* and known to be found on a

pathogenicity island and presumably offers them some competitive benefit[65]. By determining how this machinery impacts invasion of pathogenic bacteria, we may come to better understand how to prevent invasion of pathogenic species into healthy communities.

We aim to characterize the impact of this inhibitory mechanism on community assembly. Doing this will allow us to evaluate if CDI's ability to inhibit the target is dependent on population densities and the ratio of CDI to a target population.

CDI as a Proof-of-Principle Example for Uncovering Microbial Interactions

Microbial interactions are complex and difficult to predict. While we can often identify whether one species facilitates or inhibits the growth of another species, identifying the molecular mechanism of such interactions remains challenging. Bacteria within communities each have complex systems of consumption and production, which can mediate interactions with other members. To better understand the coexistence of these communities, the interactions must be teased apart. Determination of microbial interaction mechanisms is an arduous process, making the production of a more systematic and predictive process for understanding the impact of different interactions on community composition desirable. This could be used to greatly enhance our understanding of what mechanisms lead to microbial community coexistence.

The mechanism of interactions is essential to understanding the microbial community itself. Currently, knowing the genetic makeup of two species it is not enough to know how they will interact[31 38]. In some cases, knowledge of the genetic makeup of the constituent species in a community can give insight into the potential for certain molecules to be consumed or produced. – such as with the production of an essential amino acid by members of a community that contains an auxotroph[14,71,72]. However, predicting the interplay of various community interactions offers a difficult challenge as a single microbe can exhibit various complex interactions with each member of the community[6238]. If we can better predict the composition and scope of interactions, we can start engineering communities for waste management[73], bioremediation[74], and disease management[75] as well as a host of other areas that could benefit from engineering microbial communities. By understanding how different types of interactions (facilitation/inhibition) and the type (contact dependent/independent) we are closer to teasing apart the dynamics of a microbial community that could lead to functional coexistence in engineered systems.

A tool to predict interactions to better categorize interactions among 2-species microbial communities is the first step in understanding the mechanisms of coexistence in more complex systems. Previous work as used Tn-Seq on co-infecting microbes to determine how the genetics requirements of *E. coli* changes in monoculture and coculture conditions[76]. This demonstrates how the *E. coli* genome is tractable for comparison of monoculture and coculture conditions. There has also

been community level work done with predicting the changing requirements of *E. coli* in an increasing complex community[77]. This work demonstrated how *E. coli* can be used as a readout to track changes in a community, even if the other members are not well characterized. I plan to devise a method to uncover microbial interaction mechanisms. We aim to determine how cell-level interactions among bacteria lead to community-level functions. We propose to use transposon insertion sequencing (Tn-Seq) to identify genes that are strongly impacting coexistence and population fitness. Tn-Seq works by introducing a random insertion within a genome, that will disrupt gene function at the loci of insertion. This allows us to evaluate how knockouts of individual components change impact the interactions experienced between two partners, and thus their ability to coexist. I propose to use transposon insertion sequencing (Tn-Seq) to elucidate which genetic components are important in interaction between two species.

Methods

Bacteria strains, growth, and media

Experiments were performed using *E. coli* K12, *E. coli* EC93, or *E. coli* BW2076715 (Table 3). The growth conditions for bacterial strains were streaked out on Luria Broth (LB) agar plates with the appropriate antibiotics (Table 3) and stored overnight at 37°C. Then a single colony was inoculated into either LB liquid media or M9[78] media and put in a shaking incubator at 37°C. M9[78] is a minimal, define media, while LB is a rich, undefined media. All fluorescence experiments using a microplate reader were performed in M9 media unless otherwise noted because it is optically clearer. For antibiotic conditions, see Table 3.

Fluorescence labeling of *E. coli* strains

Zyppy Plasmid Miniprep kit were used as described in their protocols (Zymo Research D4036) to isolate plasmids pDiGc-Red and pAF1Fluorescence: YFP (Table 4). Then electroporation was used to introduce the plasmids into the desired *E. coli* strains (EC93 and K12 respectively). The electroporation was performed with a BTX ECM 399 - Harvard Apparatus with a voltage 1.25kV. The confirmation of plasmid uptake was done via plating on antibiotic plates (See Table 3 for antibiotic resistance associated with plasmids) and fluorescence microscopy.

