UNIVERSITY OF CALIFORNIA SAN DIEGO

SAN DIEGO STATE UNIVERSITY

VIRULENT AND TEMPERATE VIRUSES: THE 2 FACES OF EVIL

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2021

The dissertation of María Isabel Rojas Mencías is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

University of California San Diego

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2021

DEDICATION

To my mother Inés, my brother Pablo, and my father Raúl for encouraging me to always stay true to myself, sparking my passion for learning, and supporting unconditionally me while I persevere in the pursuit of life adventures.

EPIGRAPH

“Sometimes truth is stranger than fiction”

Brett Gurewitz - Bad Religion

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LIST OF ABBREVIATIONS

BIS: Bacteroidetes injection system
CFU: colony forming unit
IBD: inflammatory bowel disease
PCR: polymerase chain reaction
PFU: plaque forming unit
Phage: a virus that infects Bacteria or Archaea
Prophage: a phage that has inserted its genome into the bacterial genome (lysogen)
RT-LAMP: reverse transcriptase loop-mediated isothermal amplification
RT-qPCR: real-time quantitative PCR
VLP: viral-like particle

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ACKNOWLEDGMENTS

Thank you to my advisors and mentors Dr. Forest Rohwer and Dr. Anca Segall for constantly supporting my learning and growth as a scientist and an individual. Forest opened my eyes to the fascinating worlds of phage and the coral reef and inspired me to dare to think unconventionally. Anca, generously gave me the opportunity to join her lab mid-way into graduate school, taught me the wonders of bacterial genetics and physiology, and was a role model for how to be a woman in science.

Thank you to Dr. Barbara Bailey for supporting my learning and my growth, patiently teaching me how to properly address statistical questions, and encouraging me to be critical about my science.

Thank you to Dr. Juris Grasis for being a supportive mentor, guiding me on how to navigate my first years of graduate school and my post-graduate decisions, and being a great friend.

Thank you to Dr. Nicholas Shikuma for including me in his research group and remaining my mentor beyond my time in his lab, including me in exciting collaborations, and always being open to communicate with candor and honesty.

To Dr. Mark Little for being a committed partner during these stranger-than-fiction years and companion during the lockdown months, inspiring me to marvel at the mysteries of biology and build better hypotheses, and for often highlighting the magic of Baculoviridae.

To my dear friends Brandon Reyes, Dr. Ana Cobián, Dr. Adrian Cantú, Rasha Al Sawad, and Hamza Hajama for the unconditional friendship and love, and showing me how to be a wonderful scientist while remaining fun and kind.

Thank you to the funding agencies and donor that supported me during my PhD: Moore foundation, National Science Foundation, Dr. Jo Ann Lane.

Chapter 1, in full, is published in Marine Disease Ecology (First Edit.), 2020. Oxford Press. Mark Little*, Maria I. Rojas*, Forest Rohwer. The dissertation author was the co-primary investigator and co-first author of this paper.

Chapter 2 is coauthored with Maria I. Rojas, Hamza Hajama, Ashley R. Wolf, Adrian V. Cantu, Ana G. Cobián-Güemes, Mark Little, Michael L. Patnode, Matthew C. Hibberd, Daniel A. Cuevas, Janaki L. Guruge, Barbara Bailey, Jeffrey I. Gordon, Anca Segall, Forest Rohwer. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is published in Jove, 2021. Maria I. Rojas, Giles SS, Little M, Baron R, Livingston I, Dagenais RT, Baer J, Cobián-Güemes AG, White B, Rohwer F 2020. The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is being prepared for submission for publication of the material. Maria I. Rojas, Little M, Giles SS, Baron R, Bloomquist A, Sanchez-Quinto A, Cantú VA, Van der Greer A, Cobián-Güemes AG, Burkeen G, Livingston I, Aquino J, Jon R, Baer J, Dagenais RT, Bailey BA, Vaidya N, Rohwer F. The dissertation author was the primary investigator and author of this paper.

