Title

Observing horizontal gene transfer of the iron uptake/siderophore transport island in the cheese microbiome

Permalink

https://escholarship.org/uc/item/04g2w47c

Author

Belk, Gillian

Publication Date

2018

UNIVERSITY OF CALIFORNIA SAN DIEGO

Observing horizontal gene transfer of the iron uptake/siderophore transport island in the cheese microbiome

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Gillian Torrey Belk

Committee in charge:

Professor Rachel Dutton, Chair
Professor Kit Pogliano, Co-Chair
Professor Matt Daughtery

2018

The Thesis of Gillian Torrey Belk is approved, and it is accepted in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2018

EPIGRAPH

"Nothing in Biology makes sense except in the light of Evolution"

Theodosios Dobzhansky

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

RUSTI	iRon Uptake Siderophore/Transport Island
ProteoRUSTI	iRon Uptake Siderophore/Transport Island in Proteobacteria
ActinoRUSTI	iRon Uptake Siderophore/Transport Island in Actinobacteria
ICE	Integrative and conjugative element
BLAST	Basic Local Alignment Search Tool
ORF	Open Reading Frame

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Throughout my masters I had immense help from so many wonderful and dedicated people. First and foremost, I want to thank Dr. Rachel Dutton. It has been a pleasure working in yout lab for the past two and a half years, and I am thankful that you agreed to take my on as a Master's student. Through you I have begun to see cheese, and the possibility of microbiome research in a completely new light. Thank you, Rachel.

I would especially like to thank Dr. Gary Heussler for all of his help, guidance, and patience answering all of the questions I have about the intricate details of bacterial genetics. It has been a pleasure working with you trying to understand the intricacies of *Vibrio casei* together. You have been an intricate part of this thesis and thanks again for all of your support.

To the rest of the Dutton Lab, thank you for supporting me and helping me throughout this whole process. A special shout-out to Christina Saak and Manon Morin. Thank you both for letting me bounce ideas off of you and for pushing me to see different angles and approaches to my work. A very special thanks to Steven Villareal, Vanessa Lundsten, Carla Espinosa, and Vanessa Belk for assisting me both with my lab work, and with the prep work for my experiments. To the Dutton Lab, thank you and you all will be deeply missed.

Thank you to my committee members, Dr. Kit Pogliano and Dr. Matt Daughtery. I deeply appreciate you being a part of this process.

To the professors that I have had over the years, thank you for keeping me inspired to do biology. A very special shout out to Goran Bozinovic, Joe Pogliano, and Elio Schaechter for going above and beyond and pushing me to think about science, microbiology, and the future of biotech in an amazing, different, and hopeful light.

Thank you to my family and friends who have kept me sane over the course of my masters. To my sisters, my parents, friends and all of the rest of my relatives, thank you for being by my side through everything and patiently listening to me as I tell you about how amazing the microbes of cheese are for the billionth time. Also, thank you for coming over to the lab! I loved showing you around! To my loving boyfriend, Faris Hamdi, thank you for everything.

To my fellow master's students, Annee, Shams, John, JJ, Ichi, and all the rest, I am so happy we were able to find each other and move through this process together. You all have been so incredibly helpful and motivating to me, I cannot thank you enough. Ya'll are going to accomplish great things, and I can't wait to see where life will take you.

Thank you Dr. O, for everything.

Lastly, thank you to Gimlet Media, Crooked Media, Radiotopia, and Freakonmics for keeping me company as I do my bench work. Thank you to French Hip Hop and Mashrou Leila for keeping me focused as I write my thesis.

VITA

2017	Bachelor of Science in General Biology, University of California San Diego Minor in History, University of California San Diego
2017-2018	Teaching Assistant, Department of Biology University of California San Diego
2018	Master of Science in Biology, Microbiology Emphasis, University of California San Diego

PRESENTATIONS

Poster Presentation at the Biology Research Showcase 2018 for the Division of Biological Sciences at UC San Diego

ABSTRACT OF THE THESIS

Observing horizonal gene transfer of the iron uptake/siderophore transport island in the cheese microbiome

by

Gillian Torrey Belk

Master of Science in Biology
University of California San Diego, 2018
Professor Rachel Dutton, Chair
Professor Kit Pogliano, Co-Chair

Horizontal Gene Transfer (HGT) is the transfer of genes from one organism to another. It is a vital tool that allows various microbes to adapt rapidly to new environments by exchanging new genetic functions. Previously, our lab identified a large mobile genetic region putatively involved in iron acquisition (termed RUSTI for iRon Update Siderophore Transport Island) which we believe is horizontally transferred in the cheese community. The RUSTI region is on a much larger mobile genetic region called an integrative and conjugative element (ICE). We termed the

RUSTI-containing ICE for Proteobacteria the ProteoRUSTI ICE. To test if RUSTI is indeed horizontally transferred we co-cultured a *Vibrio casei* carrying the ProteoRUSTI ICE with three cheese-rind isolated Proteobacteria: *Psychrobacter, Vibrio sp.,* and *Pseudoalteromonas*. These bacteria were found to not carry the ProteoRUSTI ICE. We unfortunately did not observe an HGT event between *Vibrio casei* and the ProteoRUSTI ICE negative bacteria. However, this masters laid the ground work on how to design and organize HGT experiments in the cheese rind. These findings are important for the development of our model because they highlight the characteristics of RUSTI in various ways. Creating a better understanding of how mobile genetic elements such as RUSTI behave in species-species interactions provides us insight into how microbes and their genes may interact with one another in more complex microbial communities.

INTRODUCTION

The importance of microbiomes

A microbiome is a collection of microbes living together in a community, and these multispecies microbiomes encompass nearly every environment on Earth [1]. Microbiomes impact our gut directly by enhancing our ability to absorb certain nutrients from our food [2, 3]. Others, such as microbiomes in plant's rhizospheres help plants retain nutrients such as iron and phosphorus, and microbiomes of corals help them uptake and recycle nitrogen [4, 5]. Microbiomes also impact a host of other ecosystems which we are just now beginning to understand [4-6]. Our understanding of the impact and the extent of interactions within microbial communities is largely nonexistent. This is mostly because these communities are highly complex consisting of hundreds to thousands of unique microbial species [1]. Of these microbial species, nearly 80% are unculturable in a traditional lab setting. This may be because some microbes are anaerobic, rely on other bacteria they co-evolved with, cannot consume the carbon sources usually available in traditional lab media, or simply cannot live outside of exact natural environment (like high pressure of the deep sea) in the lab [7, 8]. Being unable to easily culture the vast numbers of microbes within microbiomes constrains our understanding of intricate interspecies interactions within these wide-ranging communities [7].

Problems with current model microbiomes

Models for microbiomes are needed to study the extent to which microbial communities impact their environment and vice versa. Current approaches focus largely on attempting to replicate gut microbiomes because its direct impact on human health. Often these model gut microbiomes only study a limited number of bacteria from the community. In a study done by D'hoe et al. they created a model gut microbiome using human feces isolated *Faecalibacterium prausnitzii, Vlautia hydrogenotrophica,* and *Roseburia intestinalis* [9]. The model gut bacteria were placed in stationary glass bottles supplemented with medium for colon bacteria (mMCB) to replicate the gut microbiomes. Although this is a useful tool for studying the basic dynamics of gut microbes, because only three species of microbes were used it negates the complexities of a gut microbiome that consists of thousands of species. Additionally, it fails to truly replicate the gut environment because of the lack of villi, or villi like structures for the microbes to attach to. Because of this, it would be difficult to conclude any spatial structure or ecological specializations of the microbes in a multispecies community. Other attempts to recreate model microbiomes have used *C. elegans* colonized with *E. coli* to represent host-gut microbes interactions [10]. Once again, this model is very useful to study basic dynamics of host-gut microbial interactions, but the lack of a multispecies microbial community prevents a complete understanding of all of the interactions that would be occurring. A model microbiome that is representative of all the microbes in the system and replicates the natural environment is needed to study the multispecies microbial nature of microbiomes.

Cheese as a model microbiome

Our lab developed the use of the cheese rind microbiome as a model system [11]. We hypothesize that a cheese rind microbiome comprises of all of the necessary aspects for studying multispecies microbial interactions in a microbiome. Cheese rinds include a limited number of microbes ranging from a few dozen to three. The limited number of microbes in the cheese rind and their ability to be cultured makes studying the cheese rind more manageable than other systems. We can also replicate the cheese environment in the lab, and the basic microbial profiles from the *in vitro* communities and the *in situ* communities are remarkably similar. This flexibility allows us to take apart and recreate the cheese rind community to build a better understanding of how interspecies microbes interact together in a microbiome.

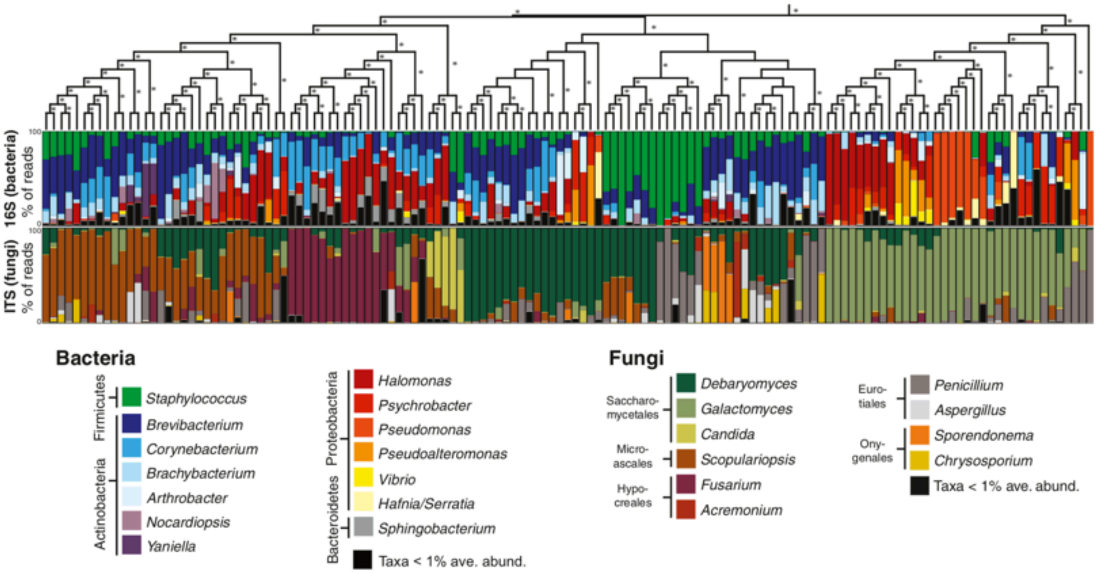

Figure 1: **Main Bacterial and Fungal Genera in Cheese.**
The above figure demonstrates the ratios of the number of bacteria and fungi in a given cheese sample over 137 cheese samples. Only 14 bacterial and 10 fungal genera were identified to live in the cheese rind at over 1% abundance. Adapted from Wolfe et al.

Simplicity of a cheese microbiome

Work done previously by Wolfe et al. found that the microbiomes of cheese rinds are relatively simple compared the microbiomes of other ecosystems. It was found that there are only 14 bacterial and 10 fungal genera found in over 1% abundance throughout cheese rinds, with an average of 6.5 bacterial species and 3.2 fungal species per microbiome [11]. This was determined by sequencing the rinds of 137 different cheese rinds from 10 different countries and comparing the cheese associated bacteria and fungus of each cheese to one another (Figure 1). The simplicity of this system allows us to easily experiment on all of the microbes in a given cheese microbiome.

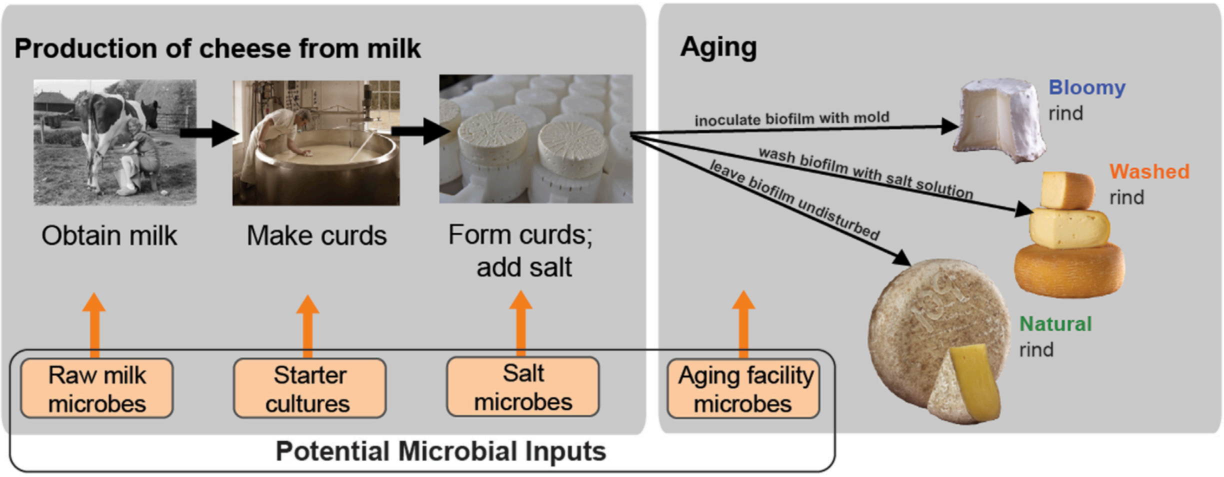

Figure 2: Introduction of Microbes in the Cheese Making Process. The above figure shows the general cheesemaking process from the milk to making the curds, to the final aging process. The figure also demonstrates where the microbes in a cheese rind originate. Nearly 60% of bacterial species and 25% of fungal species on a cheese rind come from the environment during the aging process of cheese. Adapted from Wolfe et at

With a few dozen players, the cheese microbiome still retains a level of complexity that is apt to study intricate abiotic and biotic interactions of microbial communities. In the aging process of cheese, the properties of cheese such as pH, moisture, and salinity change over time and they are influenced by and influence the microbes living in and on the cheese. These abiotic factors in the cheese making process are unique for each cheese type and are more important than the geographical location where the cheese was made. This is because the method for making distinct cheese types does not change with geographical location. So as long as cheese making techniques are kept consistent, the rind will develop in a similar fashion because the procedure for making different cheese introduces distinctive conditions within the rind. As a cheese ages, the changes in abiotic factors affect the adaptation of in this environment. In the beginning of the cheese making process starter microbes, mostly consisting of *Streptococcus* and *Lactobacillus* species, are added to the fresh curds [12]. These bacteria immediately start fermenting the lactose sugars available in the developing cheese. However, starter cultures only make up a fraction of the microbial diversity in cheese (Figure 2). It was found nearly 60% of bacterial species and 25% of fungal species on a given cheese rind do not come from the starter cultures [11]. Instead, these microbes come from the surrounding environment, the cheese making process (such as washing the cheese with a salt brine solution), and other aging cheeses in the facility [12]. The introduction of environmental bacteria to the cheese community creates a situation where the new microbes must develop their own unique niche on cheese working with or against the microbes already present in the community. [11]

The addition of non-starter culture bacteria into the cheese rind allows for interesting studies on microbial succession and rapid adaption to new environments. Additionally, because cheese is a completely man-made microbial system we have the ability to change nearly every variable of the process. With this level of control, we can adjust the abiotic and biotic factors to see how and why the abiotic factors can affect the microbial community and how the microbial community can change the abiotic factors of a cheese rind.

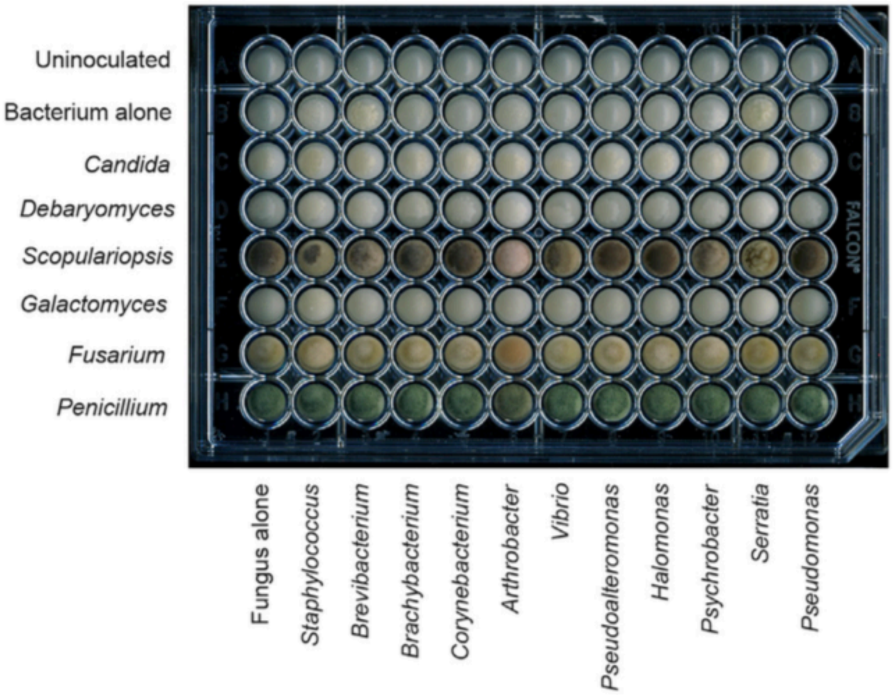

Figure 3: All Microbes of Cheese are Culturable. Above are all some of the most common cheese fungal species and bacterial species grown pairwise on CCA. This demonstrates that representative microbes from all abundant genera in a cheese microbiome are able to be cultured in a traditional lab setting. Adapted from Wolfe et al.

Culturability of the cheese microbiome

As mentioned previously, in most microbiomes the microbiota are unculturable or difficult to culture in a traditional lab environment [7]. In contrast, representative species from all fungal and bacterial genera associated with cheese communities are culturable, can be grown on basic lab media, and do not need any additional or unique culturing techniques (Figure 3). We mimic a cheese environment in the lab by using a cheese-based medium known as Cheese Curd Agar (CCA). Our CCA is made from cheese curds from Jasper Hill Farms, xanthan gum, salt, and agar. Further details on how to make CCA are in the Methods section. CCA allows us to recreate a cheese-based environment inside the lab, however, unlike a slow cheese making process CCA allows us to directly control the nutrients and other abiotic factors such as pH inside the mock cheese environment. Additionally, unlike classic cheese, CCA can be sterilized and poured into petri dishes or 96 wells. This makes it much easier to perform experiments on the cheese microbes in the lab.

Isolating bacterial species from fungal species and vice versa is also simple to do in the mock cheese environments. Addition of anti-fungals or antibiotics to the media select for all of the bacterial or fungal species on the cheese. The ability to easily separate the microbes from one another allows us to replicate the microbiome species by species much more readily. Because the cheese microbes can be grown and separated from each other so easily in a lab, it allows us to perform for studies that can demonstrate how cheese microbes interact with each other and evolve.