Microscopy

All microscopy was performed with a Leica DMi8 inverted microscope. Confirmation of fluorescence was performed both by examining colonies on agar plates as well as liquid cultures from LB & M9 being plated onto microscope slides.

Microplate reader

An Mx or H4 microplate reader from BioTek with Gen5 software was used for all microplate reader analysis. The conditions for the microplate reader were as follows: outer wells were filled with diWater to limit evaporation from edge effects on experimental wells. Then the plate loaded with an OD600 of 0.01. The growth of the cells via OD600 was monitored at 5 min intervals for 24hrs as well as the fluorescence at a gain of 100 for each respective excitation and emission spectra (Table 4).

Analysis of Communities

For determining each well's growth rate and carrying capacity, the data from the microplate reader is exported from Gen5 software and imported to MATLAB ® for analysis. For each well, we used wells that are uninoculated media to estimate the background OD and fluorescence corresponding to that well. After subtracting the background, we picked data points for each growth curve that were between OD values of 0.002 and 0.02 to avoid noise at low ODs and saturation at high ODs. A linear function was then fit into the log of OD values using the 'polyfit' function in MATLAB ®. The slope of this line was reported as the growth rate for that well. Maximum OD in each well was reported as a proxy for carrying capacity.

Transposon library construction

Transposon libraries were constructed using plasmid protocol[79]. When colonies had grown overnight, individual colonies were isolated and placed into individual wells on a 384-well plate in LB without antibiotics in order to isolate different insertions. These plates then had 17% glycerol added to them to freeze in the -80°C freezer. Through this process 3.5, 384-well plates were made of individual isolates, with none of the outer wells being used for concerns of edge effects (such as an increased evaporation rate) in the microplate readers.

Preparation of Mutant Library Coculture

A liquid handling robot was used to isolated 5uL of each well of the 384 well microreader plates from the Tn-Seq library each containing a different insertion. This was then pipetted into a new 384-well plate via the liquid handling robot which would be run in an Mx or H4 microplate reader to monitor OD600 of the community and fluorescence of the individual strains with previously described protocol. Then the comparison of the fluorescence of the mutant library target (K12) and CDI+ effector strain (EC93) was compared to determine impact of the inhibition for each isolated from the Tn-Seq library.

Results and Discussion

The molecular machinery of CDI has been previously studied in detail, however the impact on population dynamics has not been well-described. The impact of CDI is unclear in community assembly. We aim to characterize the effect of this inhibition on community assembly. CDI dynamics will also elucidate the role of negative interactions in community coexistence and stability.

We aim to describe how the relative ratio and density of CDI cells compared to target cells impacts the inhibition of a target cell. For example, it is unclear if a high density of CDI cells will continue to inhibit the target or if they will instead self-contact repeatedly, using energy to produce the immunity protein and replenish the toxin. This would offer the target some relief from the inhibition.

For this approach, we use two-species communities comprised of an *E. coli* strain that exerts CDI and a target *E. coli* K12 strain. The two species are labeled with different fluorescent markers. This allows us to quantitatively measure how the community's populations change over time. The fluorescence intensity at varying ratios and density of the populations are measured. Then using this data, we quantify the impact of the CDI+ effector cells on the target cells.

I hypothesized that CDI+ effector cell's ability to inhibit the target is dependent on population densities and the ratio of CDI to a target population. I anticipate that has the ratio of CDI increases that inhibition should increase, however, it is unclear if a high density of CDI cells will continue to inhibit the target

or if they will instead self-contact repeatedly, using energy to produce the immunity protein and replenish the toxin. This would offer the target some relief from the inhibition.