Chapter 5, in full, is published in mSystems, 2020. Rojas MI*, Cavalcanti GS*, McNair K, Benler S, Alker AT, Cobián-Güemes AG, Giluso M, Levi K, Rohwer F, Bailey BA, Beyhan S, Edwards RA, Shikuma NJ. The dissertation author was the co-primary investigator and co-first author of this paper.

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ABSTRACT OF THE DISSERTATION

Virulent and Temperate Viruses: The Two Faces of Evil

by

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Doctor of Philosophy in Biology

University of California San Diego, 2021
San Diego State University, 2021

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This work presents evidence that temperate and virulent viruses impact the ecology, evolution, and health state of their bacterial and eukaryotic hosts across ecosystems including the ocean, human gut, and urban environment.

Temperate viruses can switch between a lysogenic and a lytic lifestyle. In the lytic cycle, phage lyse the host cell shortly after infection to release the progeny into the

environment. In the lysogenic cycle, phage integrate into the bacterial genome to replicate with it during cell division. Here, it is shown how bacteriophage, viruses that infect bacteria, are key drivers of evolution in the marine environment and presents ongoing work on a mechanism that regulates prophage lysogenic-to-lytic the switch in the human gut.

Part of this research was conducted amidst a global pandemic caused by a virulent virus; this inspired the assembly of a workflow that combines community building with molecular biology to identify environmental reservoirs of SARS-CoV-2 and other eukaryotic RNA viruses. Using these methods, SARS-CoV-2 RNA was detected on surfaces of the urban environment and the risk of infection by contact with contaminated fomites in San Diego County was predicted. The viral RNA community present on these surfaces was also characterized.

Lastly, with the aim to uncover novel virus-derived systems where bacteria interact with their eukaryotic host, this thesis presents the identification of a new phage tail-like contractile injection system in *Bacteroides*, that was found prevalent in the gut of healthy adults.

Chapter 1 : Bacteriophage can drive virulence in marine pathogens

Introduction

Marine ecosystems around the globe are in dramatic decline caused by anthropogenic impacts such as pollution, overfishing, climate change, and increasingly prevalent diseases in ecologically important macro-organisms (Bateman et al. Chapter 1, Bojko et al. Chapter 6, Burge and Hershberger Chapter 9). Many marine disease ecologists are reconsidering the strategies used to understand and investigate the etiology of these diseases. Recent studies incorporate the notion that an array of stressors can disrupt natural holobiont communities, leading to a variety of detrimental ecological outcomes often potentiated by microbial pathogenesis (Egan and Gardiner 2016; Morrow et al. 2018; Bateman et al. Chapter 1; Morton et al. Chapter 3). While microbial diseases can be caused by a variety of organisms, most bacteria involved in pathogenicity in marine environments contain horizontally acquired elements that are largely overlooked and play essential ecological and evolutionary roles. These elements are often carried by phage genomes integrated in the genome of the bacterial host. The expression of these prophage-encoded genes can confer pathogenicity and dysbiosis, the latter defined by an unbalanced composition of the host-associated microbial community.

Bacteriophages, or simply “phages,” are viruses that infect bacteria and have the unique ability to undergo one of two lifestyles, lytic or lysogenic. In the lytic cycle, upon infection, the phage uses the machinery of the bacterial host to replicate, synthesize new viral particles, and release its progeny, often killing the bacterial host by cell lysis (Echols 1972). During the lysogenic cycle, the phage genome is integrated into the bacterial genome and its

replication occurs only as part of the normal cell cycle (reviewed in Young 1992). Interestingly, different environmental and cellular cues can trigger the switch from one cycle to the other (Wommack and Colwell 2000).