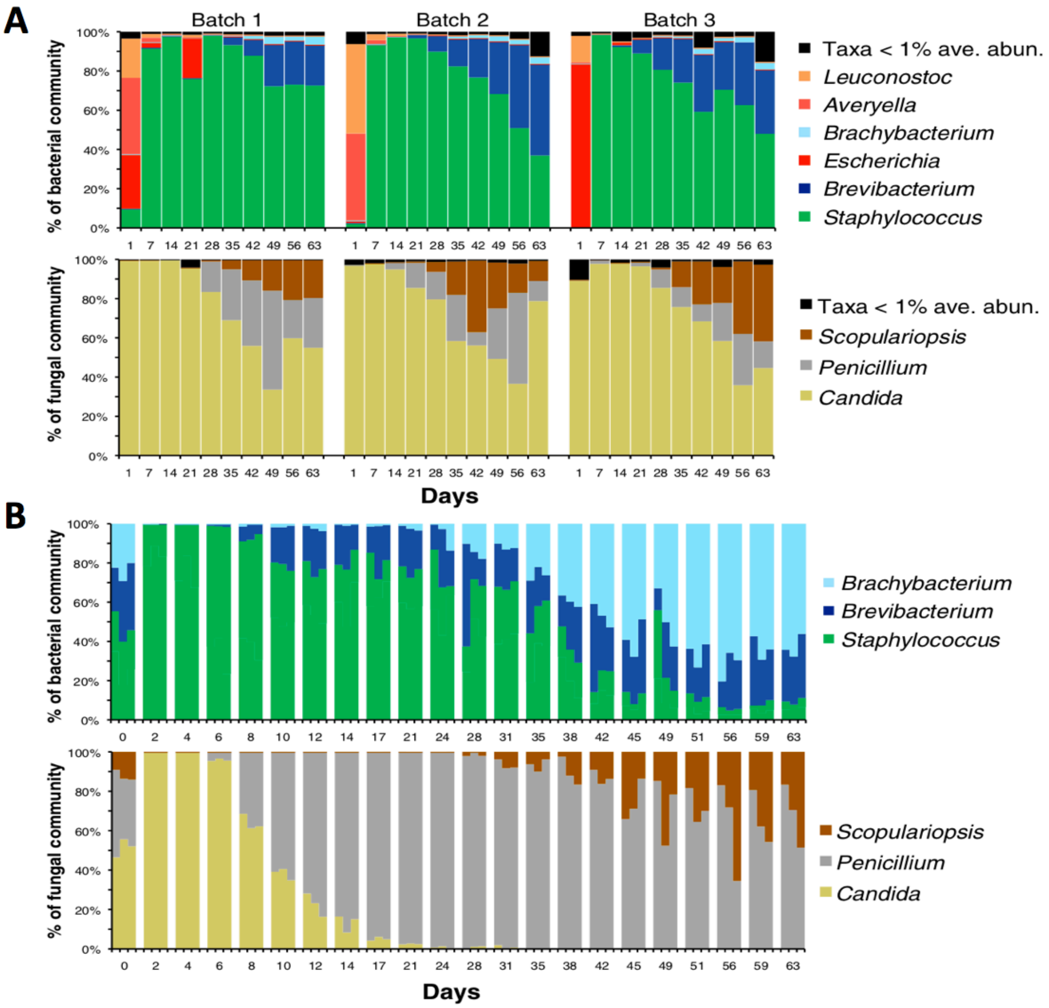

Figure 4: Succession of Microbes on *In Situ* Reflects Succession of Microbes *In Vitro*.
(A) A succession experiment was performed on a natural rind cheese from the Jasper Hill Farms, and samples were collected every day for 63 days during the development of the cheese. Samples from the 63 days were collected and sequenced using amplicon sequencing. After the data was collected from the in vivo experiment, the succession experiment was replicated in vitro on CCA. (B) The bacterial and fungal species from the amplicon sequencing of the natural rind cheese were inoculated on a CCA 96 well plate at uniform amounts of approximately 200 CFUs. The wells were harvested for 63 days, the bacterial and fungal samples were analyzed to determine how the community changes on the mock cheese environment. Adapted from Wolfe et at

Reproducibility of the cheese microbiome

One of the strengths of our system is that our model microbiome on CCA is almost identical to the original cheese microbiome. This phenomenon was determined by comparing the succession of microbes adapting to the cheese environment on a natural rind cheese from Jasper Hill Farms to the succession of microbes on CCA (Figure 4). The succession of bacteria and fungi on the natural rind cheese and CCA developed at similar ratios. The largest difference between the *in situ* and *in vitro* study was that the microbes *in vitro* on CCA appeared to reach the matured state of the cheese faster than that of the natural rind cheese. This may be because the surface to volume ratio is higher in the *in vitro* system, causing faster deacidification.

The cheese microbiome is therefore an ideal model to study species-species interactions of microbes because of the simplicity, culturability, and reproducibility of the system.

Understanding microbial interactions by studying Horizontal Gene Transfer

The cheese environment encourages rapid adaptation

Cheese can be a challenging environment for microbes to live for a variety of reasons. As mentioned previously, the abiotic and biotic factors of the environment of cheese changes over the course of the aging process. For both hard and soft cheeses, the pH of the cheese can vary wildly. In hard cheeses, the pH of the environment is consistently acidic [12]. In mold ripened, or soft cheese, the pH is initially low, but becomes more neutral as the cheese ages [12]. The acidification of the cheese environment is in part due to the starter bacteria such as various *Lactobacillus* species, consuming lactose and creating lactic acid as a by-product [12]. The pH in mold-ripened cheese becomes neutral over time because the microbes living on the rind exhaust the lactate on the surface of the developing cheese within a few days, and this rapid reduction of lactate raises the pH of the cheese [12]. These changes in pH select for microbes that can survive in a variety of pH ranges all over the spectrum. The aging cheese also creates an environment where microbes must constantly adapt to changes in salinity. For example, in hard cheeses, such as Parmigiano Reggiano, the high salinity levels lead to a steady decline in the number of microbes in the community as the cheese ages [12]. The changes in pH and salinity of the environment are due to an ever-changing microbial landscape where different microbes acidify or neutralize the pH and or encourage changes in salinity [12].

The rind of a cheese consists of the densest number of microbes in the cheese environment. The microbial composition of the rind is dependent on the rind type of the cheese: bloomy rind, natural rind, or washed rind (Figure 2). Bloomy rinds from cheeses such as Brie and Camembert consist of a dense rind abundant in fungal species [11, 12]. A natural rind is left to be naturally inoculated by environmental microbes and are representative of some Cheddar cheeses [11, 12]. Lastly, washed rind is washed with a salt brine during the aging process and is a common practice in some French cheese [11, 12]. The type of rind affects the succession of microbial composition, which in turn affects the available nutrients. For example, in mold-ripened soft cheese, yeast such as *Geotrichum candidum* will initially hydrolyze the proteins and fats in the cheese [12]. After the initial breakdown of macronutrients, the *Penicillium* species can begin to grow all across the surface of the cheese [12]. This growth can be seen by the cheese makers as a white filamentous layer on the top of the aging cheese [12]. Both the molds and the yeast secrete a variety of proteases and lipases that further breakdown the fats and proteins in the community [12]. This breakdown of nutrients directly affects the microbes and creates secondary metabolites for other microbes to digest. These interactions are further expanded with the addition of environmental bacteria moving into the cheese rind environment.

Properties of the milk itself can make it a hostile environment to microbial life. Mammalian milk hosts a variety of antimicrobial properties that sequester vital nutrients, so that pathogenic microbes cannot access them and subsequently cause infections [13]. Lactoferrin, one of these antimicrobial compounds found in milk, sequesters iron away from potentially pathogenic microbes [13, 14]. In Da Silvia et al, they found that the addition of lactoferrin to Minas Frescal cheese reduced the amount of *Staphylococcus aureus* below their detection limit while cheese without the lactoferrin resulted in a *S. aureus* contamination [14]. Because lactoferrin and other iron sequestering compounds lower the amount of iron available for the microbes that live on cheese is highly limited. To survive in a cheese environment the microbial community must adapt to an environment limited in micronutrients.

Horizontal gene transfer is prominent in bacteria

Horizontal Gene Transfer (HGT) is the transfer of genes from one microbe to another [15]. This exchange of genetic information allows bacteria to acquire new functions that can help them adapt more rapidly to novel environments [16]. One of the most extensively studied adaptations of bacteria is the rapid exchange of antibiotic resistance genes via HGT in environments with antibiotics [17-23]. Bacteria can also acquire functions via HGT that are beneficial to living in environments with changing temperature, moisture, salinity, pH, nutrient availability, as well as general biotic factors from their microbial neighbors [22].

There are three common ways by which genes are transferred: transformation, transduction, or conjugation. Transformation is the uptake and integration of naked DNA from the environment and is the only form of HGT that is led mostly by the recipient cell [17, 24]. Both Gram-positive and Gram-negative bacteria use what is known as a transformation pilus (Tfp) to transport environmental DNA into the cell [24]. The free floating DNA could come from a number of sources including dead or alive neighboring cells [25]. Often, bacteria undergo the naturally ability to absorb free floating DNA when given the correct environmental cues [24]. *Vibrio cholera*, for instance, is known to become competent only when around chitin molecules [26]. The second form of HGT is transduction, the transfer of bacterial genes via bacteriophages [19, 20]. A complete bacteriophage infection cycle can occur within a bacterium in as little as 40 minutes [27]. During such a rapid infection, mistakes are often made and sometimes a part of the host DNA is packaged with the viral DNA [19, 20]. When the bacteriophage injects itself into other bacteria with a mixture of phage and previous host DNA, sometimes the original host DNA is integrated into the new host's chromosome [19, 20]. If the new host survives the infection, then it would have gained genetic information from the previous host [19, 20]. Lastly, conjugation is a form of genetic transfer that requires physical contact of the donor bacterium and the recipient bacterium [21]. Often, but not always, conjugation involves the transfer of a plasmid from one bacterium to another [21]. Transposons, integrative and conjugative elements (ICE), and plasmids, are all elements that are transferred via conjugation [22, 23].

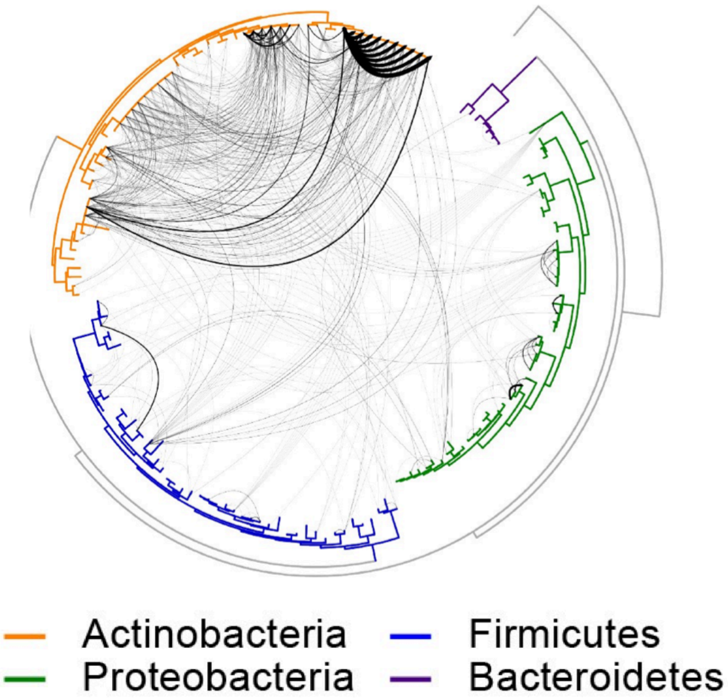

Figure 5: Horizontal Gene Transfer is Prominent in Cheese Isolated Bacteria. The plot represents the prominence of HGT within the cheese community. Each black line in the circos plot represents a putative HGT event. From the plot we can see that most of the HGT appears to happen inter phyla, but some HGT events are intra phyla. Approximately 4700 genes are thought to be horizontally transferred. Adapted from Bonham et at.

Horizontal gene transfer is prominent in the cheese communities

Previous research by Bonham et al. discovered bioinformatic evidence that HGT is common with cheese-associated bacteria [28]. This discovery was determined by looking at the genomes of 165 different cheeses from around the world. Out of these 165 cheese isolated bacteria, nearly four fifths (78.8%) were identified to have at least one putative horizontally transferred gene [28]. In total, almost 4700 genes putatively transferred genes were identified in the cheese-associated bacteria (Figure 5). Nearly 88% of the genes transferred were within a single phylum. However, approximately 12% of the genes did transfer across various phyla. To better identify these genes, they were further clustered into 264 individual groups based on the proximity within 5,000 base pairs of one putative horizontally transferred gene with another.

Iron-related genes are highly transferred in the cheese community

The 264 gene clusters were further analyzed to identify which specific functions of the genes are putatively transferred based on homology with other characterized genes. Most of the gene functions (53%) were not able to be assigned to a specific function. However, out of the remaining 47% of genes that could be assigned specific functions, 12% were involved with the uptake of various nutrients such as amino acid and lipid transport systems. It is understandable that genes associated with the breakdown and uptake of fats and proteins would be present in cheese because the metabolism of cheese after the lactate is digested is mostly associated with the breakdown of fats and casein and uptake of fats and proteins [12]. Eleven percent of the genes were associated with metal ion, iron siderophore and vitamin B12 transport systems. Cheese is a micronutrient limited environment, and evidence of horizontally transferred genes that potentially intake micronutrients reflect the needs of bacteria in the environment. The clusters of iron-related genes are further organized into phyla specific iron gene clusters and were found in 29 individual Actinobacteria species and 9 individual Proteobacteria species. Although these iron related genes came from different phyla there were some similarities between them including iron-related genes such as predicted siderophore uptake systems. Siderophores are small (1<kDa) molecules that are ferric iron chelators which are secreted from the cell into the environment and then reabsorbed [29]. The siderophore uptake genes in the iron gene cluster were also accompanied by varying numbers of ATPase subunits, AraC-like transcriptional regulators, surface binding proteins, and phosphonate genes depending on the phyla the iron gene cluster was associated with. These

clusters were named the iRon Uptake Siderophore/Transport Islands or RUSTI. The RUSTI region changed slightly depending if it was associated with the Actinobacteria (ActinoRUSTI) or Proteobacteria (ProteoRUSTI). The ActinoRUSTI region consists of mostly siderophore transport genes mentioned above, and the ProteoRUSTI region consists of phosphonate metabolism genes and iron/siderophore uptake genes. In both ActinoRUSTI and ProteoRUSTI, the iron regions only encode for iron/siderophore uptake systems and do not encode for siderophore synthesis genes. This leads us to hypothesize that this region is helping the bacteria take advantage of the siderophores other microbes in the cheese community excrete as public goods.

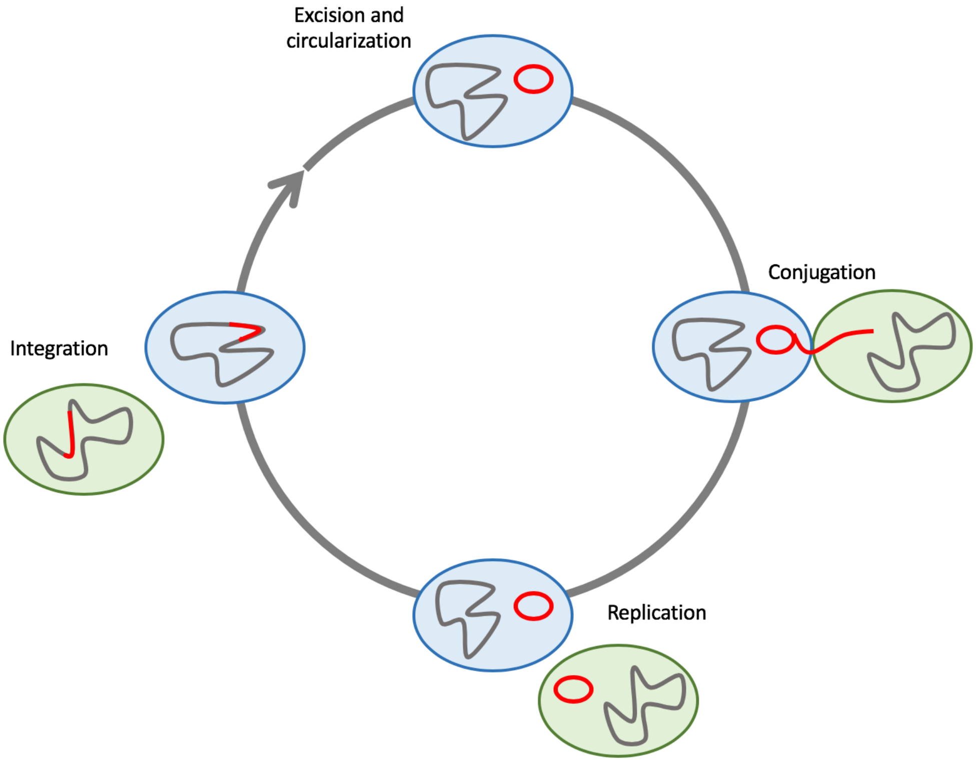

Figure 6: ICE Transfer.
Above is a schematic of an ICEs lifecycle. An ICE integrates itself into the host's chromosome. When induced to transfer, the ICE will excise itself from the chromosome and circulate. It will then transfer itself to another bacteria via conjugation. The replicate of the original ICE will circularize in the new host. Then both ICEs will integrate back into their hosts chromosome.

The ProteoRUSTI is on a putative integrative and conjugative element

In this work, we focus on the RUSTI gene cluster associated with Proteobacteria, specifically in the cheese isolate *Vibrio casei*. We hypothesize that the ProteoRUSTI is located on a putative Integrative and Conjugative Element or (ICE). An ICE is a mobile genetic element that can self-transfer but unlike a plasmid it cannot independently self-replicate, so it propagates by inserting itself into the host chromosome (Figure 6) [30]. The most commonly studied ICEs associated with various *Vibrio* species are what are known as SXT-type ICEs. These ICEs were isolated from *Vibrio cholera* after a cholera epidemic in southeast Asia [30]. The SXT ICE encoded for a variety of antibiotic resistance genes including sulfamethoxazole which gave it its name [31]. Although the putative ICE found in *Vibrio casei* does not contain any antibiotic resistance genes it carries other similar features to SXT ICEs. The putative ProteoRUSTI ICE is integrated at serine tRNA complex, where other SXT-like ICEs have been known to integrate into the *Vibrio* chromosome. The putative ProteoRUSTI ICE also consists of conjugative machinery (T4S) and integrases as well as various regulatory genes, such as *setR* [23]. Additionally, the RUSTI region could be classified as a variable region that is common on SXT ICEs.

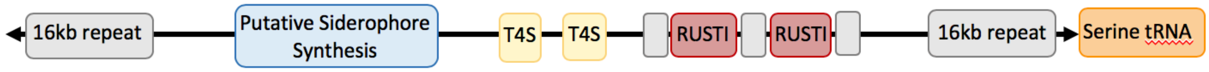

Figure 7: Main Functional Groups on the ProteoRUSTI ICE.
Above is a cartoon representative of the ProteoRUSTI ICE not drawn to scale. The putative integrative and conjugative element contains various unique features including the 16 kilobase repeats that flank the edges of the ICE. The other rectangles in grey represent other repeated regions inside the ICE. The putative ProteoRUSTI ICE consists of various T4 secretion systems and a region that may encode for siderophore synthesis proteins. The RUSTI region was found to be duplicated on the ProteoRUSTI ICE . It is not known if the RUSTI region uptakes the siderophore synthesized elsewhere on the putative ICE. The ProteoRUSTI ICE possibly integrates at the Serine tRNA site. Not shown is a putative toxin/antitoxin system that may explain the ICEs relative stability in the cheese community even with such large repeats flanking either side.

The ProteoRUSTI ICE has a few unique features such as the fact that the ProteoRUSTI ICE is 230 kilobases long, or approximately 100 kilobases longer than the longest known SXT ICE (Figure 7). Unlike the SXT ICE, the ProteoRUSTI ICE does not consist of any of the known core SXT elements such as antibiotic resistance or various regulatory and integration genes [31]. The ProteoRUSTI ICE also contains variety of repeats. There is a 16 kilobase repeat flanking either edge of the putative ICE. The RUSTI regions are also flanked by a large 2.6kb repeat. These features have not been seen on other ICEs before and more research needs to be done to determine the functions of these unique features.