Characterizing the target population and the CDI+ effector population

To distinguish the target and CDI+ effector cell in a two-species community, we electroporated each strain with a different plasmid containing distinct fluorescence protein genes (Table 3). The target expresses YFP while the CDI+ expresses dsRed. This use of fluorescence monitoring will allow for the individual populations to be distinguished from one another in a 2-species community. First, we characterized each strain's growth rate and relative fluorescence units (RFU) in monoculture conditions (Fig. 10), and then compared the fluorescence overlap between the two fluorophores (Fig. 11) to ensure in a coculture there would not be overlap of the excitation and emission spectra of the YFP and dsRed fluorophores. We determined that these population can be distinguished from one another because of the negligible overlap between expression of dsRed and YFP (Fig. 11).

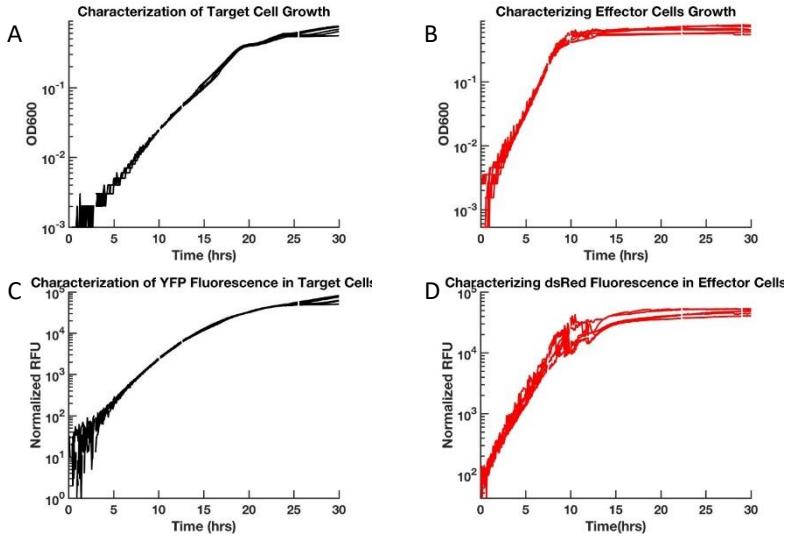

Figure 10 | Characterization of target and effector monocultures. The growth of the monocultures of A) target cells (black) and B) effector cells (red) was monitored until stationary phase was reached. The normalized relative fluorescence units (RFU) of the monocultures of C) YFP expressing target cells (black) and the D) dsRed expressing effector cells (red) was monitored until stationary phase was reached.

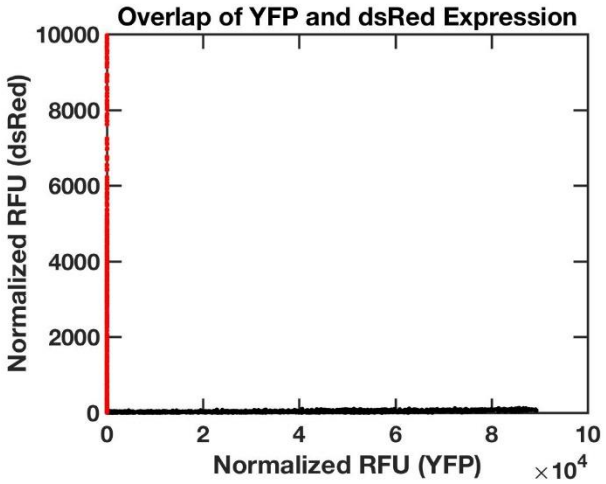

Figure 11 | Comparison of fluorescence overlap of dsRed and YFP fluorophores. Level of emission of target YFP fluorescence in the dsRed wavelength and level of emission of effector dsRed fluorescence in the YFP wavelength. Normalized relative fluorescence units (RFU) of the monocultures of YFP (black) expressing target cells and dsRed (red) expressing effector cells was monitored.

In order to determine if the fluorescence can be used to predict OD600 in a culture we compared the respective RFU to the OD600 in monocultures, then

determined the variation from linear correlation (Fig. 12). The RFU of both YFP and dsRed can be used to infer OD600 of their respective populations therefore the corresponding OD600 of each of the two populations can be determined while in two-species communities.