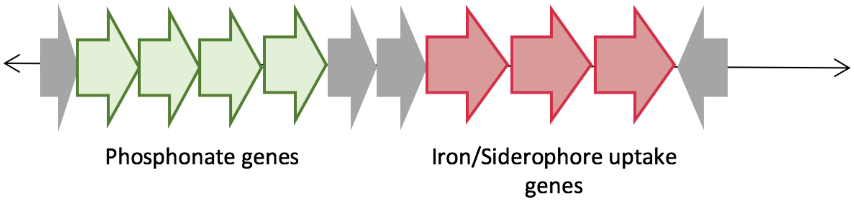

Figure 8: The RUSTI Region.
The RUSTI region of the ProteoRUSTI ICE consists of phosphonate genes and iron/siderophore uptake genes. It is still unknown the exact function of the phosphonate genes, and what siderophore the iron/siderophore uptakes genes uptake.

Properties of the ProteoRUSTI

The ProteoRUSTI element itself consists of mostly phosphonate genes and iron/siderophore uptake genes (Figure 8). Phosphonate is a common form of phosphorus in a marine environment, however, it is not known to exist in cheese [32]. It is unclear if these phosphonate genes have a role in the cheese community or if they are vestigial genes from when *Vibrio casei's* ancestor lived in a marine environment. The ProteoRUSTI region also encodes for a siderophore uptake system. At this moment, we do not know what siderophore this uptake system is absorbing, but we hypothesize the siderophores are excreted by *Vibrio casei's* neighbors, possibly helping the host cheat off of its neighbors.

In this experiment we aim to better characterize the rate of transfer of the ProteoRUSTI ICE. We hypothesize by creating a better understanding of when, how, and to whom this ICE is transferred in the cheese community, it can help us extrapolate how mobile genetic elements move throughout microbial communities.

RESULTS

Construction of strains for measuring horizontal gene transfer of the ProteoRUSTI region

Our experimental goal was to detect the horizontal gene transfer (HGT) of the ProteoRUSTI ICE to better characterize how this mobile genetic element moves through the cheese community. Our experimental strategy was to use two different strains of *Vibrio casei,* one which would be used as a donor of the ICE, and one would be the recipient. This experiment required the construction of genetically distinguishable donor (with an antibiotic-marked RUSTI), and recipient strains (with a separate antibiotic cassette at a neutral site), so that HGT could be detected. Although *Vibrio casei* is easily culturable, genetic tools for working with this cheese isolate have not been characterized. Thus, construction and validation of vectors use in this organism required significant efforts. Here, I describe my work towards establishing plasmids and strains for use in HGT assays.

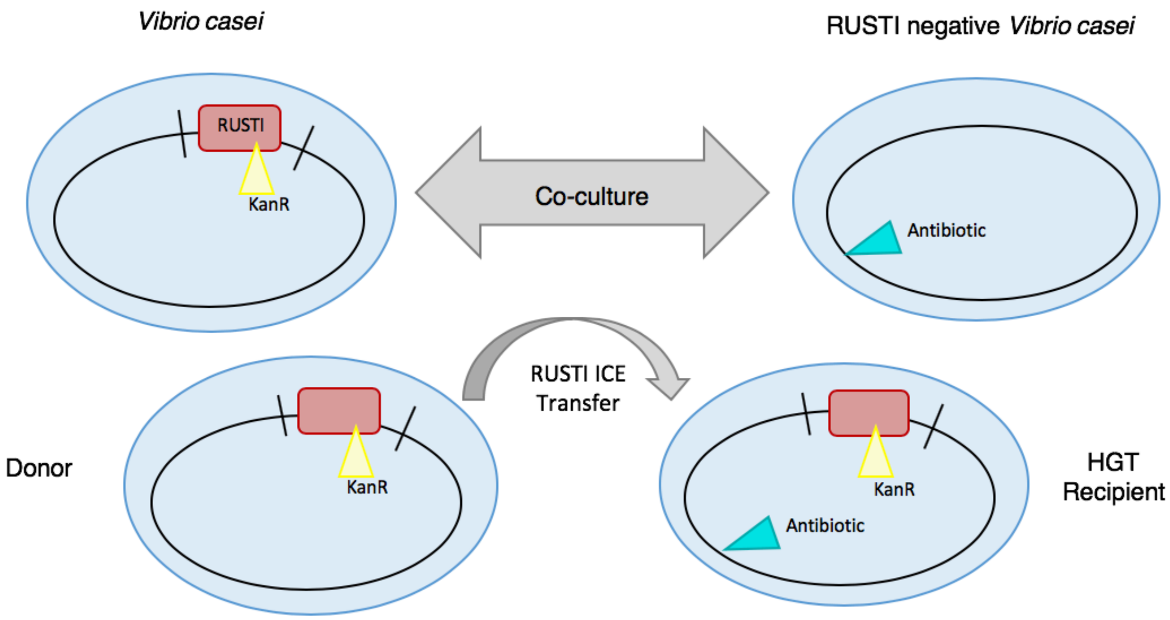

Figure 9: Experimental Approach of the Transfer of the ProteoRUSTI ICE from *Vibrio casei* to another *Vibrio casei* strain.

Two strains of *Vibrio casei* are needed in this experiment. One with an antibiotic resistant marker in the RUSTI region, and another strain that lost the ProteoRUSTI ICE with an antibiotic resistance in the chromosome. The two strains would be co-cultured together. In the case of a horizontal gene transfer event the ProteoRUSTI ICE with the Kanamycin resistance would be transferred to the other strain of *Vibrio casei.*

In order to detect HGT, the donor strain would have an antibiotic resistance marker inserted into the RUSTI region of the ProteoRUSTI ICE. For the recipient strain, we wanted to eliminate the ProteoRUSTI region so that we could observe its acquisition *de novo*. Since ICEs are mobile elements capable of excising from the chromosome, we reasoned that *V. casei* could be induced to lose the ProteoRUSTI ICE by exposing cells to stressful conditions normally associated with ICE mobilization. Subsequently, an additional antibiotic resistance marker would be placed into a neutral site on the chromosome. We would be able to identify the transfer of the ProteoRUSTI ICE between the *Vibrio casei* strains because it would lead to the recipient strain being resistant to both antibiotics and easily identified on double selection plates (Figure 9). We hypothesized that observing HGT between two clones would be the most efficient way to observe HGT of the

ProteoRUSTI ICE because horizontally transferred genes must be passed to cells which are capable of being recognized by the donor cell, and then the DNA must recombine at specific site within the host genome (in this case, likely the serine tRNA). Thus, using an isogenic strain background would make successful HGT more likely [17].

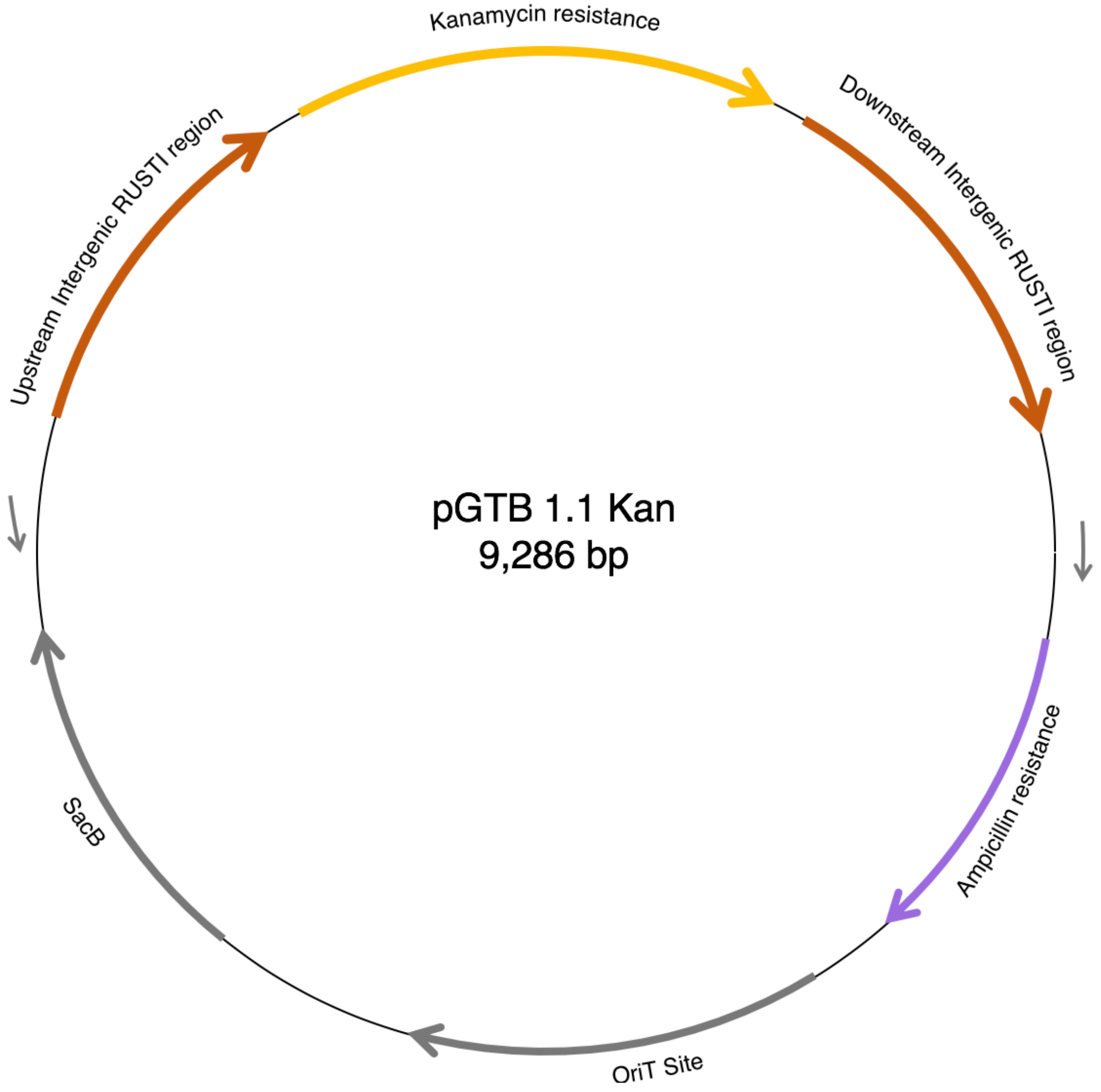

Figure 10: Vector Inserting Kanamycin Resistance into the RUSTI Region.
Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert Kanamycin resistance into an intergenic region of the RUSTI region on the ProteoRUSTI ICE. This vector was created.

Creation of a vector for inserting Kanamycin resistance into the RUSTI region

The first mutant that was constructed was the *Vibrio casei* strain with the antibiotic resistance in the RUSTI region of the ProteoRUSTI ICE. We chose to insert the antibiotic resistance into the RUSTI region instead of another area of the ProteoRUSTI ICE for a few reasons. First, we knew that this RUSTI region was commonly transferred region in the ProteoRUSTI bacteria living on cheese. Second, we did not know the exact ends of the ProteoRUSTI ICE, therefore inserting an antibiotic resistance gene in the known RUSTI region was determined to be the best approach. The antibiotic resistance marker we chose to insert into the RUSTI region was Kanamycin because *Vibrio casei* is not naturally resistant.

We engineered a *Vibrio casei* strain with Kanamycin resistance inside an intergenic site within the RUSTI region on the putative *Vibrio casei* ICE. This site is a 1000 base pair intergenic region between a hypothetical protein upstream and Rossman fold nucleotide binding protein downstream. This appeared to be an ideal site to insert the Kanamycin resistance genes because both genes were annotated as divergent therefore we predicted that integration of a Kanamycin resistant gene would not interfere with the transcription of the genes. To insert the Kanamycin resistance marker, we utilized an allelic exchange vector originally designed for use in *V. cholerae.* This vector, pGP704-*sacB*, was used because it is a known suicide vector in *Vibrio cholerae*, which is in the same Genus as our *Vibrio casei*. Additionally, a Postdoc in our lab, Dr. Gary Heussler, had successfully used pGP704-*sacB* to knock out a gene in *Vibrio casei*, so we knew that the vector worked in our *Vibrio*. Gibson Assembly was used to construct our vector and subsequently transform *E. coli* with it (Figure 10, for details see Materials and Methods). *E. coli* was then used to transfer the vector to the *Vibrio casei* via conjugation. Once the vector was integrated into the

Vibrio casei chromosome a counterselection on Sucrose plates was performed to successfully generate a stable chromosomally-integrated Kanamycin resistance gene.

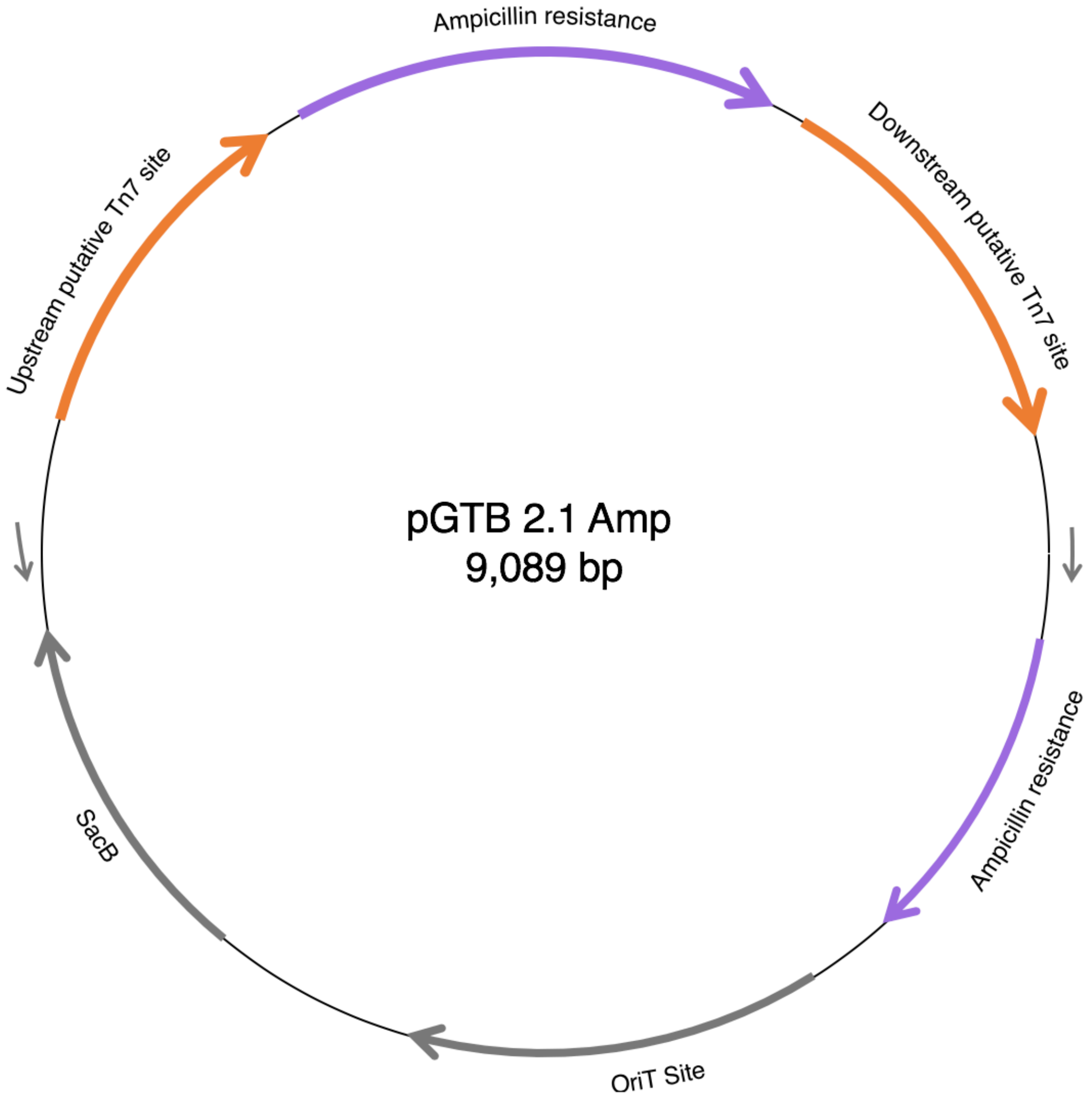

Figure 11: Vector Inserting Ampicillin Resistance into the Putative Tn7 att site. Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert Ampicillin resistance into the putative Tn7 att site. Upstream is the *rarD* gene and downstream is the *glmS* gene. This vector was never created.

Creation of a vector for inserting Ampicillin resistance into the putative Tn7 att site

After the completion of the *Vibrio casei* strain with the Kanamycin resistance in the RUSTI region we began construction a separate *Vibrio casei* strain with another antibiotic resistance marker. The technique to induce the *Vibrio casei* to lose the ProteoRUSTI ICE was still being optimized by a Postdoc in the lab, however construction of the *Vibrio casei* strain with another antibiotic resistance marker in a neutral site continued. We chose to insert an Ampicillin resistance gene as our additional antibiotic resistance marker because it encoded for a different type of antibiotic resistance than the Kanamycin (beta-lactam versus aminoglycoside respectively). We chose to insert an Ampicillin resistance gene in the chromosome of *Vibrio casei* at the putative Tn7 att site because it is a highly conserved integration site in Proteobacteria [33]. The Tn7 att site was originally discovered as the natural integration site of the Tn7 transposon, but has since been utilized as a general integration site for engineered genetic regions in Proteobacteria [33]. The Tn7 att site is often found upstream of the *glmS* gene [33]. The *glmS* gene sequence from *Vibrio cholera* was blasted to find its homolog in *Vibrio casei* [33]. The *glmS* homolog was identified in *Vibrio casei* just downstream from a gene encoding for an outer membrane protein annotated as *rarD*. The intergenic area between *rarD* and *glmS* was identified as the putative Tn7 att site for *Vibrio casei*. An allelic exchange vector was created to insert the Ampicillin resistance marker into the putative Tn7 att site (Figure 11, see Materials and Methods for further details). However, we were never able to construct the vector from the Gibson Assembly.

We hypothesize that this may be due to a problem with the vector design and the homology of the Ampicillin resistance on the pGP704-*sacB* backbone with the Ampicillin resistance marker. We hypothesize that this may be due to a problem with the vector design. Although there were

100 base pairs from the Ampicillin resistance gene on the pGP704-*sacB* backbone amplicon, it is possible that the exonucleases from the Gibson Assembly degraded the amplicon to this point. This would have exposed the Ampicillin resistance genes on the backbone to that of the Ampicillin resistance marker. This homology could have inhibited the correct vector from being assembled.