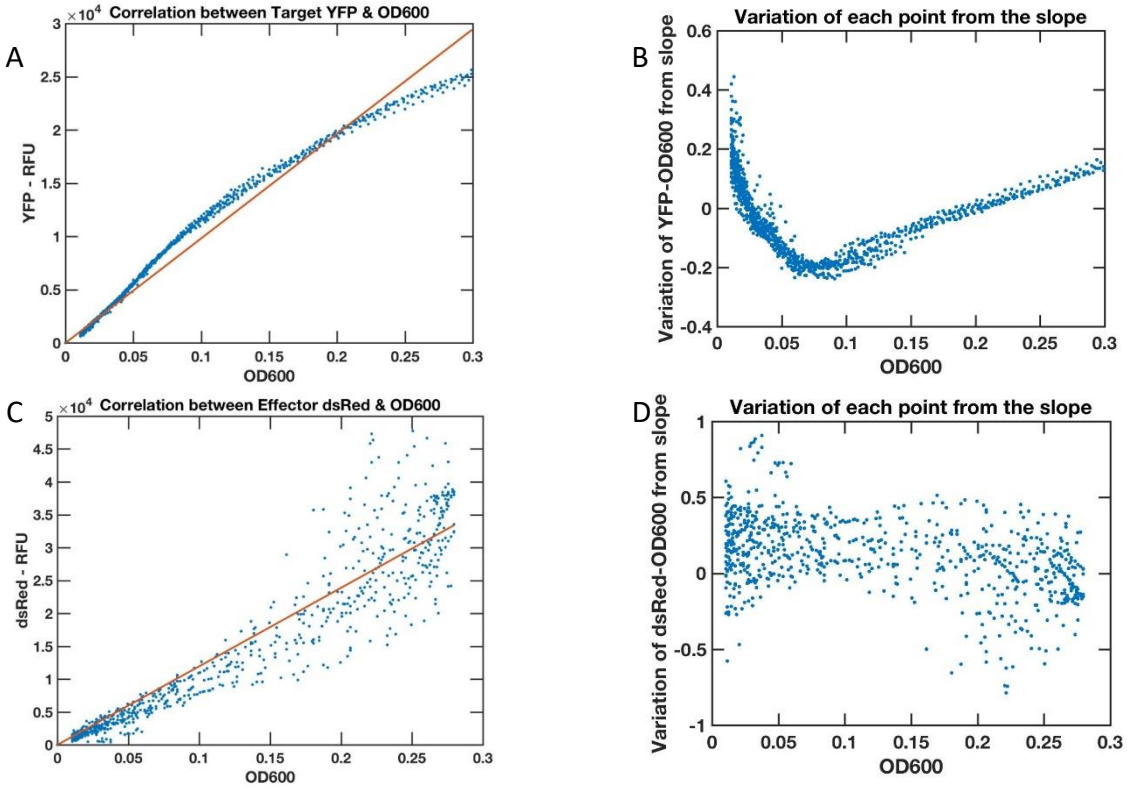

Figure 12 | Predictive correlation of RFU and OD600. A/C) Comparison of OD600 to RFU for YFP and dsRed (respectively) B/D) Variation from linear correlation in A and C (respectively). Linear correlation of RFU to OD600 for YFP and dsRed

Target cells inhibition by CDI is density dependent in two-species cocultures

When performing two-species cocultures, we find the target proliferates when CDI+ effector population levels are low (Fig. 13). When the population of CDI+ effector cells reaches a certain threshold the target population is inhibited while the CDI+ effector population levels stay relatively the same as they are in monoculture.