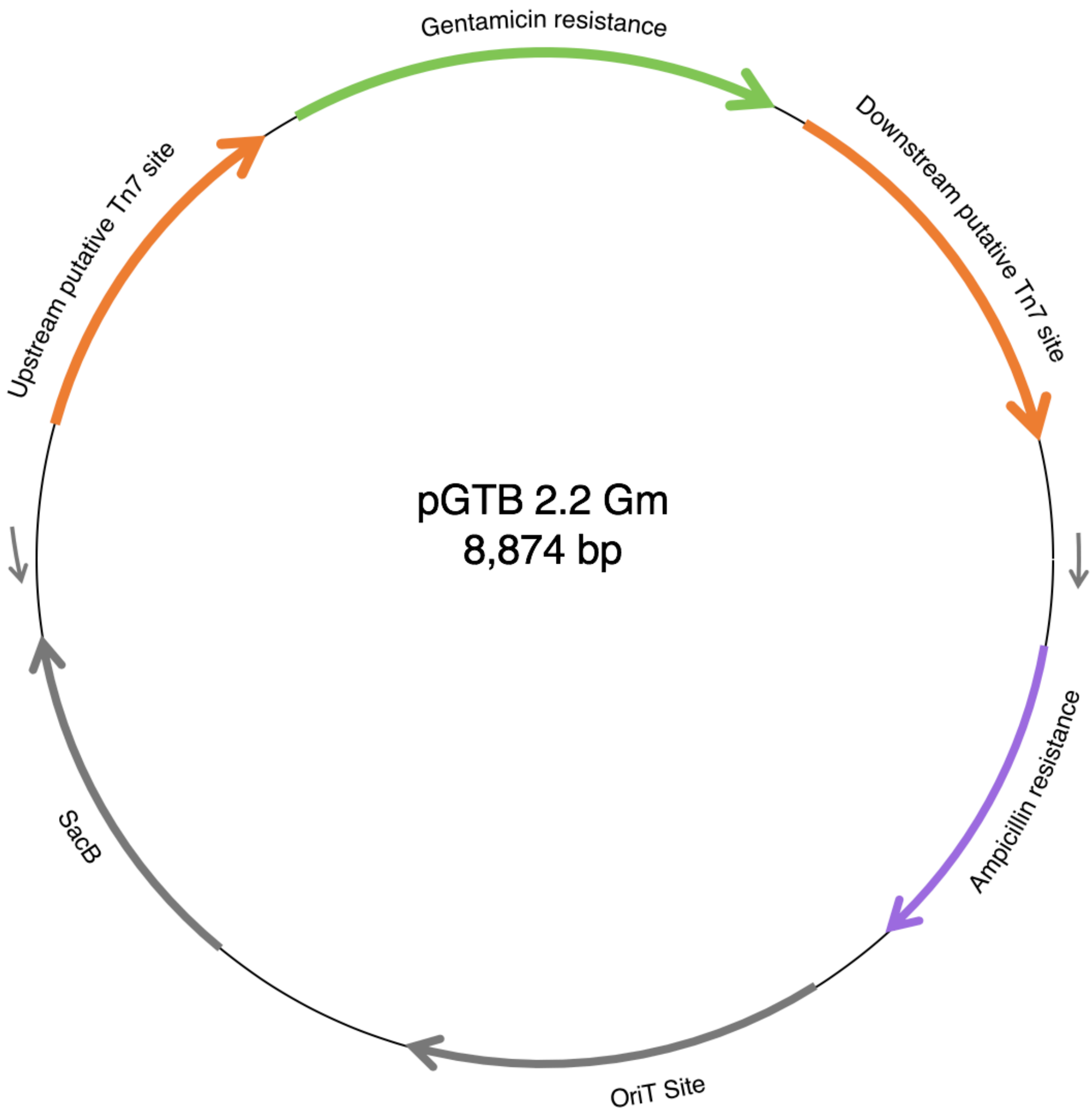

Figure 12: Vector Inserting Gentamicin Resistance into the Putative Tn7 att site. Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert Gentamicin resistance into the putative Tn7 att site. Upstream is the *rarD* gene and downstream is the *glmS* gene. This vector was created.

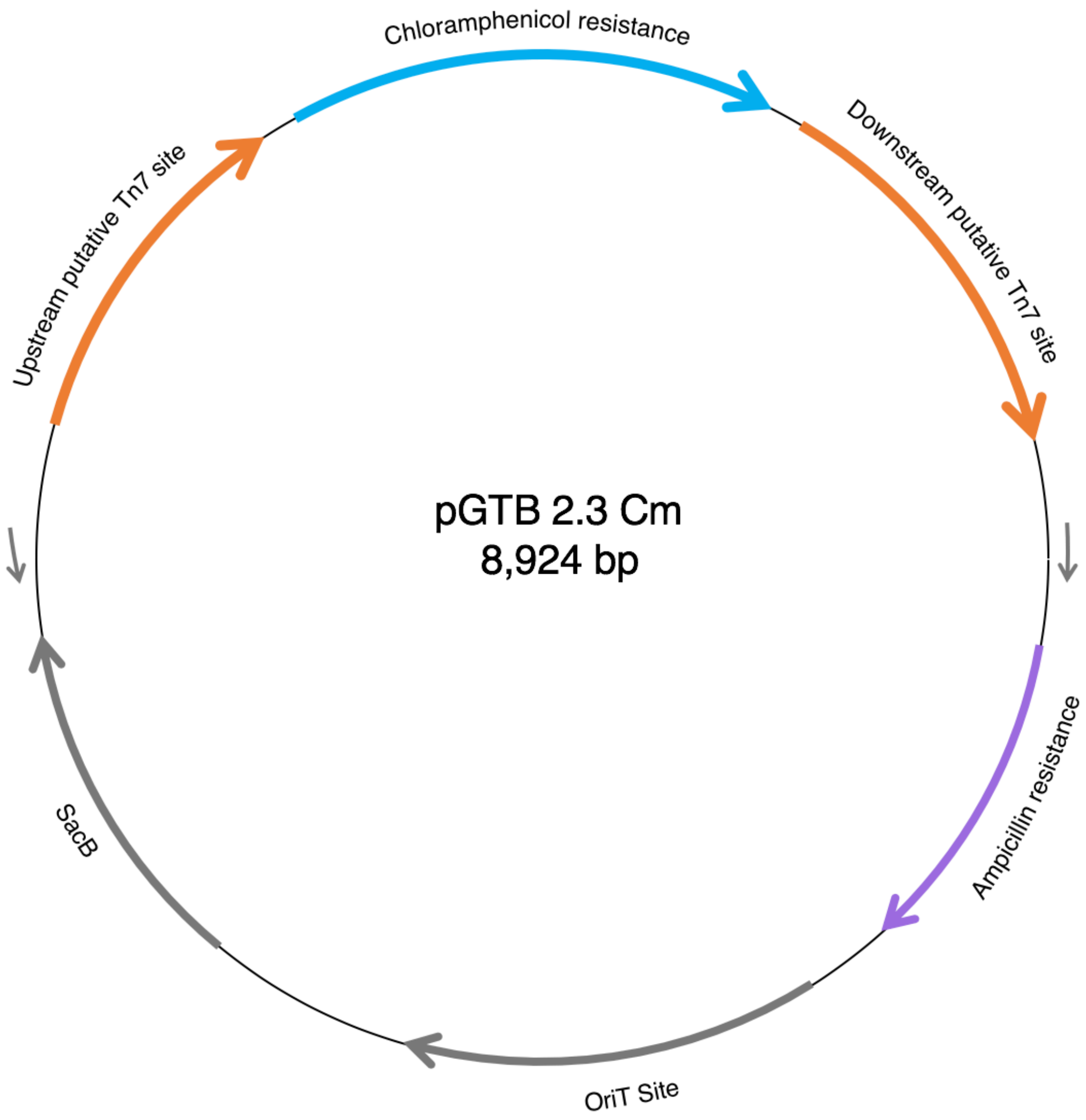

Figure 13: Vector Inserting Chloramphenicol Resistance into the Putative Tn7 att site. Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert Chloramphenicol resistance into the putative Tn7 att site. Upstream is the *rarD* gene and downstream is the *glmS* gene. This vector was created.

Creation of vectors for inserting Gentamicin resistance or Chloramphenicol resistance into the putative Tn7 att site

To circumvent homology issues during Gibson Assembly from occurring, we redesigned the pGP704-*sacB* vector to replace the Ampicillin resistance marker with a Gentamicin resistance marker or a Chloramphenicol resistance marker. Because the same vector was used, but the antibiotic resistances were changed, both vectors were still designed to chromosomally insert the antibiotic resistance genes at the putative Tn7 att site of *Vibrio casei* via allelic exchange. Gibson Assembly was utilized to generate both vectors (Figure 12 and Figure 13, see Materials and Methods for details). Both vectors were successfully generated. Unfortunately, multiple attempts of generating merodiploids in *Vibrio casei* via conjugation with *E. coli* were unsuccessful. After attempting the conjugation under several different conditions with no success (see Materials and Methods) we concluded that inserting genes into the putative Tn7 att site is likely lethal to *Vibrio casei*.

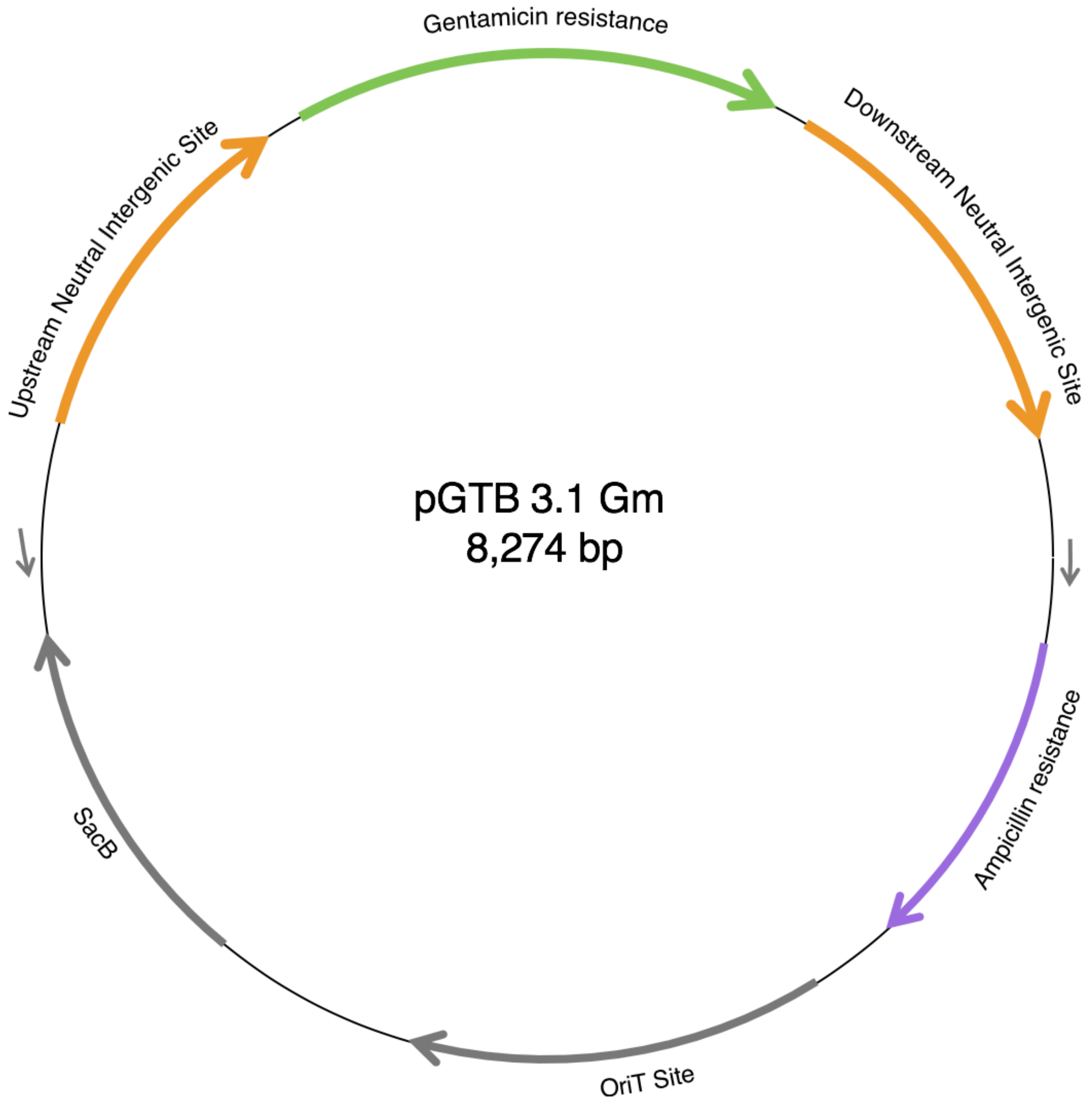

Figure 14: Vector Inserting Gentamicin Resistance into the Neutral Site. Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert Gentamicin resistance into the neutral site on the *Vibrio casei* chromosome. Upstream is a gene that encodes for Glutathione S-transferase and downstream is a gene that encodes for a hypothetical protein. This vector was created.

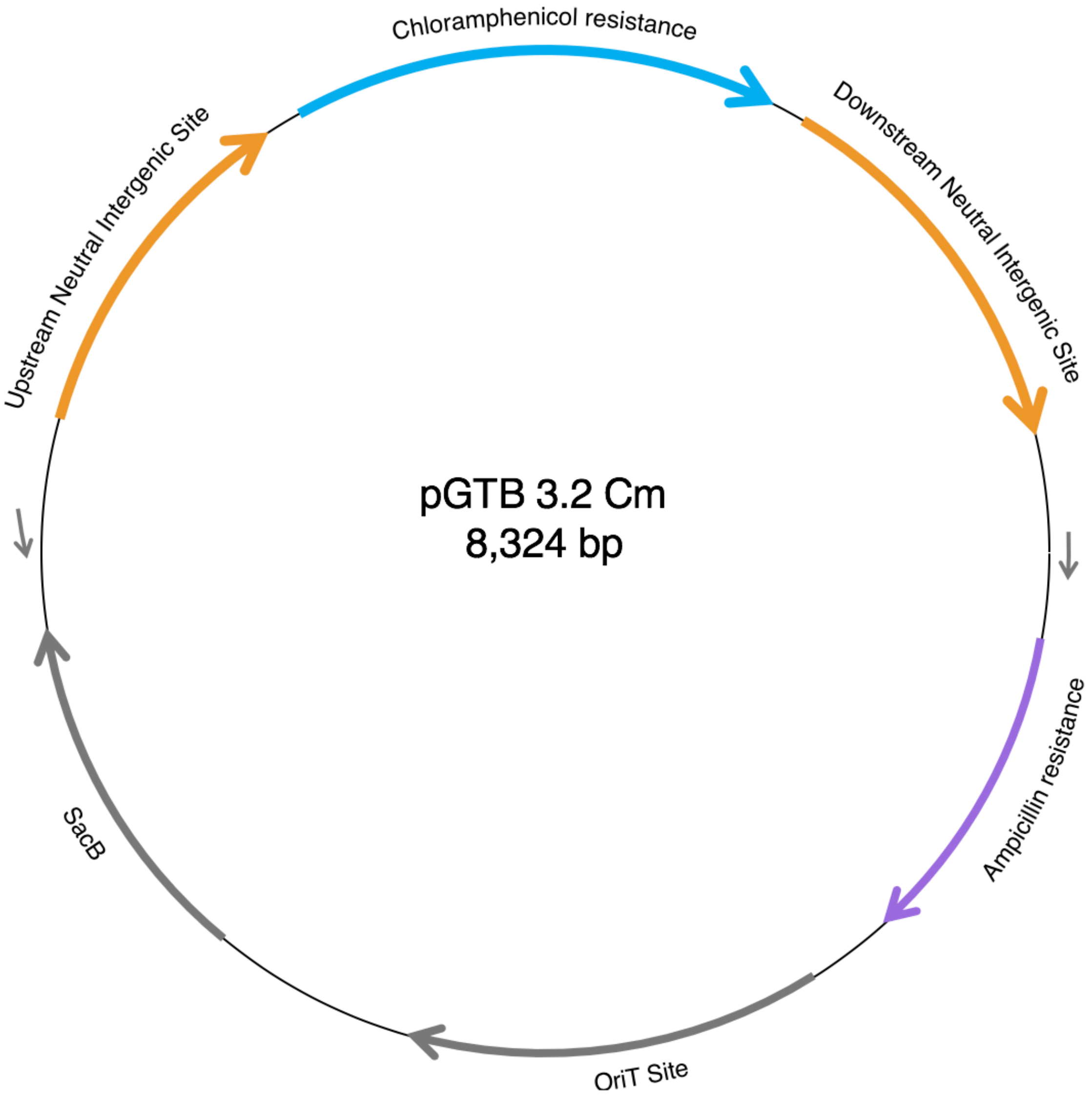

Figure 15: Vector Inserting Chloramphenicol Resistance into the Neutral Site. Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert Chloramphenicol resistance into the neutral site on the *Vibrio casei* chromosome. Upstream is a gene that encodes for Glutathione S-transferase and downstream is a gene that encodes for a hypothetical protein. This vector was created.

Creation of vectors with Gentamicin resistance or Chloramphenicol resistance into a neutral intergenic site

Due to the failure of attempts to insert a resistance marker at the Tn7 att site, we next attempted to generate a *Vibrio casei* strain with a Gentamycin resistance or Chloramphenicol resistant marker on a different part of the chromosome. The *Vibrio casei* JB196 genome was analyzed in order to identify potential intergenic regions, not inside the ProteoRUSTI ICE. Our criteria for a neutral site was an intergenic region between two divergent, non-housekeeping genes. We identified three locations on the Vibrio casei chromosome that would be potential insertion sites for the antibiotic resistance gene: between Glutathione S-transferase upstream and a hypothetical protein downstream, a L-aspartate oxidase upstream and a S-(hydroxymethyl) glutathione dehydrogenase downstream, and peptidase upstream and a chorismite mutase I downstream. Vectors to integrate either a Chloramphenicol or Gentamicin resistance marker in each of the three sites were generated using Gibson Assembly. When transforming with S17-1 *E. coli* the first vector between the genes predicted to encode a Glutathione S-transferase and a hypothetical protein was the most successful, so we decided to continue only with this vector. Subsequent *E. coli* were used to insert the first vector into the *Vibrio casei* via conjugation. After the vector successfully integrated into the *Vibrio casei* chromosome, a counter selection was performed to ensure the right insertion was selected for. The *Vibrio casei* clones with Gentamicin resistance or Chloramphenicol resistance were a success (Figure 14 and 15).

Summary of vector and strain construction

The previous steps highlight the significant effort made to characterize and understand the various genetic tools needed to tag the *Vibrio casei* chromosome with several genetic markers. As mentioned previously, the tools needed to genetically modify *Vibrio casei* had not been well characterized, so the simple act of inserting an antibiotic resistance marker into the chromosome had to be designed by taking into consideration experiments done with *Vibrio cholerae* as well as by trial and error. We were able to successfully create a *Vibrio casei* with a Kanamycin resistance in the RUSTI region as well two *Vibrio casei* strains with a Gentamicin and Chloramphenicol resistances in a neutral site.

Experimental detection of HGT

Our original experimental strategy to observe transfer of the ProteoRUSTI ICE between two *Vibrio casei* clones required a *Vibrio casei* isolate that had naturally lost the putative ProteoRUSTI ICE. Previous work in the lab had identified such isolates based on a UV-induced ICE excision protocol. Briefly, *Vibrio casei* cultures were exposed to UV radiation at varying intensities, grown in LB for 6 hours at room temperature, and plated for single colonies. These colonies were then screened for ICE excision via PCR with primers amplifying several regions of the putative ICE. ICE negative strains were identified based on lack of amplification. However, approximately at the same time as the completion of the Gentamicin mutant and the Chloramphenicol mutant, we found the putative ICE negative *Vibrio casei* isolates identified were false negatives and no ICE negative *Vibrio casei* strains had been isolated. For this reason, it was necessary to change our experimental approach.

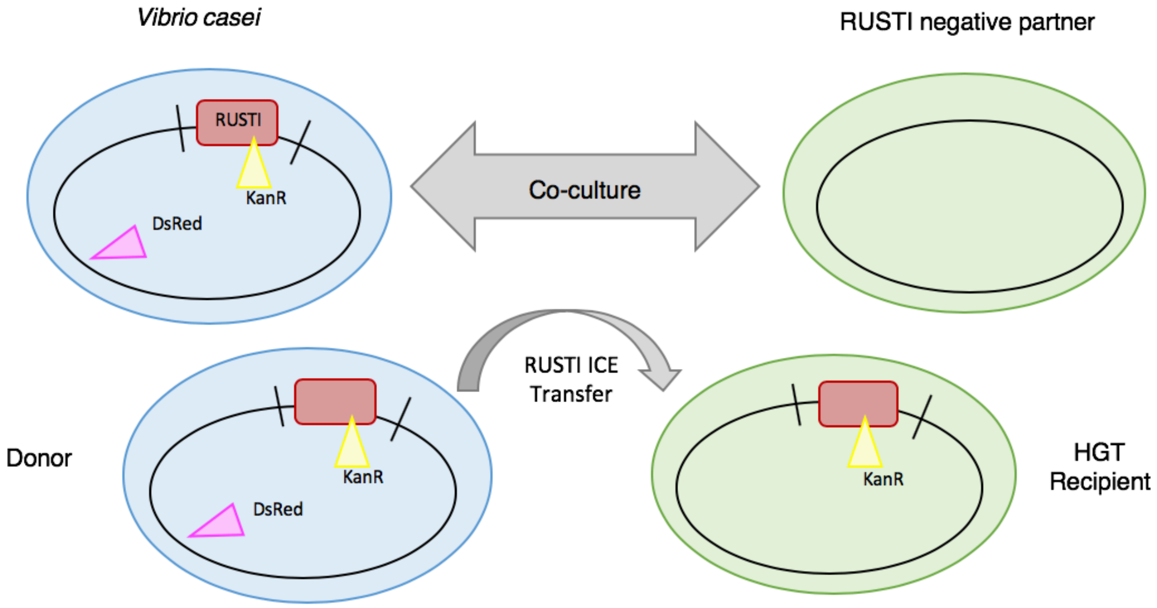

Figure 16: Experimental Approach of the Transfer of the ProteoRUSTI ICE from Vibrio casei to Naturally ProteoRUSTI Negative Cheese Community Members.

A strain of *Vibrio casei* with an antibiotic resistant marker in the RUSTI region, and a fluorescent marker in the chromosome. The *Vibrio casei* would be co-cultured with bacteria isolated from the cheese community that naturally do not have the ProteoRUSTI ICE. In the case of a horizontal gene transfer event the ProteoRUSTI ICE with the Kanamycin resistance would be transferred to the cheese community members. An HGT event would be recognized as non-fluorescing colonies on a Kanamycin selection plate.

Experimental approach with Vibrio casei and naturally ProteoRUSTI ICE negative cheese bacteria

Our new experimental approach involved observing the horizontal gene transfer of the ProteoRUSTI ICE from *Vibrio casei* to any microbial member of the original Washed Moses Sleeper cheese community where the *Vibrio casei* was isolated from. We hypothesized that *Vibrio casei* evolved with members of the cheese community and would therefore be likely to engage in HGT with community members it had co-evolved with. Thus, we sought to isolate a set of bacterial species that normally co-occur with *Vibrio casei*. The bacterium was originally isolated from a washed-rind cheese produced in Vermont, named Washed Moses Sleeper. Frozen stocks of the cheese rind were stored at -80C and were available for further isolations. We concluded that we could isolate bacteria that naturally did not have the ProteoRUSTI ICE (termed ProteoRUSTI ICE negative bacteria) from the original cheese community. Once isolated, the ProteoRUSTI ICE negative bacteria would be co-cultured with *Vibrio casei* to encourage horizontal gene transfer.

The new approach consisted of co-culturing the naturally ProteoRUSTI ICE negative bacteria with a *Vibrio casei* mutant tagged with a variety of identifying genetic markers that allow detection of HGT. *Vibrio casei* was tagged with both an antibiotic resistance and fluorescence marker to identify a transfer of the ProteoRUSTI ICE to from the *Vibrio casei* donor to the cheese community bacteria recipient (Figure 16). Horizontal gene transfer recipients could be distinguished from the *Vibrio casei* donor because they will have acquired antibiotic resistance but will not fluoresce on antibiotic selection plates. This is in contrast to the *Vibrio casei* donors, which would also grow on the antibiotic media but would be fluorescent. The naturally ProteoRUSTI ICE negative bacteria have not been sequenced and therefore at the moment targeted genetic manipulation is not possible.

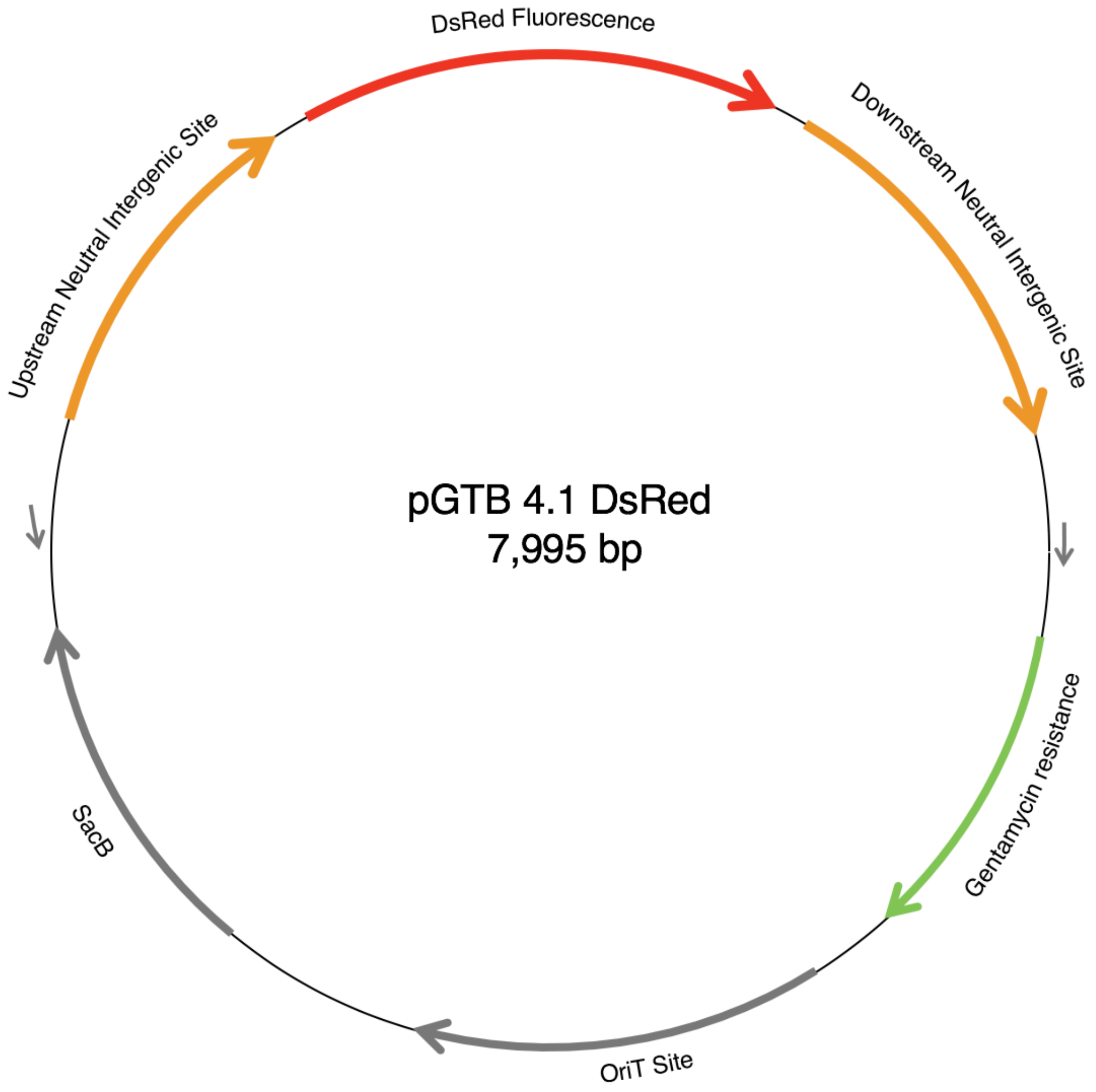

Figure 17: Vector Inserting DsRed Fluorescent Marker into the Neutral Site. Depicted is a suicide vector adapted from *Vibrio cholerae* is used to insert DsRed fluorescent marker into the neutral site on the *Vibrio casei* chromosome. Upstream is a gene that encodes for Glutathione S-transferase and downstream is a gene that encodes for a hypothetical protein. This vector was created.

Creation of a vector for inserting DsRed fluorescence into a neutral intergenic site

We decided to utilize DsRed as the fluorescence marker to be integrated onto the chromosome of the strain of *Vibrio casei* tagged with the Kanamycin resistance marker in the RUSTI region. As discussed, this double mutant would allow us to distinguish between *V. casei* and a partner species that acquired kanamycin resistance through horizontal transfer of the RUSTI region. We inserted a DsRed fluorescent marker into the same neutral intergenic site previously used to insert Gentamicin and Chloramphenicol antibiotic resistance markers. This site was chosen again because insertion of Gentamicin and Chloramphenicol markers had no detectable effect on growth or viability of the *Vibrio casei* mutants.

However, we did alter the pGP704-*sacB* vector backbone that would deliver the DsRed fluorescent marker to the *Vibrio casei* through allelic exchange. We switched the Ampicillin resistance marker for a Gentamicin resistance marker in the backbone because *Vibrio casei* has a low natural resistance to beta-lactam antibiotics that complicates the use of ampicillin as a genetic marker. To construct this vector, we used Gibson Assembly techniques. The vector was transformed into competent *E. coli* which were subsequently used to transfer the vector into *Vibrio casei* via conjugation. A counterselection was performed to successfully generate a stable chromosomally integrated DsRed fluorescent marker (Figure 17, see Materials and Methods for more details).

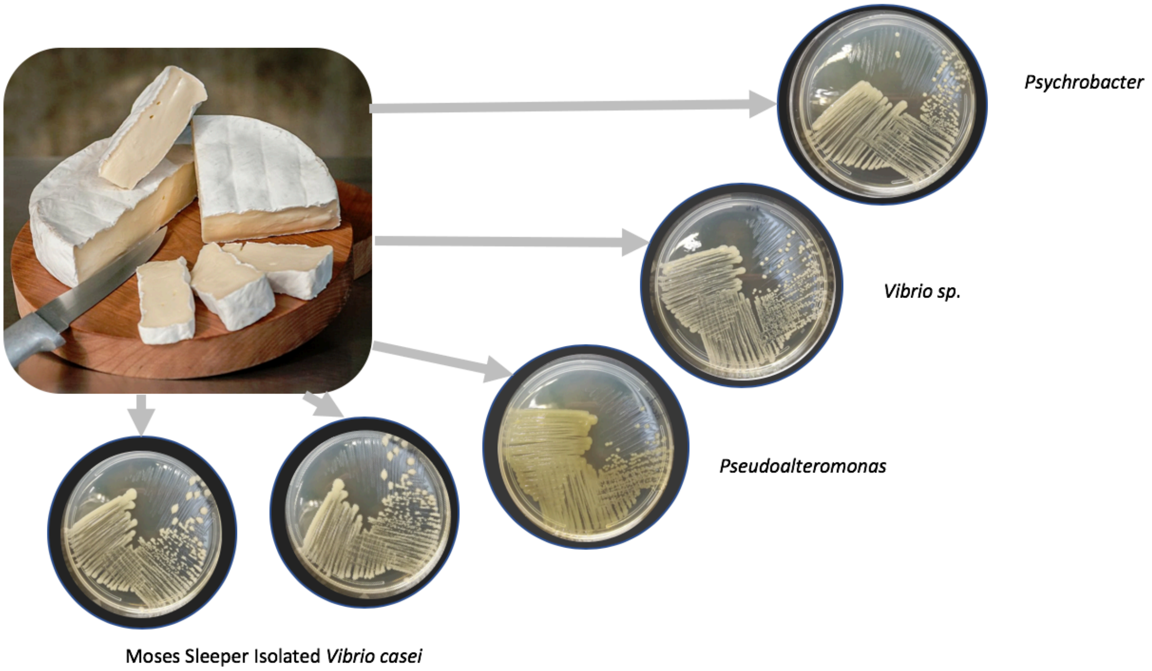

Figure 18: Isolated Bacteria from Moses Sleeper Cheese Community. Thirteen bacteria were isolated from the Moses Sleeper cheese community. This is the same cheese community our WT *Vibrio casei* was isolated from. We identified two additional *Vibrio casei* strains with the ProteoRUSTI ICE. Nine Proteobacteria were isolated and identified to naturally not contain the ProteoRUSTI ICE. Two gram-positive bacteria were isolated as well. A *Psychrobacter sp.*, a *Vibrio* species without the ProteoRUSTI ICE, and a *Pseudoalteromonas sp.* were used for the duration of the experiment.

Isolating naturally ProteoRUSTI ICE negative bacteria from the Washed Moses Sleeper Community

After the creation of the *Vibrio casei* double mutant we needed to isolate naturally ProteoRUSTI negative bacteria from the cheese community. To isolate ProteoRUSTI ICE negative bacteria from the Washed Moses Sleeper community we took a frozen sample of the cheese (isolated in 2011, see Material and Methods). The sample was serially diluted and plated onto both BHI medium with cycloheximide and PCAM with cycloheximide to select against fungi and ensure we isolated only bacterial species from the community. From these plates we identified

13 unique colony morphologies. Once isolated, the 16s gene of all of the unique colony morphologies were amplified via PCR. We aimed to identify the Proteobacteria to use them in the HGT experiment because HGT occurs more often in bacteria that are in the same phyla [17]. After the Proteobacteria were isolated a PCR was performed to determine if the bacteria contained the ProteoRUSTI ICE naturally or not. The two bacteria that were identified as containing the ProteoRUSTI ICE were additional *Vibrio casei* species. The other bacteria that were identified as not containing the ProteoRUSTI ICE were mostly *Psychrobacter species*, an additional *Vibrio sp.*, *Pseudoalteromonas species,* and one Gram-positive species. *Psychrobacter sp.* (1), the additional *Vibrio sp.* (11), and a *Pseudoalteromonas sp* (13B) were used for the rest of the experiment (Figure 18).

Table 1: Thirteen unique bacterial phenotypes isolated from the Washed Moses Sleeper cheese community

Number	Phenotype	Bacteria	Used further?
1	Beige, circular, raised, entire, 3-4 mm	*Psychrobacter alimentarius*	Yes
2	Beige, circular, raised, undulated, 3-4 mm	*Psychrobacter alimentarius*	No
3	Orange-like, circular, raised/convex, entire, 5 mm	*Pseudoalteromonas sp.*	No
4	Slightly transparent/light green, irregular, flat, lobate, 5-6 mm, fried egg look	*Vibrio sp.*	No
5	Beige, irregular, concave, lobate, 10 mm, spreads and surrounds other colonies	*Vibrio casei*	Yes
6	White, circular, raised, entire, 1 mm	*Psychrobacter alimentarius*	No
7	Neon yellow, circular, raised, entire, 5 mm	*Micrococcus yannanesis*	No
8	Yellow, transparent, circular, crater form, entire, 6 mm	*Pseudoalteromonas sp.*	No
9	Light orange, pink, circular, round, entire, 3 mm	*Psychrobacter alimentarius*	No
10	Slightly green, flat, undulate, irregular, 5 mm	*Pseudoalteromonas sp.*	No
11	Yellow, entire, embonate, 5 mm	*Vibrio sp.*	Yes
12	Neon yellow, circular, round, entire, 2 mm	*Glutamicibacter sp.*	No
13 A	White colonies, irregular, flat, undulate, 7 mm	*Vibrio casei*	Yes
13 B	Yellow, transparent, circular, round, 3 mm	*Pseudoalteromonas sp.*	Yes

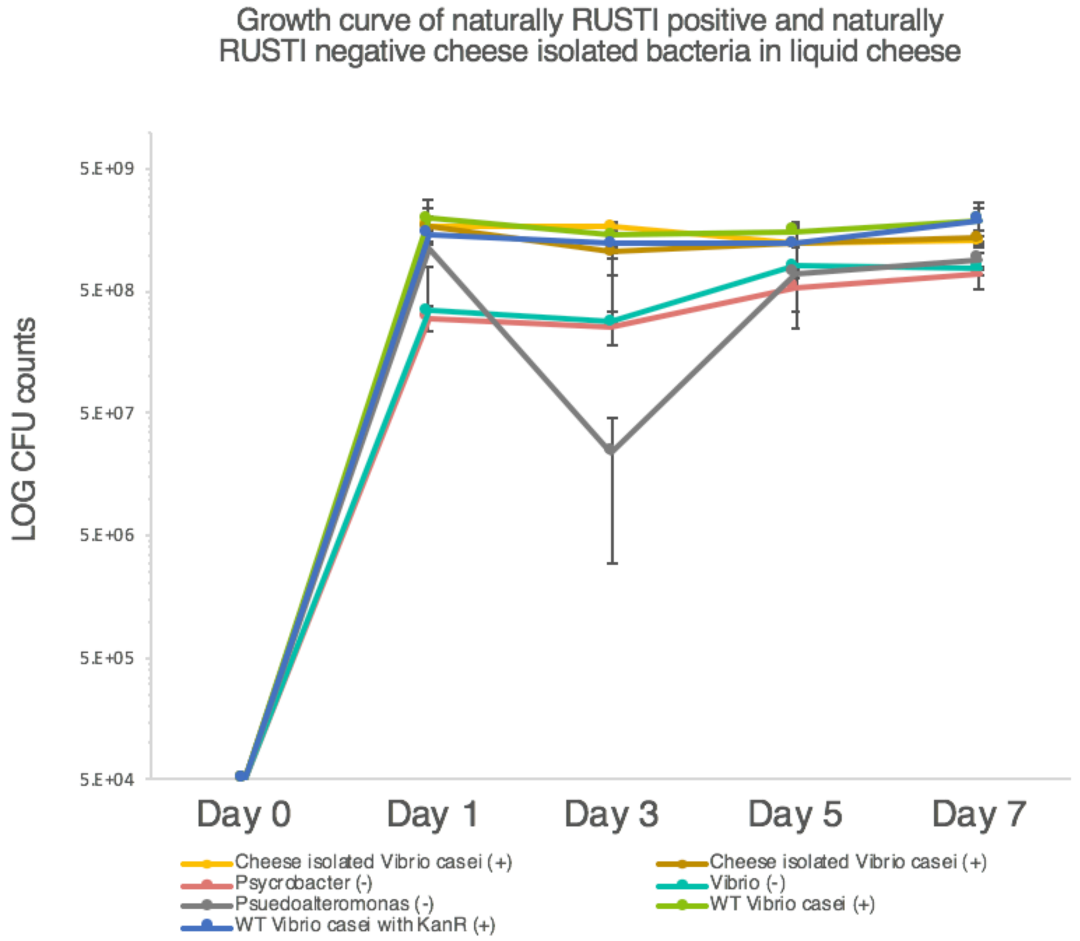

Figure 19: Growth Curve of Naturally ProteoRUSTI ICE Negative and Positive Bacteria. A growth curve was performed with the WT *Vibrio casei*, the *Vibrio casei* strain with the Kanamycin resistance in the RUSTI region, the two isolated *Vibrio casei* strains from the Moses Sleeper community, and the naturally ProteoRUSTI negative *Psychrobacter sp.*, a *Vibrio sp.*, and a *Pseudoalteromonas sp*. There appears to be no dramatic difference in growth between all of the bacteria.

Growth curve of naturally ProteoRUSTI ICE negative bacteria and various strain of Vibrio casei

A growth assay was performed with the new *Vibrio casei* isolated from the Washed Moses Sleeper, all three naturally ProteoRUSTI ICE negative bacteria, the WT *Vibrio casei*, and the

Vibrio casei with the Kanamycin resistance gene in the RUSTI region of the ICE. Each sample was grown in beveled Erlenmeyer flasks with 30 mL of liquid cheese for a week (see Materials and Methods for more details). The goal was to assay if there were any significant growth differences between the different microbes in the cheese community. As illustrated in Figure 19, the naturally ProteoRUSTI ICE negative bacteria have similar growth dynamics in comparison to the ProteoRUSTI ICE positive bacteria.