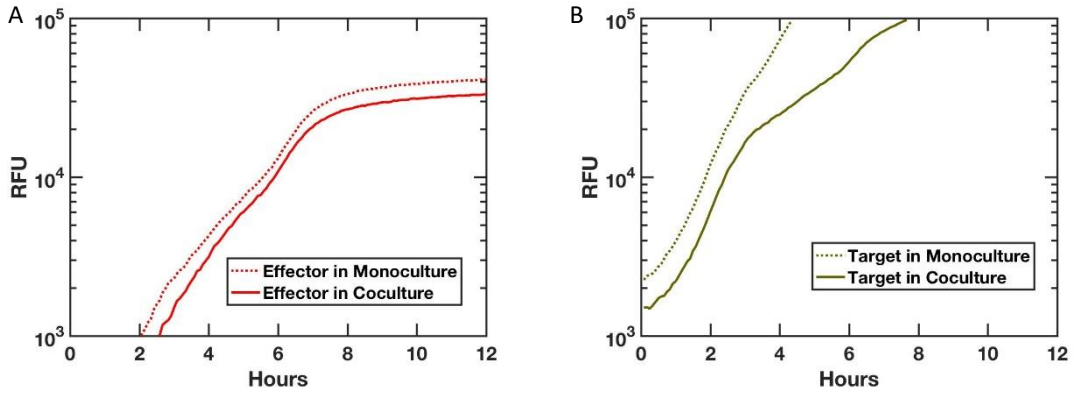

Figure 13 | Experimental data of monoculture (dashed lines) and two-species communities (solid lines). A comparison of relative fluorescence units (RFU) for both monocultures and two-species communities shows that CDI+ effector cells (red) inhibit the target cells (yellow) at a 1:1 ratio of Target to CDI+ effector cells.

The inhibition only occurred at a high density of CDI+ effector cells. When the CDI+ effector cells were at lower density, there was little to no inhibition occurring (Fig. 14). This indicates that the CDI strain is effective at inhibition in the two-species communities and that the effect is density dependent.

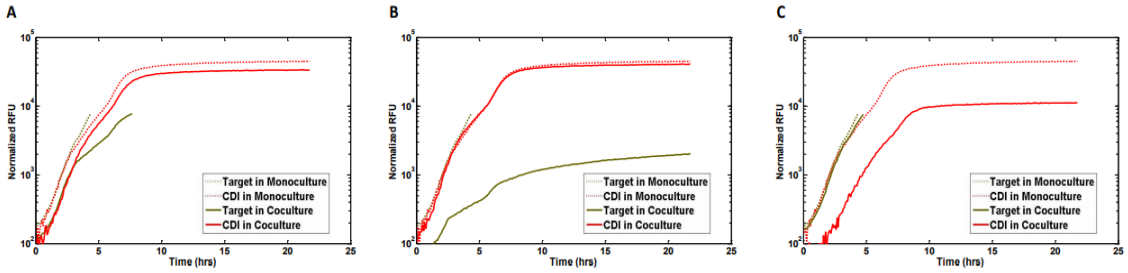

Figure 14 | Determining density dependence. A comparison of relative fluorescence units (RFU) for both monocultures and two-species communities shows that target cells (YFP) are inhibited by the CDI cells (dsRed). (A) A 1:1 ratio of Target to CDI cells. (B) 1:10 and (C) 10:1

Using Tn-Seq to understand microbial interactions

In Tn-Seq, random insertions are added across the genome, allowing us to predict how such mutations change the interactions between two partners. Examining the fitness of different mutants of a Tn-Seq library in cocultures with their partner species, these mutations will stand out by comparing Tn-Seq results of cocultures with those of monocultures without interactions. To evaluate this methodology, I construct *E. coli* communities that have an interaction that is inhibitory (through contact-dependent inhibition).

Our approach offers several advantages: (1) the use of synthetic communities with known interspecies interactions in a controlled environment minimizes confounding factors in assessing the Tn-Seq methodology; (2) candidate genes identified using Tn-Seq can be readily assessed based on the known molecular mechanisms of contact-dependent inhibition interactions; and (3) since *E. coli* can be easily manipulated, we can directly confirm Tn-Seq findings by constructing the identified mutations.

Assessment of the Mutant library

In the previous section, it was shown that the CDI+ effector cells inhibit the target cells in a density dependent manner (Fig. 14). This known simple inhibition mechanism will be used to assess the effectiveness of using Tn-Seq to understand cellular mechanisms of interaction. The predicted results for the mutant is the disruption of transmembrane proteins is expected to impart resistance for target (Fig. 9)[70,80,81].

In order to determine if the creation of the mutant library was successful, we performed a comparison of the ancestor and clones from the library. The constructed library shows increased phenotypic variation in growth rate and carrying capacity compared to the ancestral strain, indicating successful insertions in the mutant library (Fig. 15). When we performed coculture experiments by combining the target and the effector in the same well and monitoring the fluorescence levels, we found that the coculture indicates the target is resistant to the CDI+ effector (Fig. 16), which can be determined by the increase in RFU by some insertions – indicating that they are no longer inhibited by the CDI+ effector cells. These isolates would be sequenced to confirm they have insertions in the transmembrane proteins used by CDI+ effector cell to inhibit the target.

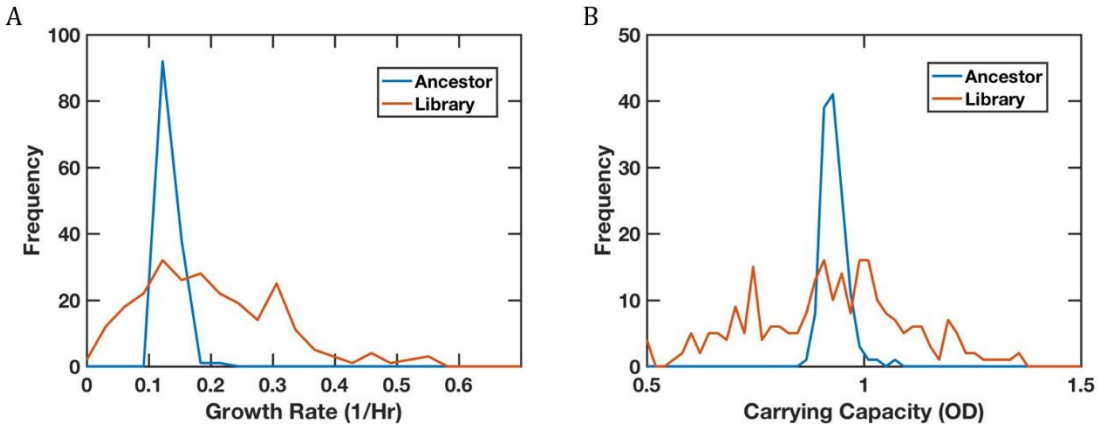

Figure 15 | Comparison of ancestor and library. Monocultures of the ancestor and the library's A) growth rate and B) carrying capacity were compared.