Horizontal Gene Transfer Experiment

Horizontal Gene Transfer experiment on PCAM

The *Vibrio casei* tagged with Kanamycin resistance in the RUSTI region of the ProteoRUSTI ICE and DsRed was co-cultured with its partner bacteria *Psychrobacter, Vibrio sp., and Pseudoalteromonas* on cheese curd agar wells. On days 1, 3, 5, and 7 the wells were extracted, diluted, and plated onto PCAM and PCAM with Kanamycin. Unexpectedly, the *Vibrio casei* tagged with Kanamycin resistance and DsRed did not fluoresce when grown on PCAM. Since the *Vibrio casei* did not fluoresce on the PCAM we were unable to determine if the cells growing on the selection plate were either the original *Vibrio casei* donor strain or a partner bacterium that had received the kanamycin resistance marker via HGT of the RUSTI region. We hypothesize the lack of fluorescence from the DsRed in *Vibrio casei* is due to the stress of the selection medium which may have influenced the expression of the fluorescing of the DsRed protein.

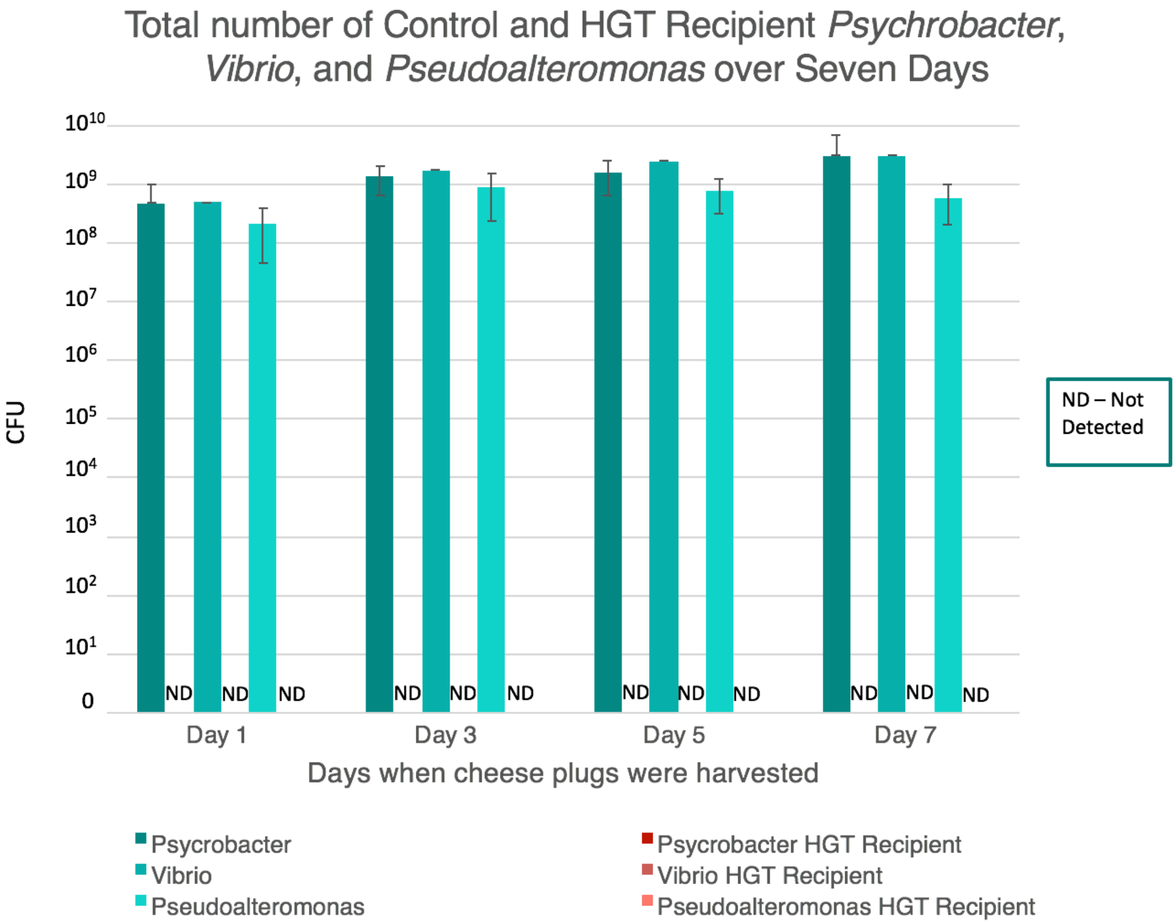

Figure 20: HGT of the ProteoRUSTI ICE was Not Detected.
HGT of the ProteoRUSTI ICE was not detected across all experimental conditions. The control plates across all conditions had the naturally ProteoRUSTI negative bacteria present but known were found in the experimental conditions.

Horizontal gene transfer experiment on LB

To address the lack of fluoresce on the PCAM media we performed the same experiment, but instead of plating the cells onto PCAM we plated them on LB medium. The double tagged *Vibrio casei* was co-cultured with its partner bacteria *Psychrobacter, Vibrio sp., and Pseudoalteromonas* on cheese curd agar wells. On days 1, 3, 5, and 7 the wells were extracted, diluted, and plated onto LB and LB with Kanamycin.

Over the course of the experiment, we did not detect any evidence of an HGT event (Figure 20). Over all conditions we did not observe any non-fluorescing Kanamycin resistant bacteria; all the bacteria present on the LB with Kanamycin plates were expressing the DsRed fluorescence marker. There are a variety of explanations for why we did not detect a horizontal gene transfer event, and this includes a limited range of detection and general compatibility problems with the bacteria. This is described in further detail in the Discussion.

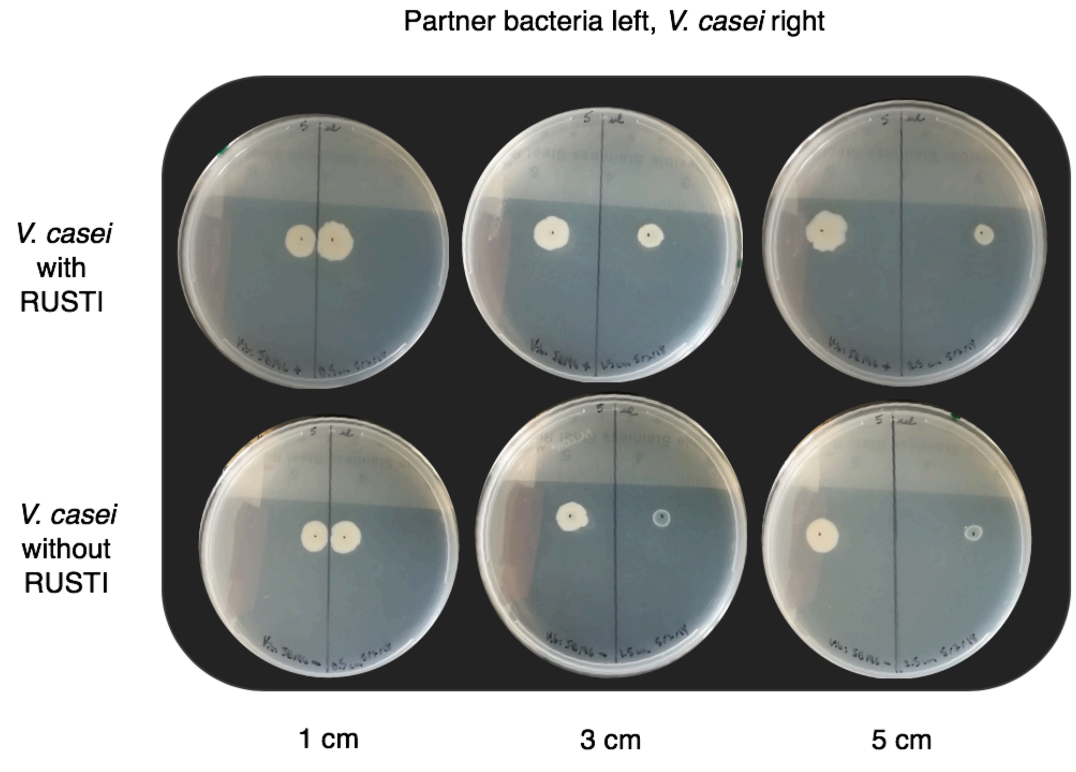

Figure 21: Growth Defect of WT *Vibrio casei* and *Vibrio casei* Strain Without the RUSTI Region. A serendipitous phenotype was discovered on PCAM where WT *Vibrio casei* and *Vibrio casei* strain without the RUSTI region grew into smaller colonies when they were grown farther apart from the other members of the cheese community. The *Vibrio casei* strain without the RUSTI region had a more pronounced growth defect. Both strains of *Vibrio casei* did not have a growth defect when near other members of the cheese community.

Serendipitous phenotype of Vibrio casei near partner bacteria

Phenotypic differences of Vibrio casei with and without the RUSTI region near partner bacteria

A serendipitous discovery was made during the HGT experiment on PCAM. An interesting *Vibrio casei* phenotype was observed on the PCAM control plate without any antibiotics. On the control plate the *Vibrio casei* only grew into large, fluorescing colonies when it was grown in proximity to the naturally RUSTI negative bacteria on the same plate. As distance increased between the *Vibrio casei* and partner naturally RUSTI negative cheese community bacteria, the *V. casei* colony grew smaller in diameter and less dense.

To investigate this intriguing phenotype, we grew the wild type *Vibrio casei* without any antibiotic or fluorescent markers with the three partner bacteria, *Psychrobacter, Vibrio sp,* and *Pseudoalteromonas*. A *Vibrio casei* with the RUSTI region knockout, but still an intact ProteoRUSTI ICE was also grown next to the three naturally ProteoRUSTI bacteria to determine if the RUSTI region on the ProteoRUSTI region affected this phenotype in any way. The ProteoRUSTI ICE positive *Vibrio casei* and the three naturally ProteoRUSTI bacteria were grown 1 cm, 3 cm, and 5 cm apart on a PCAM plate. Pictures of the colonies were taken on days 3 and 6. We found the RUSTI negative *Vibrio casei* had a growth defect compared to the WT *Vibrio casei*. When 3 cm and 5 cm away from any of the community partners RUSTI negative *Vibrio casei* grew smaller and clear compared the WT *Vibrio casei* which, although was still smaller than the *Vibrio casei* that grew next to the partner bacteria, did not grow clear (Figure 21). These growth defects in both *Vibrio caseis* were masked when they were grown next to any of the community members. Additionally, there is no difference in growth of the RUSTI negative *Vibrio* *casei* and the WT *Vibrio casei* when grown next to community members.

DISCUSSION

This thesis was a testament to try to understand and characterize how the RUSTI element was horizontally transferred throughout the cheese community. Previous research done by our lab has highlighted that the RUSTI element is common throughout the cheese rind bacterial communities. By characterizing how and when genes, such as the RUSTI element, transfer throughout the cheese community it can provide better insight into how other environmental beneficial genes are transferred in more complex microbial communities. This masters work highlighted the development of many tools to help better work with this novel system as well as some insight into how horizontal gene transfer of the RUSTI region may impact the cheese community.

To create a better understanding of HGT in the cheese community we employed a variety of different approaches. Our first strategy involved using two *Vibrio casei* clones to observe HGT in the cheese community. Our second strategy was to observe HGT of the ProteoRUSTI ICE from *Vibrio casei* to other members of the cheese community. In our first strategy, we needed to implement novel genetic techniques to the cheese isolated *Vibrio casei*. This bacterium had never been genetically manipulated before, so we based our techniques on the related and well documented *Vibrio cholerae*. Our cheese isolate and *Vibrio cholerae* fall within the same genus; therefore, we expected tools developed in *Vibrio cholera* to be appropriate for our isolate. We found that some genetic tools from *Vibrio cholerae* did not work while others did. For example, integrating genes at the Tn7 att site works well for *Vibrio cholerae* but proved to be lethal for our *Vibrio casei*. However, allelic exchange techniques from *Vibrio cholerae* proved to be immensely helpful in manipulating the genome of *Vibrio casei*. For our second strategy we wanted to observe

HGT of the ProteoRUSTI ICE from *Vibrio casei* to *Psychrobacter, Vibrio sp., and Pseudoalteromonas*. After co-culturing *Vibrio casei* that contains the ProteoRUSTI ICE with other members of the cheese community which naturally do not contain the ProteoRUSTI ICE, we expected that the ProteoRUSTI ICE from *Vibrio casei* would transfer to any of the other cheese community members. Unfortunately, we did not witness any transfer. However, we did witness an interesting emerging phenotype over the course of the experiments that could give some insight into the importance of RUSTI region on the ProteoRUSTI ICE in pairwise experiments.

Our working with *Vibrio casei* took a lot of unexpected turns. In many ways the bacteria are very similar to other *Vibrio,* but in other ways they are very unique. As mentioned earlier, the Tn7 att site is an active region for transfer in many Proteobacteria including *Vibrio cholera.* This site is located just upstream of the *glmS* gene. We suspect the lethality of the putative Tn7 site in *Vibrio casei* is due to the gene upstream of *glmS*. At the 3' end of the putative Tn7 site is a gene responsible for the expression of RarD, a cell membrane protein in the drug/metabolite transporter (dmt) superfamily. We speculate that the insert of a gene in this location caused the overexpression of RarD, which disrupted homeostasis inside the cell and thereby killing it. Alternatively, insertion of a gene at the Tn7 site could have led to the downregulation of the cell membrane protein, preventing the *Vibrio casei* from uptaking important metabolites and upsetting homeostasis that leads to cell death.

Working with novel bacteria poses a challenge. When performing genetics on novel bacteria, it is helpful to cross reference existing genetic tools from a well-documented related species, but one can expect that these tools will have to be modified for the novel organism. As we strive to better understand how the microbes within microbiomes interact with each other, it will be imperative for us to expand our traditional thoughts on genetic manipulation tools in order to use novel bacteria. Other technologies such as those involving Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) may prove to be a great asset for more targeted and direct manipulation of the genome. Our aim is that if we can understand the microbial interactions of native cheese bacteria we might be able to extrapolate these interactions to other microbiomes. Model organisms enable us to understand biological processes in great detail, but we are limited by the fact that our model organisms are rarely seen in natural environments. Therefore, it will be worth the struggle and the effort to manipulate novel bacteria because working directly with native bacteria can provide us a more direct understanding of the microbial interactions that transpire within microbiomes.

In our second strategy to better understand HGT in the cheese community, we co-cultured *Vibrio casei* with three ProteoRUSTI ICE negative bacteria native to the cheese community: *Psychrobacter, Vibrio sp., and Pseudoalteromonas.* Our bioinformatic evidence revealed the RUSTI region is especially prolific in the cheese community and therefore we expected HGT to occur over the course of the experiment. However, we did not see any HGT events in *Psychrobacter, Vibrio sp., and Pseudoalteromonas*, which lead us to a few different explanations. First, it is possible that our limit of detection for an HGT event was too low. When counting CFUs, on an average, 150 bacteria at a time were counted. If the ProteoRUSTI ICE transfer was a rare event, we would have not detected it with this approach. An alternative to this approach would have been to introduce a GFP or mCherry fluorescent tag onto ProteoRUSTI ICE and then record the transfer events using flow cytometry. It is also possible that our hypothesis about when the transfer event would occur was incorrect. We hypothesized that the transfer of the ProteoRUSTI ICE would occur within a week; however, in reality the transfer event could occur in a cheese community at any time over the months long aging process, and the rate of transfer could also increase over the course of maturation. If we were to replicate our model, but change the extraction times, we may be able to observe an HGT event with our current limit of detection. Second, an HGT event may not have been not witnessed because of general compatibility issues with the other bacteria. When entering the *Psychrobacter, Vibrio sp., and Pseudoalteromonas* the ProteoRUSTI ICE could have been degraded by CRISPR proteins or restriction enzymes before it was integrated into the chromosome. *Psychrobacter, Vibrio sp., and Pseudoalteromonas* may have also lacked the proper sites for the ProteoRUSTI to integrate into the chromosome. Additionally, if the RUSTI ICE were to integrate into any of the *Psychrobacter, Vibrio sp., and Pseudoalteromonas* it is possible that the new host may not have the correct DNA polymerases to transcribe the ICE.

Although we did not see HGT of the ProteoRUSTI ICE, a serendipitous result led us to still hypothesize that the RUSTI region is important for life on cheese. It was found that *Vibrio casei* appeared to have a growth defect when not grown next to its partner bacteria from the cheese community. To test if this phenotype was RUSTI related we performed an experiment where we grew WT *Vibrio casei* and *Vibrio casei* without a RUSTI region pairwise with *Psychrobacter, Vibrio sp., and Pseudoalteromonas* at three different distances. We found that at the farther distances both *Vibrio caseis* had a growth defect and the colonies grew smaller when away from the partner bacteria. The *Vibrio casei* without the RUSTI region had a more pronounced growth defect and where the colonies grew smaller and clearer. The clear phenotype of the *Vibrio* without RUSTI maybe caused by the bacteria growing in a less dense colony. We hypothesize that pH may be the cause of the phenotypic differences of *Vibrio* compared to their counterparts. The *Vibrio casei* may be fermenting the sugar, dextrose, from the PCAM and releasing organic acids in its fermentation of dextrose. Other *Vibrio* species such as the O1 classical *Vibrio cholera* is known to acidify the media [34]. O1 classical *Vibrio cholera* is hypothesized to release organic acids byproducts of 2,3-Butanediol fermentation [34]. The O1 classical *Vibrio cholera* will acidify the media even if it is detrimental to the growth of the colony, similar to our *Vibrio*. This phenotype may be common among *Vibrio*s because *Vibrio*s naturally come from a marine environment where excreted acids would be diluted in the environment. This phenotype is not witnessed when the WT *Vibrio casei* are near the partner bacteria or the RUSTI negative *Vibrio casei* because the partner bacteria may be degrading the casein proteins in the milk powder of the PCAM media releasing nitrogenous molecules that would raise the pH. How exactly RUSTI relates to this phenotype remains to be determined.

Although we did not characterize the rate of transfer of HGT in the cheese community, this thesis created and implemented a system to study a the ProteoRUSTI ICE in the cheese rind community that had never been done before. This thesis helped develop a system to insert genes into the *Vibrio casei* RUSTI region of the ProteoRUSTI ICE as well as on the chromosome. With the genetic tools designed in this thesis it will be easier to manipulate *Vibrio casei.* Additionally, both the ProteoRUSTI ICE and the cheese rind community are new to microbiology, and nearly all the tools and experiments to study them had to designed and implemented by integrating prior knowledge from other microbes and previous research done in the lab. This thesis has laid the groundwork for the implementation of many other HGT experiments for better characterizing microbial interactions in the cheese microbiome.

Future Directions

This Master's thesis laid the ground work for many follow up experiments to explore horizontal gene transfer of the RUSTI region in the cheese community. A new approach to observing horizontal gene transfer of the ProteoRUSTI ICE would be to sequence the genomes of all the Proteobacteria associated with the Washed Moses Sleeper community. When all of the Proteobacteria in the cheese community are sequenced we would be able to perform genetics in the bacteria and be able to add an additional antibiotic resistant marker. Already in the lab one of the *Psychrobacter sp.* may have a Gentamycin resistance marker on the Tn7 att site. With an antibiotic selection marker on both bacteria we could more easily identify potential HGT events of the ProteoRUSTI ICE from the *Vibrio casei* to the naturally RUSTI negative bacteria on double selection plates. Testing a variety of extraction times ranging the length of the natural aging process of the cheese can also give us insight into if and how the rate of HGT changes throughout the aging process of the cheese. We could also repeat this experiment with other Proteobacteria from other cheese communities because the bioinformatic evidence provides into the proliferation of the RUSTI region throughout a variety of different cheeses.