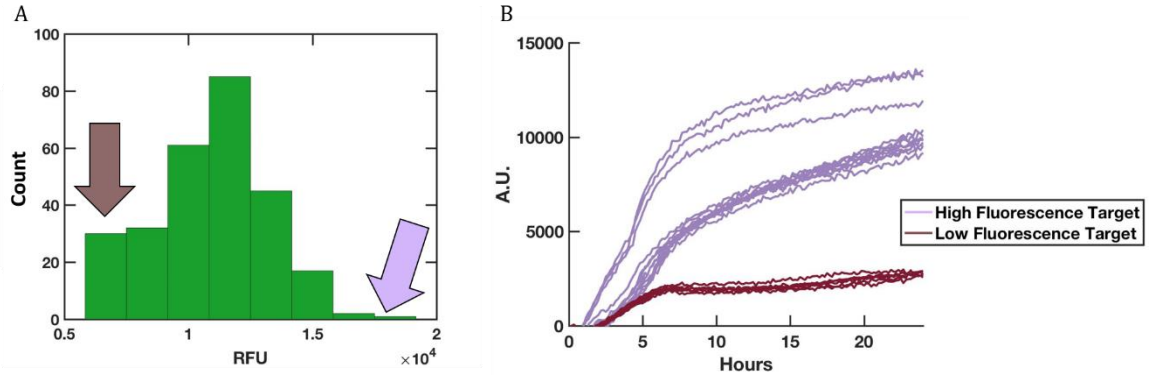

Figure 16 | Coculture community to explore targets resistant to CDI+ effector. A) Indicates the distribution of RFU strength for a 384-well plate with a coculture of mutant target library and CDI+ effector cells. Each well contained an individually selected colony from the Tn-Seq mutant library. B) The RFU of high (purple) and low (brown) fluorescence levels of the mutant target library while being cocultured with the CDI+ effector cells.

Current Results and Future Steps

During the assessment of the mutant library there were inconsistencies discovered (Fig. 17). A coculture of CDI+ effector and target cells were monitored for the fluorescence of the target to determine mutants of interest. Mutants with an increased level of fluorescence at the population level are mutants of interest, since this indicates they are no longer inhibited by the CDI+ effector cells. These identified mutants would then be sequenced to determine if the insertions were in known areas of the genomes associated with CDI inhibition. In figure 17 the assessments (A/B) are both from the same Tn-Seq library, but due to differences in starting concentration of target cells, we are unable to determine accurate mutants of interest. This inconsistency is an issue that needs to be addressed before moving forward with using Tn-Seq to understand mechanisms of interaction between microbes. In order to address the inconsistent starting OD600, the effect of density and how to control for it when screening for altered interactions needs to be considered (Fig. 18). Using GFP fluorescence to monitor the library in coculture, it was observed that the starting ratio of effector to target was causing an increase in fluorescence for some mutants, leading to their false identification as mutants of interest. As a result, it seemed like a high percentage of mutants were now resistant to CDI. One solution would be to not use 384-well plates since the cell density in individual wells will vary. The use of a random-barcode library would alleviate this issue by making trackable insertions[82]. Another future direction is to examine contact independent interactions to ensure both types can be predicted with this

method. Amino acid exchange is one example of such an interaction that could be used as an initial proof-of-concept for the prediction of these interactions[37].

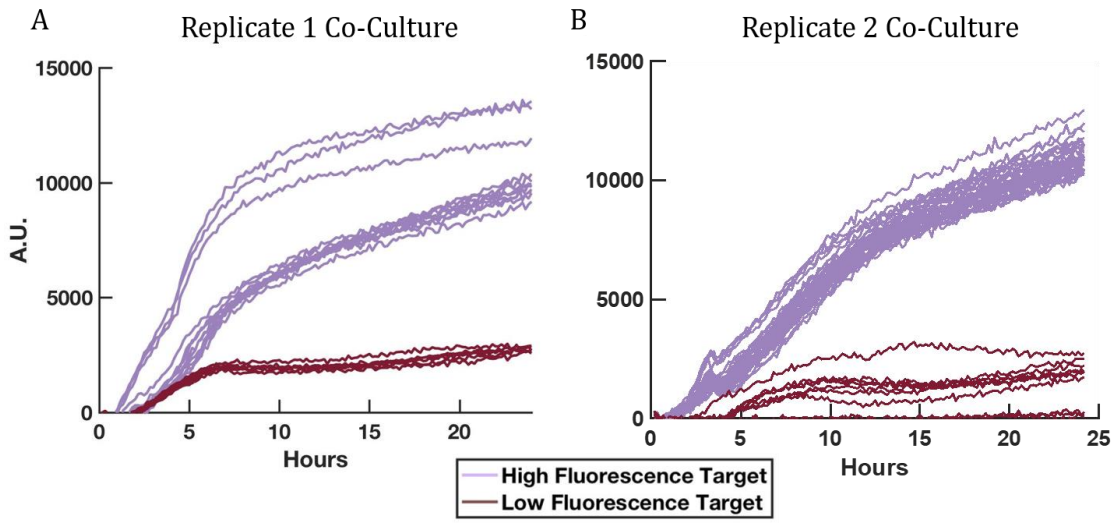

Figure 17 | Verifying targets of interest from Tn-Seq mutant library. A/B) The RFU of high (purple) and low (brown) fluorescence levels of the mutant target library while being cocultured with the CDI+ effector cells. The same targets of interest were compared in A and B.

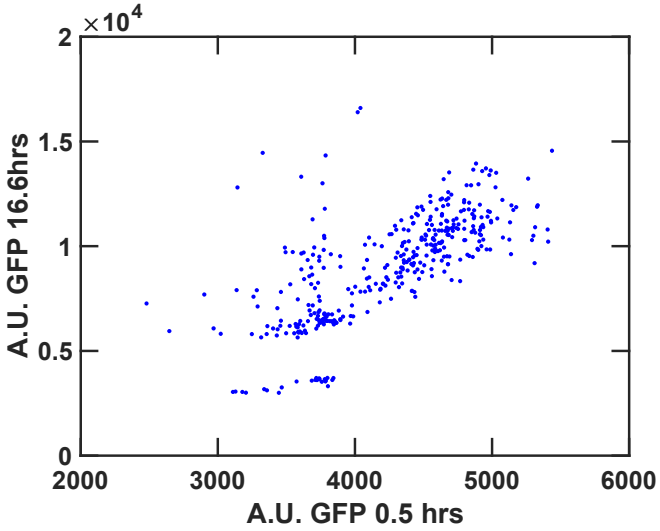

Figure 18 | Comparing initial and final RFU for individual wells Tn-Seq mutant library. The GFP fluorescence was monitored for the same wells in the beginning of growth (0.5hr) and at the end of growth (16.6hr).

Tables

Table 1 | Default parameters for computational model.

Parameter	Variable Name	Default
average consumption links	Qc	0.5
average production links	Qp	0.5
average consumption rate (fmole per cell)	At	0.15
average production rate (fmole per cell per hr)	Bt	0.1
Max interaction strength (1/hr)	ri0	0.2
Samples being screened	Ns	500
Mediator Diffusion (cm^2/hour)	Dmed	5e-6*3600
Cell Dispersal(cm^2/hour)	Dcell	5e-8*3600
Number of Species	Nc	10
Mediators	Nm	5
fraction of positive interactions	fpi	0.1
Community Height (cm)	Z	0.5

Table 2 | Functions used in simulations

Function Name	Purpose
NetworkConfig_Binomial	Binomial network configuration
DistInteractionStrengthMT_PA	Interaction matrix based on strength probability distribution
WellmixedInteraction_DpMM_ExMTC	Well-mixed model for growth of interacting species
Spatial1DInteraction_DpMM_ExMTC_SKD	1D spatial model for growth of interacting species

Table 3 | List of strains used in this study.

Species	Strain	Plasmids	Abx	CDI?	Type
E. coli	EC93 CH6449	None	None	Yes	Effector
E. coli	K-12 (MG1655)	None	None	No	Target
E. coli	EC93 CH6449	pDiGc-Red (derived from pDiGc)	Carbenicillin (100 ug/ml)	Yes	Effector
E. coli	K-12 (MG1655)	pAF1Fluorescence: YFP	Carbenicillin (100 ug/ml)	No	Target
E. coli	BW2076715	pJA1 transposon plasmid	Ampicillin (100ug/ml) + Kanamycin (50 ug/ml)	No	N/A
E. coli	K-12 (MG1655)	None	Chloramphenicol (25 ug/ml)	No	Target

Table 4 | List of fluorescent plasmids used in this study.

Plasmid Name	Fluorophore	Excitation/Emission	Antibiotic Resistance
pDiGc-Red (derived from pDiGc)	dsRed	Excitation = 560nm Emission = 587nm	Carb
pAF1Fluorescence: YFP	YFP	(1)Excitation = 514nm; (1)Emission = 527nm; (2)Excitation = 470nm; (2)Emission = 515nm	Ampicillin (100 ug/ml)
pJA1 transposon plasmid	N/A	N/A	ampicillin resistance marker, kanamycin resistance marker is contained within the transposon

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