If this approach becomes to be too challenging, another approach would be to see if horizontal gene transfer of the ProteoRUSTI ICE from the *Vibrio casei* to a model organism such as *E. coli*. Working with a well characterized organism can provide us a variety of insights uncharacterized bacteria cannot. For example, another project in our lab characterized the upregulation and downregulation of various genes in *E. coli* using a combination of RB-TnSeq and RNASeq analyses while the *E. coli* was grown with bacterial and fungal members of the cheese community [35]. By using a model organism, Morin et al were able to characterize what genes and pathways are important to the *E. coli* when grows pairwise with a variety of cheese community members or in a full cheese community [35]. This level of deep understanding cannot be done currently in our cheese bacteria or fungus because they their genomes are not nearly as well characterized as *E. coli*'s. However, a caveat about with working with *E. coli* it is hard to see how the microbes would interact with one another when an outside organism is not around.

If in either HGT experiment were able to see transfer of the ProteoRUSTI ICE is would be interesting to determine what exact elements of the ProteoRUSTI ICE or the RUSTI region makes it such a prolific mobile genetic element. We could use the *Vibrio casei* with RUSTI knockout and see if that affects the rate of transfer at all. We could also knock out individual genes in the RUSTI region such as the phosphonate genes to determine if they are truly vestigial genes from when *Vibrio casei*'s ancestors lived in the ocean, or do they have some role in the cheese environment. This can also help us characterize how important environmental genes are transferred inside microbial communities and what are the most important aspects of those genes for transfer experiments.

MATERIALS

Bacterial Strains

The following strains were used throughout these experiments. The *Vibrio casei* strain JB196 was isolated from a Washed Moses Sleeper cheese from Jasper Hill Farms in Vermont. This strain was engineered in the following ways to generate additional mutants. A Kanamycin resistance gene was inserted chromosomally within the RUSTI region of the ProteoRUSTI ICE. For two additional mutants, a Gentamicin and a Chloramphenicol resistance marker were both inserted in the large chromosome at base pair 1,994,570 of *Vibrio casei.* A *Vibrio casei* double mutant was created by inserting a DsRed fluorescent marker into the same stable intergenic region listed below within the JB196::KmR strain . Several additional cheese isolated strains were isolated from frozen samples of Washed Moses Sleeper cheese rind (isolated 10/26/2010). These include three naturally ProteoRUSTI ICE negative strains: *Psychrobacter, Vibrio sp., and Pseudoalteromonas.* Two different *E. coli* strains were used for cloning purposes, S17-1 and MFD*pir*.

Table 2: Bacterial Strains Used

Strain	Strain notes	Citation
WT *Vibrio casei* JB196		
JB196::KmR	*Vibrio casei* with Kanamycin resistance	
JB196::GmR	*Vibrio casei* with Gentamycin resistance	
JB196::CmR	*Vibrio casei* with Chloramphenicol resistance	
JB196::KmR, DsRed	*Vibrio casei* with Kanamycin resistance and DsRed	
Psychrobacter sp.	Does not contain the ProteoRUSTI ICE. This species has not been sequenced.	
Vibrio sp.	Does not contain the ProteoRUSTI ICE. This species has not been sequenced.	
Pseudoalteromonas sp.	Does not contain the ProteoRUSTI ICE. This species has not been sequenced.	

Table 2 Continued: Bacterial Strains Used		
E. coli S17-1	*thi pro hsdR- hsdM+* Δ*recA* RP4-2::TcMu-Km::Tn7	
MFD*pir E. coli*	MG1655 RP4-2-Tc:: Mu1::*aac(3)IV*- *aphA*- *nic35*- Mu2::zeo *dapA*::(erm-pir) Δ*recA*	Ferrières et al. 2010

Types of media

Strains used were grown as indicated in either LB, BHI, Plate Count Agar with Milk and salt (PCAM), liquid cheese medium, SOC, and cheese curd agar (CCA).

Table 3: Media Used

Media	Amount	Ingredients	Notes
PCAM	1L	5 g Bacto Tryptone, 2.5 g Bacto Yeast Extract, 1 g Dextrose, 1 g Whole Milk Powder, 10 g NaCl, 15 g Bacto Agar	Ensure Whole Milk Powder dissolves
CCA	500mL	50 g frozen cheese curd, 2.5 g xanthan gum, 15 g NaCl, 8.5 g Bacto Agar	Neutralize pH with 10M NaOH

Antibiotics used and the concentrations

When indicated, strains were grown with the following concentrations of antibiotics. Ampicillin at 100 ug/ml and 250 ug/ul, Gentamicin at 10 ug/ml, Chloramphenicol at 50 ug/ml, and Kanamycin at 50 ug/ml.

Primers used

Table 4: Primers Used

Code	Primer Name	Primer Sequence	Notes
1	pGEH13_1 1	GCAGGATATCTGGATTGACGATGTTATTGAGATTCTTCTTTCCATCAG	Amplify upstream RUSTI region (for KanR)
2	pGEH13_1 2	TACCGTCGACCACTTCAGAGCAGATCATGATTAGAGTTTG	Amplify upstream RUSTI region (for KanR)
3	pGEH13_1 3	CTCTGAAGTGGTCGACGGTATCGATAAGCTAGCTTAATTAG	Amplify termination site (for KanR)
4	pGEH13_1 4	GTTCTTCTAACGAGCTCATCGTCCGGGCCG	Amplify termination site (for KanR)
5	pGEH13_1 5	GATGAGCTCGTTAGAAGAACTCGTCAAGAAGGCGATAG	Amplify KanR
6	pGEH13_1 6	CTTTATTGGCCAGAGCGCTTTTGAAGCTCACG	Amplify KanR
7	pGEH13_1 7	AAGCGCTCTGGCCAATAAAGCGCTTTTAGCGCG	Amplify downstream RUSTI region (for KanR)
8	pGEH13_1 8	TATCTAGAACCGGTGGAGCCTTTGGGGTAATCAAGGAATATTCCATTTC	Amplify downstream RUSTI region (for KanR)
9	pGEH14_1	GCAGGATATCTGGATGGAATGACCGCACGTTACTGGTTTGAG	Amplify upstream putative Tn7 region (for AmpR)
10	pGEH14_2	TACCGTCGACAGATCGCTGCCATTTTAGTATGATACTTTAATAC	Amplify upstream putative Tn7 region (for AmpR)
11	pGEH14_3	GCAGCGATCTGTCGACGGTATCGATAAGCTAGCTTAATT	Amplify termination site (for AmpR)
12	pGEH14_4	GCATTGGTAACGAGCTCATCGTCCGGGCC	Amplify termination site (for AmpR)
13	pGEH14_5	GATGAGCTCGTTACCAATGCTTAATCAGTGAGGCACCTAT	Amplify AmpR
14	pGEH14_6	CGTACCGATGCGCGGAACCCCTATTTGTTTATTTTTCTAAATAC	Amplify AmpR
15	pGEH14_7	GGGTTCCGCGCATCGGTACGCTGCCATTTCTAACCC	Amplify downstream putative Tn7 region (for AmpR)
16	pGEH14_8	TATCTAGAACCGGTGTATAAACAATGCACACACAACCTAATACGTTTCTC	Amplify downstream putative Tn7 region (for AmpR)
17	NEW (w/ Gm) pGEH14_4	CGCCACCTAACGAGCTCATCGTCCGGGCC	Amplify termination site (for GmR)
18	NEW (w/ Gm) pGEH14_5	GATGAGCTCGTTAGGTGGCGGTACTTGGGTCGATATC	Amplify GmR (for Tn7 vector)
19	NEW (w/ Gm) pGEH14_6	CGTACCGATGTTGACATAAGCCTGTTCGGTTCGTAAAC	Amplify GmR (for Tn7 vector)
20	NEW (w/ Gm) pGEH14_7	CTTATGTCAACATCGGTACGCTGCCATTTCTAACCC	Amplify downstream putative Tn7 region (for GmR)
21	NEW (w/ Chlor) pGEH14_4	GATTCATTAACGAGCTCATCGTCCGGGCC	Amplify termination site (for CmR)
22	NEW (w/ Chlor) pGEH14_5	GATGAGCTCGTTAATGAATCGGCCAACGCGCG	Amplify CmR (for Tn7 vector)

Table 4 Continued: Primers Used

23	NEW (w/ Chlor) pGEH14_6	CGTACCGATGTTGACATAAGCCTGTTCGGTTCGTAAAC	Amplify CmR (for Tn7 vector)
24	NEW (w/ Chlor) pGEH14_7	CTTATGTCAACATCGGTACGCTGCCATTTCTAACCC	Amplify downstream putative Tn7 region (for CmR)
25	pGTB_1.1_Gm_1	GCAGGATATCTGGATTTAAACCAATCTCGCTCTAAGCGC	Amplify upstream region of neutral site
26	pGTB_1.1_Gm_2	TACCGTCGACGGAAATAAATCAATTTTCAGCCATTCAGACATAACA	Amplify upstream region of neutral site
27	pGTB_1.1_Gm_3	ATTTATTTCCGTCGACGGTATCGATAAGCTAGCTTAATT	Amplify termination site
28	pGTB_1.1_Gm_4	CGCCACCTAACGAGCTCATCGTCCGGGCC	Amplify termination site
29	pGTB_1.1_Gm_5	GATGAGCTCGTTAGGTGGCGGTACTTGGGTCGATATC	Amplify GmR
30	pGTB_1.1_Gm_6	AAATTTTCTATTGACATAAGCCTGTTCGGTTCGTAAAC	Amplify GmR
31	pGTB_1.1_Gm_7	CTTATGTCAATAGAAAATTTTCACTTCACATCAAACCAGAGACTATT	Amplify downstream region of neutral site
32	pGTB_1.1_Gm_8	TATCTAGAACCGGTGGATGATCTTACCAATATTTCGGCGACAGAC	Amplify downstream region of neutral site
33	pGTB_1.1_Cm_4	GATTCATTAACGAGCTCATCGTCCGGGCC	Amplify CmR
34	pGTB_1.1_Cm_5	GATGAGCTCGTTAATGAATCGGCCAACGCGCG	Amplify CmR
35	pGTB 2.1 RFP 1	GAACGAATTGATCGCGCGCACCGTACGTC	removes duplicate
36	pGTB 2.1 RFP 2	CGGTGCTTAACGAGCTCATCGTCCGGGCC	For rfp vector
37	pGTB 2.1 RFP 3	GATGAGCTCGTTAAGCACCGGTGGAGTGACGACC	For rfp vector
38	pGTB 2.1 RFP 4	GTGAAAATTTTCTATTGACAGCTAGCTCAGTCCTAGG	For rfp vector
39	pGTB 2.1 RFP 5	TAGCTGTCAATAGAAAATTTTCACTTCACATCAAACCAGAGAC	For rfp vector
40	pGTB 2.1 RFP 6	CGCCACCTAACACATTTCCCCGAAAAGTGCCACCC	For rfp vector
41	pGTB 2.1 RFP 7	GGGAAATGTGTTAGGTGGCGGTACTTGGGTCGATATC	For rfp vector
42	pGTB 2.1 RFP 8	GGTCTGACAGTTGACATAAGCCTGTTCGGTTCGTAAAC	For rfp vector
43	pGTB 2.1 RFP 9	CTTATGTCAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTG	For rfp vector
44	pGTB 2.1 RFP 10	TGCGCGCGATCAATTCGTTCAAGCCGAGATCGGC	Removes duplicate
45	RUSTI Chk F	GTACGCACGCTCCAGTTAAACTGAG	Checks for the presence of the RUSTI region
46	RUSTI Chk R	ACTGTGCTAATCGGGCTATCAGTAGG	Checks for the presence of the RUSTI region
47	27f	AGAGTTTGATCCTGGCTCAG	To amplify the 16S
48	1492r	GGTTACCTTGTTACGACTT	To amplify the 16S
49	pGP704sacb28_Gib_F	CACCGGTTCTAGATACCTAGGTGAGCTCTGGTAC	For Gibson cloning into pGP704
50	pGP704sacb28_Gib_R	ATCCAGATATCCTGCAGGAATTCCTCGAGACG	For Gibson cloning into pGP704

Vectors created by Gibson Assembly

Gibson Assembly was used to construct of the vectors throughout the experiment. Suicide vectors were created to insert various genes into *Vibrio casei* via allelic exchange. All vectors had an upstream homologous region, a termination site, the insertion gene of interest, the downstream homologous region, and the pGP705*sacB* backbone. The pGP705*sacB* backbone consists of a *sacB* gene, a R6K origin of replication *oriT*, an *oriT*, and an Ampicillin resistance gene. Gibson Assembly was done using the New England Biolabs (NEB) Gibson Assembly Cloning Kit. All vectors were made as instructed from the NEB Gibson Assembly cloning Kit (20 ul of reaction for 50^{o}C for 60 minutes). A BioRad T100 Thermocycler was used to perform the Gibson Assembly. All primers are at 10 uM.

Table 5: Vectors Created

Vector	Amplicons (Primers Used)	Notes
pGTB 1.1 Kan	1-2, 3-4, 5-6, 7-8, 49-50	An allelic exchange vector was created to insert a Kanamycin resistance gene into a neutral site of the RUSTI region inside the RUSTI ICE of *Vibrio casei*
pGTB 2.1 Amp	9-10, 11-12, 13-14, 15-16, 49-50	An allelic exchange vector was created to insert am Ampicillin resistance gene at the putative Tn7 site (upstream: *rarD*. Downstream: *glmS*) of *Vibrio casei*
pGTB 2.2 Gm	1-2, 3-17, 18-19, 20-8, 49-50	An allelic exchange vector was created to insert a Gentamycin resistance gene at the putative Tn7 site (upstream: *rarD*. Downstream: *glmS*) of *Vibrio casei*
pGTB 2.3 Cm	1-2, 3-21, 22-23, 24-8, 49-50	An allelic exchange vector was created to insert a Chloramphenicol gene resistance at the putative Tn7 site (upstream: *rarD*. Downstream: *glmS*) of *Vibrio casei*
pGTB 3.1 Gm	25-26, 27-28, 29-30, 31-32, 49-50	An allelic exchange vector was created to insert a Gentamycin resistance gene at a neutral site (upstream: Glutathione S-transferase. Downstream: hypothetical protein) on the *Vibrio casei* chromosome
pGTB 3.2 Cm	25-26, 27-33, 34-30, 31-32, 49-50	An allelic exchange vector was created to insert a Chloramphenicol resistance gene at a neutral site (upstream: Glutathione S-transferase. Downstream: hypothetical protein) on the *Vibrio casei* chromosome
pGTB 4.1 DsRed	36-37, 38-39, 40-41, 42-43, 44-35, 49-50	An allelic exchange vector was created to insert a DsRed fluorescence gene at a neutral site (upstream: Glutathione S-transferase. Downstream: hypothetical protein) on the *Vibrio casei* chromosome also tagged with a Kanamycin resistance gene in the RUSTI region. The Ampicillin resistance gene in the pGP705*sacB* was also replaced with a Gentamycin resistance gene

METHODS

Plasmid and Strain Construction

Vibrio with Kanamycin resistance gene marker in the RUSTI region in the ProteoRUSTI ICE in Vibrio casei

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 1.1 Kan, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 1.1 Kan vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 4 ul of pGTB 1.1 Kan vector. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37°C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with 100 ug/mL Ampicillin and incubated overnight at 37°C.

Plasmid purification and transformation of MFDpir

The transformed S17-1 *E. coli* were grown overnight in 2 mL of LB medium and 2 uL of Ampicillin (100 mg/ uL) shaking at room temperature.

A Qiagen Miniprep Kit was used to extract the plasmid from the S17 *E. coli.* MFDpir *E. coli* were electroporated with the extracted plasmid as described above, however the media was additionally supplemented with 75 ul of DAP (300 uM).

Mating Vibrio casei with MFDpir

Transformed MFD*pir E. coli* were grown overnight in 2 mL of LB supplemented with 6 uL of DAP (300 uM), and 2 uL of Ampicillin (100 mg/mL). Wild-type *Vibrio casei* were grown overnight in culture tubes with 2 mL of LB. The overnight cultures of MFD*pir E. coli* and the *Vibrio casei* were spun down and 40 ul were spotted at 1:1 and 7:3 ratios on LB-agar supplemented with DAP (300 uM) and incubated at room temperature for 16 hours. The spots were collected and plated onto LB-agar with Ampicillin (100 ug/mL) and incubated for 48 hours at room temperature. Resulting colonies were then re-patched onto LB-agar with Ampicillin (100 ug/mL) and incubated at room temperature overnight before further use.

Counterselection

Vibrio casei merodiploid from the mating was grown overnight in 2 mL of LB. The cells were 10-fold serial diluted 5 times and 5 dilutions were plated on LB agar, LB agar supplemented with 5% Sucrose, and LB agar supplemented with 5% Sucrose with Kanamycin (50 ug/mL) counterselection plates. The LB and the 5% Sucrose plates were used as controls.

Strain is completed

Vibrio casei from the 5% Sucrose with Kanamycin counterselection plates were patched onto LB with Kanamycin (50 mg/ml) selection plates. A PCR was performed on the cells that grew with primers 1 and 8 to confirm the Kanamycin resistance insert in the RUSTI region of the *Vibrio casei* ProteoRUSTI ICE.

Vibrio with Ampicillin resistance gene at Tn7 att site

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 2.1 Amp, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 2.1 Amp vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 4 ul of pGTB 2.1 Amp vector. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37^{o}C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with

100 ug/mL Ampicillin and incubated overnight at 37°C.

Strain was not completed

Explanation mentioned in Results.

Vibrio with Gentamycin resistance genes at Tn7 att site

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 2.2 Gm, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 2.2 Gm vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 4 ul of pGTB 2.2 Gm vector. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37°C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with 100 ug/mL Ampicillin and incubated overnight at 37°C.

Plasmid purification and transformation of MFDpir

The transformed S17-1 *E. coli* were grown overnight in 2 mL of LB medium and 2 uL of Ampicillin (100 mg/ uL) shaking at room temperature.

A Qiagen Miniprep Kit was used to extract the plasmid from the S17 *E. coli.* MFDpir *E. coli* were electroporated with the extracted plasmid as described above, however the media was additionally supplemented with 75 ul of DAP (300 uM).

Mating Vibrio casei with MFDpir

Transformed MFD*pir E. coli* were grown overnight in 2 mL of LB supplemented with 6 uL of DAP (300 uM), and 2 uL of Ampicillin (100 mg/mL). Wild-type *Vibrio casei* were grown overnight in culture tubes with 2 mL of LB. The overnight cultures of MFD*pir E. coli* and the *Vibrio casei* were spun down and 40 ul were spotted at 1:1 and 7:3 ratios on LB-agar supplemented with DAP (300 uM) and incubated at room temperature for 16 hours. The spots were collected and plated onto LB-agar with Ampicillin (100 ug/mL) and incubated for 48 hours at room temperature. Resulting colonies were then re-patched onto LB-agar with Ampicillin (250 ug/mL) and incubated at room temperature overnight before further use.

Strain is not completed

Explanation mentioned in Discussion.

Vibrio with Chloramphenicol resistance genes at Tn7 att site

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 2.3 Cm, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 2.3 Cm vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 4 ul of pGTB 2.3 Cm vector. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37^{o}C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with 100 ug/mL Ampicillin and incubated overnight at 37^{o}C.

Plasmid purification and transformation of MFDpir

The transformed S17-1 *E. coli* were grown overnight in 2 mL of LB medium and 2 uL of Ampicillin (100 mg/ uL) shaking at room temperature.

A Qiagen Miniprep Kit was used to extract the plasmid from the S17 *E. coli.* MFDpir *E. coli* were electroporated with the extracted plasmid as described above, however the media was additionally supplemented with 75 ul of DAP (300 uM).

Mating Vibrio casei with MFDpir

Transformed MFD*pir E. coli* were grown overnight in 2 mL of LB supplemented with 6 uL of DAP (300 uM), and 2 uL of Ampicillin (100 mg/mL). Wild-type *Vibrio casei* were grown overnight in culture tubes with 2 mL of LB. The overnight cultures of MFD*pir E. coli* and the *Vibrio casei* were spun down and 40 ul were spotted at 1:1 and 7:3 ratios on LB-agar supplemented with DAP (300 uM) and incubated at room temperature for 16 hours. The spots were collected and plated onto LB-agar with Ampicillin (100 ug/mL) and incubated for 48 hours at room temperature. Resulting colonies were then re-patched onto LB-agar with Ampicillin (250 ug/mL) and incubated at room temperature overnight before further use.

Strain is not completed

Explanation mentioned in Discussion.

Vibrio with Gentamycin resistance gene at neutral, stable intergenic site

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 3.1 Gm, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 3.1 Gm vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 4 ul of pGTB 3.1 Gm vector. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37^{o}C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with 100 ug/mL Ampicillin and incubated overnight at 37^{o}C.

Plasmid purification and transformation of MFDpir

The transformed S17-1 *E. coli* were grown overnight in 2 mL of LB medium and 2 uL of Ampicillin (100 mg/ uL) shaking at room temperature.

A Qiagen Miniprep Kit was used to extract the plasmid from the S17 *E. coli.* MFDpir *E. coli* were electroporated with the extracted plasmid as described above, however the media was additionally supplemented with 75 ul of DAP (300 uM).

Mating Vibrio casei with MFDpir

Transformed MFD*pir E. coli* were grown overnight in 2 mL of LB supplemented with 6 uL of DAP (300 uM), and 2 uL of Ampicillin (100 mg/mL). Wild-type *Vibrio casei* were grown overnight in culture tubes with 2 mL of LB. The overnight cultures of MFD*pir E. coli* and the *Vibrio casei* were spun down and 40 ul were spotted at 1:1 and 7:3 ratios on LB-agar supplemented with DAP (300 uM) and incubated at room temperature for 16 hours. The spots were collected and plated onto LB-agar with Ampicillin (100 ug/mL) and incubated for 48 hours at room temperature. Resulting colonies were then re-patched onto LB-agar with Gentamycin (10 ug/mL) and incubated at room temperature overnight before further use.

Counterselection

Vibrio casei merodiploid from the mating was grown overnight in 2 mL of LB. The cells were 10-fold serial diluted 5 times and 5 dilutions were plated on LB agar, LB agar supplemented with 5% Sucrose, and LB agar supplemented with 5% Sucrose with Gentamycin (10 ug/mL) counterselection plates. The LB and the 5% Sucrose plates were used as controls.

Strain is completed, but cannot be used

Explanation mentioned in Results and Discussion.

Vibrio with Chloramphenicol resistance gene at neutral, stable intergenic site

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 4.1 DsRed, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 4.1 DsRed vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 4 ul of pGTB 4.1 DsRed. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37°C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with 100 ug/mL Ampicillin and incubated overnight at 37°C.

Plasmid purification and transformation of MFDpir

The transformed S17-1 *E. coli* were grown overnight in 2 mL of LB medium and 2 uL of Ampicillin (100 mg/ uL) shaking at room temperature.

A Qiagen Miniprep Kit was used to extract the plasmid from the S17 *E. coli.* MFDpir *E. coli* were electroporated with the extracted plasmid as described above, however the media was additionally supplemented with 75 ul of DAP (300 uM).

Mating Vibrio casei with MFDpir

Transformed MFD*pir E. coli* were grown overnight in 2 mL of LB supplemented with 6 uL of DAP (300 uM), and 2 uL of Ampicillin (100 mg/mL). Wild-type *Vibrio casei* were grown overnight in culture tubes with 2 mL of LB. The overnight cultures of MFD*pir E. coli* and the *Vibrio casei* were spun down and 40 ul were spotted at 1:1 and 7:3 ratios on LB-agar supplemented with DAP (300 uM) and incubated at room temperature for 16 hours. The spots were collected and plated onto LB-agar with Ampicillin (100 ug/mL) and incubated for 48 hours at room temperature. Resulting colonies were then re-patched onto LB-agar with Chloramphenicol (50 mg/ml) and incubated at room temperature overnight before further use.

Counterselection

Vibrio casei merodiploid from the mating was grown overnight in 2 mL of LB. The cells were 10-fold serial diluted 5 times and 5 dilutions were plated on LB agar, LB agar supplemented with 5% Sucrose, and LB agar supplemented with 5% Sucrose with Chloramphenicol (50 ug/mL) counterselection plates. The LB and the 5% Sucrose plates were used as controls.

Strain is completed, but cannot be used

Explanation mentioned in the Discussion.

Vibrio with Kanamycin resistance gene marker in the RUSTI region in the ProteoRUSTI ICE in Vibrio casei and a DsRed marker into a stable part of the chromosome

Transformation of E.coli S17-1

A drop dialysis was performed on each vector after Gibson assembly. Twenty-five mL of TE buffer was added to a 15 cm petri dish. Four uL of pGTB 3.2 Cm, the Gibson assembled vector, was pipetted onto a Millipore 0.25 um filter floating on the TE buffer. After two hours the dialyzed pGTB 3.2 Cm vector was collected. *E. coli* S17-1 was then transformed with the dialyzed vector by electroporation. The preset bacterial protocol was used from a Bio Red Gene Pulser Xcell. Electroporation was done at 1mm at 1.8 kV. Forty uL of competent S17-1 *E. coli* were electroporated with 10 ul of pGTB 3.1 Gm vector. Immediately after electroporation, cells were recovered with 1 ml of SOC medium and incubated at 37°C for one hour while spinning. After the recovery period the bacteria were plated onto pre-warmed LB agar supplemented with 50 ug/mL Gentamycin and incubated overnight at 37°C.

Mating Vibrio casei with Kanamycin resistance in the RUSTI region with S17 E. coli

S17 *E. coli* with the vector were grown overnight in culture tubes with 2 mL of LB media, and 2 uL of Gentamycin (50 mg/mL). *Vibrio casei* with Kanamycin resistance in the RUSTI region were grown overnight in culture tubes with 2 mL of LB media. The overnight cultures of S17 *E. coli* and the *Vibrio casei* with Kanamycin resistance in the RUSTI region were spotted in 1:1 and 7:3 ratios on top of each other on an LB plate. The cells were grown for 6 hours. After the allocated time the spots were collected and plated onto LB with Kanamycin (50 mg/mL) selection plates.

Counterselection

Vibrio casei merodiploid from the mating was grown overnight in 2 mL of LB. The cells were 10-fold serial diluted 5 times and 5 dilutions were plated on LB agar, LB agar supplemented with 5% Sucrose, and LB agar supplemented with 5% Sucrose with Kanamycin (50 ug/mL) counterselection plates. The LB and the 5% Sucrose plates were used as controls.

Strain is completed

Vibrio casei with Kanamycin resistance in the RUSTI region from the 5% Sucrose with Kanamycin counterselection plates was patched onto LB with Kanamycin (50 mg/ml) selection plates. A PCR was performed on the cells that grew with primers 36 and 43 to confirm the DsRed fluorescence marker in the stable, neutral intergenic site on the *Vibrio casei* chromosome.

HGT Experiment

Identifying the presence of the ProteoRUSTI ICE in the cheese community

To isolate other community members of *Vibrio casei* community, a frozen sample isolated in 2011 from a Washed Moses Sleeper cheese from Jasper Hill Farms was plated onto Plate Count Agar with Milk and Salt (PCAM) treated with cycloheximide antifungal as well as BHI media treated with cycloheximide antifungal. Thirteen bacteria with unique colony morphologies were streaked out on their respective media. To test if any of these bacteria harbored the ProteoRUSTI ICE, a PCR was performed using primers 45 and 46 designed to highlight the inside of the RUSTI region inside the ProteoRUSTI ICE. Two out of the thirteen colony morphologies were positive for the ProteoRUSTI ICE, and the others were all negative for the ProteoRUSTI ICE. All thirteen bacteria were then sequenced using 16SrRNA identification using primers 47 and 48. The two ProteoRUSTI ICE positive bacteria were identified as additional *Vibrio casei* strains. The ProteoRUSTI ICE negative bacteria were identified as Gram-positive and Gram-negative species.

The two Gram-positive species were a *Micrococcus yunnanensis* and a *Glutamicibacter* species. For the Gram-negative species there were a few *Psychrobacter* and *Pseudoalteromonas* species as well as an additional *Vibrio* species. A single *Psychrobacter* and *Pseudoalteromonas* species as well as the additional *Vibrio* species were selected for the horizontal gene transfer experiment.

Growth curve of ProteoRUSTI ICE positive and negative cheese associated bacteria

A growth assay was performed to test for inherit differences between the naturally ProteoRUSTI ICE positive WT *Vibrio casei*, cheese isolated *Vibrio casei* and the naturally ProteoRUSTI ICE negative *Psychrobacter, Vibrio sp., and Pseudoalteromonas*. Each bacterium was grown overnight in 2 mL of LB. The growth was tested by placing 10 uL of each bacterium in individual beveled Erlenmeyer flasks with 30 mL of liquid cheese (2% cheese, 3% salt, 1.5% agar, pH 7). A 10 uL sample was taken from each flask at days 1, 3, 5, and 7. The sample was diluted until 10^{-7} and plated on LB-agar plates. The number of visible bacterial colonies were counted using a counter.

Co-culturing Vibrio casei with Psychrobacter, Vibrio sp., and Pseudoalteromonas for potential HGT events

The double mutant *Vibrio casei* was co-cultured with *Psychrobacter, Vibrio sp., and Pseudoalteromonas* in a 96 well cheese curb agar plate in triplicate. Twenty thousand CFUs were plated on each well at a 1:10 dilution. Each well was extracted after 1, 3, 5, and 7 days. The wells were homogenized and diluted at 10^{-7} then plated onto Plate Count Agar with Milk and Salt (PCAM) and PCAM with Kanamycin (50 ug/mL) selection plates. However, the florescent DsRed in *Vibrio casei* did not fluoresce on the PCAM Kanamycin selection plate. We believe that this is because of the constitutive promotor was not expressing, potentially because of pH stress (3).

The experiment was repeated, but this time the homogenized samples were plated onto LB and LB with Kanamycin selection plates. The double mutant *Vibrio casei* was co-cultured with *Psychrobacter, Vibrio sp., and Pseudoalteromonas* in a 96 well cheese curb agar plate at a 1:10 dilution. Twenty thousand CFUs were plated on each well at a 1:10 dilution. Each well was extracted after 1, 3, 5, and 7 days. The wells homogenized and diluted at 10^{-7} then plated onto LB and LB with Kanamycin selection plates

Phenotype Experiment

Differences in the phenotypes of Vibrio casei RUSTI positive and negative near community partners

A serendipitous discovery was made during the experiment on PCAM. The *Vibrio casei* had a growth increase on PCAM when in the vicinity of the its ProteoRUSTI negative community members. When away from its community members on PCAM the double clone *Vibrio casei* grew into smaller colonies. To test if this phenotype WT *Vibrio casei* was co-cultured with *Psychrobacter, Vibrio sp., and Pseudoalteromonas* on PCAM plates at 1 cm, 3 cm, and 5 cm apart. To test if this phenotype is RUSTI related, *Vibrio casei* was also co-cultured with the RUSTI region inside the RUSTI ICE knocked out (RUSTI negative *Vibrio casei)* with *Psychrobacter, Vibrio sp., and Pseudoalteromonas* on PCAM plates at 1cm, 3cm, and 5cm apart. Pictures were taken at days 3 and 6 to show the difference in growth between WT *Vibrio casei* and RUSTI negative *Vibrio casei*. The pictures of the colonies were taken on days 3 and 6.

REFERENCES

1. Thompson, L.R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Locey, K. J., Prill, R. J., Tripathi, A., Gibbons, S. M., Ackermann, G., Navas-Molina, J. A., Janssen, S., Kopylova, E., Vazquez-Baeza, Y., Gonzalez, A., Morton, J. T., Mirarab, S., Zech Xu, Z., Jiang, L., Haroon, M. F., Kanbar, J., Zhu, Q., Jin Song, S., Kosciolek, T., Bokulich, N. A., Lefler, J., Brislawn, C. J., Humphrey, G., Owens, S. M., Hampton-Marcell, J., Berg-Lyons, D., McKenzie, V., Fierer, N., Fuhrman, J. A., Clauset, A., Stevens, R. L., Shade, A., Pollard, K. S., Goodwin, K. D., Jansson, J. K., Gilbert, J. A., Knight, R., *A communal catalogue reveals Earth's multiscale microbial diversity.* Nature, 2017. **551**(7681): p. 457-463.

2. O'Hara, A.M. and F. Shanahan, *The gut flora as a forgotten organ.* EMBO Rep, 2006. **7**(7): p. 688-93.

3. Chaparro, J.M., Sheflin, Amy M., Manter, Daniel K., Vivanco, Jorge M., *Manipulating the soil microbiome to increase soil health and plant fertility.* Biology and Fertility of Soils, 2012. **48**(5): p. 489-499.

4. Alegria Terrazas, R., Giles, C., Paterson, E., Robertson-Albertyn, S., Cesco, S., Mimmo, T., Pii, Y., Bulgarelli, D., *Plant-Microbiota Interactions as a Driver of the Mineral Turnover in the Rhizosphere.* Adv Appl Microbiol, 2016. **95**: p. 1-67.

5. Radecker, N., Pogoreutz, C., Voolstra, C. R., Wiedenmann, J., Wild, C., *Nitrogen cycling in corals: the key to understanding holobiont functioning?* Trends Microbiology, 2015. **23**(8): p. 490-7.

6. Krajmalnik-Brown, R., Ilhan, Z. E., Kang, D. W., DiBaise, J. K., *Effects of gut microbes on nutrient absorption and energy regulation.* Nutr Clin Pract, 2012. **27**(2): p. 201-14.

7. Lagier, J.C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., Levasseur, A., Rolain, J. M., Fournier, P. E., Raoult, D., *Culturing the human microbiota and culturomics.* Nature Reviews Microbiology, 2018.

8. Wade, W., *Unculturable bacteria--the uncharacterized organisms that cause oral infections.* J R Soc Med, 2002. **95**(2): p. 81-3.

9. Kevin D'hoe, S.V., Karoline Faust, Frédéric Moens, Gwen Falony, Didier Gonze, Verónica Lloréns-Rico, Lendert Gelens, Jan Danckaert, Luc De Vuyst, Jeroen Raes, *Integrated culturing, modeling and transcriptomics uncovers complex interactions and emergent behavior in a synthetic gut community.* bioRxiv, 2018.

10. Han, B., Sivaramakrishnan, P., Lin, C. J., Neve, I. A. A., He, J., Tay, L. W. R., Sowa, J. N., Sizovs, A., Du, G., Wang, J., Herman, C., Wang, M. C., *Microbial Genetic Composition Tunes Host Longevity.* Cell, 2018. **173**(4): p. 1058.

11. Wolfe, B.E., Button, J. E., Santarelli, M., Dutton, R. J., *Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity.* Cell, 2014. **158**(2): p. 422-433.

12. Patrick F Fox, P.L.H.M., Timothy M Cogan, Timothy P Guinee, *Cheese: Chemistry, Physics and Microbiology*. Third Edition ed. Major Cheese Groups, ed. E.A. Press. Vol. Volume 2. 2004, London: Academic Press.

13. Ellison, R.T., 3rd, *The effects of lactoferrin on gram-negative bacteria.* Adv Exp Med Biol, 1994. **357**: p. 71-90.

14. Da Silva, A.S., Honjoya, E. R., Cardoso, S. C., De Souza, C. H., Costa Mde, R., De Santana, E. H., Aragon-Alegro, L. C., *Antimicrobial action of lactoferrin on Staphylococcus aureus inoculated in Minas frescal cheese.* Arch Latinoam Nutr, 2012. **62**(1): p. 68-72.

15. J. A. Heinenmann, R.A.B., *Horizontal Gene Transfer.* In Reviews in Cell Biology and Molecular Science, 2006.

16. Wiedenbeck, J. and F.M. Cohan, *Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches.* FEMS Microbiol Rev, 2011. **35**(5): p. 957-76.

17. Thomas, C.M. and K.M. Nielsen, *Mechanisms of, and barriers to, horizontal gene transfer between bacteria.* Nat Rev Microbiol, 2005. **3**(9): p. 711-21.

18. Ellison, C.K., Dalia, T. N., Vidal Ceballos, A., Wang, J. C., Biais, N., Brun, Y. V., Dalia, A. B., *Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in Vibrio cholerae.* Nat Microbiol, 2018.

19. Jiang, S.C. and J.H. Paul, *Gene transfer by transduction in the marine environment.* Appl Environ Microbiol, 1998. **64**(8): p. 2780-7.

20. Zeph, L.R., M.A. Onaga, and G. Stotzky, *Transduction of Escherichia coli by bacteriophage P1 in soil.* Appl Environ Microbiol, 1988. **54**(7): p. 1731-7.

21. Jain, R., Rivera, Maria C., Moore, Jonathan E., Lake, James A., *Horizontal Gene Transfer in Microbial Genome Evolution.* Theoretical Population Biology, 2002. **61**(4): p. 489-495.

22. Frost, L.S., Leplae, R., Summers, A. O., Toussaint, A., *Mobile genetic elements: the agents of open source evolution.* Nat Rev Microbiol, 2005. **3**(9): p. 722-32.

23. Wozniak, R.A. and M.K. Waldor, *Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow.* Nat Rev Microbiol, 2010. **8**(8): p. 552-63.

24. Johnston, C., Martin, B., Fichant, G., Polard, P., Claverys, J. P., *Bacterial transformation: distribution, shared mechanisms and divergent control.* Nat Rev Microbiol, 2014. **12**(3): p. 181-96.

25. Redfield, R.J., *Evolution of bacterial transformation: is sex with dead cells ever better than no sex at all?* Genetics, 1988. **119**(1): p. 213-21.

26. Meibom, K.L., Blokesch, M., Dolganov, N. A., Wu, C. Y., Schoolnik, G. K., *Chitin induces natural competence in Vibrio cholerae.* Science, 2005. **310**(5755): p. 1824-7.

27. Wang, I.N., *Lysis timing and bacteriophage fitness.* Genetics, 2006. **172**(1): p. 17-26.

28. Bonham, K.S., B.E. Wolfe, and R.J. Dutton, *Extensive horizontal gene transfer in cheese-associated bacteria.* Elife, 2017. **6**.

29. Miethke, M. and M.A. Marahiel, *Siderophore-based iron acquisition and pathogen control.* Microbiol Mol Biol Rev, 2007. **71**(3): p. 413-51.

30. Zakharova, I.B. and D.V. Viktorov, *Conjugative integrative elements (ICEs) of microorganisms.* Mol Gen Mikrobiol Virusol, 2015. **33**(3): p. 9-16.

31. Burrus, V., J. Marrero, and M.K. Waldor, *The current ICE age: biology and evolution of SXT-related integrating conjugative elements.* Plasmid, 2006. **55**(3): p. 173-83.

32. Villarreal-Chiu, J.F., J.P. Quinn, and J.W. McGrath, *The genes and enzymes of phosphonate metabolism by bacteria, and their distribution in the marine environment.* Front Microbiol, 2012. **3**: p. 19.

33. Parks, A.R. and J.E. Peters, *Transposon Tn7 is widespread in diverse bacteria and forms genomic islands.* J Bacteriol, 2007. **189**(5): p. 2170-3.

34. Yoon, S.S. and J.J. Mekalanos, *2,3-butanediol synthesis and the emergence of the Vibrio cholerae El Tor biotype.* Infect Immun, 2006. **74**(12): p. 6547-56.

35. Morin, M., E.C. Pierce, and R.J. Dutton, *Changes in the genetic requirements for microbial interactions with increasing community complexity.* bioRxiv, 2018.