Through infection, phages have the capability to move host genes between bacteria. When fragments of the host chromosome are packaged within the viral particles, bacterial DNA is shared through infection to a recipient bacterial cell. This horizontal transfer is termed transduction, and it can be the generalized transfer of a DNA sequence from a random position in the bacterial genome, or the specialized transfer of DNA from a specific location in the bacterial chromosome. In either case, the amount of packaged DNA is limited by the size of the viral capsid and hence of the original viral genome (Cui et al. 2014). In addition to phages, other mechanisms of horizontal gene transfer between bacteria occur via the transfer of transposons and plasmids. Transposons are DNA sequences that can jump from one location in the bacterial genome to another, generating gene duplications or truncating genes when their insertion interferes with the coding sequence of the gene. Plasmids, on the other hand, are small DNA molecules, often circular and double-stranded, that can replicate independently of the bacterial genome and can be transferred between bacterial hosts through conjugation.

Phages, generalized transducing agents, transposable elements, and plasmids are some of the major drivers of microbial evolutionary processes and therefore likely play a key role in microbial pathogenicity and dysbiosis. This chapter focuses on the role of phage-encoded elements in the context of the etiology of economically and ecologically relevant marine pathogenesis and dysbiosis. This section also provides a meta-analysis of all known, fully sequenced, marine bacterial host-associated pathogenic and non-pathogenic genomes that

serve as a baseline for understanding how the ecology of horizontal gene-transfer carried out by phage contributes to the evolution of marine pathogens.

Transduction in the marine environment occurs at high rates and has been suggested to have the minimum capacity to move 10^{24} genes from viruses to host per year globally (Rohwer and Thurber 2009). Within the past 5 years, the influence of horizontally acquired genetic elements from viruses has gained traction in the field of marine disease ecology. Recent analyses of the genomes of multiple pathogenic *Vibrio* strains revealed prophage-encoded elements that contribute to the pathogenicity of the bacteria (Weynberg et al. 2015, Box 4.1). These tripartite eukaryote–microbe–phage interactions likely determine many marine disease mechanisms. When a bacterium incorporates in its genome a viral genome, or acquires a prophage through infection, it is called a lysogen. Viral replication and survival occur through bacterial cell division, thus producing more lysogens as progeny. Phages that can initiate their incorporation into the chromosome of the host are known as temperate phages. It is well known that temperate phages have considerable gene repertoires that may enhance bacterial host fitness, and since phages are the most abundant biological entities on the planet, with an estimated 4.80×10^{31} phages on Earth, it is reasonable to predict that these viruses strongly influence the unfolding of marine pathogenesis across a variety of organisms and ecosystems (Cobián Güemes et al. 2016).

While prophages encompass around 25 percent of phages in the global phage gene pool, only forty-one prophage-mediated phenotypes have been observed or experimentally demonstrated (Bondy-Denomy and Davidson 2014; Casjens 2005). Prophages or temperate phages can enhance the fitness of their bacterial hosts in a variety of ways **(Figure 1.1A-E)**, such as (1) conferring metabolic capacities through the acquisition of photosynthetic genes in

Cyanobacteria (Rohwer and Thurber 2009), (2) encoding functional proteins such as anti-CRISPR systems in *Pseudomonas aeruginosa* which allow the bacteria to outcompete other bacteria (Bondy-Denomy et al. 2014), and (3) exclusion factors like the Imm protein of the famous phage T4 in *Escherichia coli* that prevents other phages from infecting the lysogen (Lu and Henning 1994; Obeng et al. 2016). Horizontally acquired mutualistic viruses therefore allow lysogens to broaden their ecological niche space **(Figure 1.1A-E)**. In many instances, the prophage can encode exotoxins that directly affect the host **(Figure 1.1B)**. In addition, some prophage-encoded proteins have been shown to inhibit predation from bacterivorous protists **(Figure 1.1D)**. We are only starting to shed light on the functional roles of integrated phage, but the fact that most bacterial genomes harbor about one to two prophages (Casjens 2003) indicates that these are significant players in a plethora of ecological dynamics. An assessment of temperateness, or the ability to initiate lysogeny or a lyosgenic conversion of viruses in seawater, revealed that within phage communities, 80 percent of the members contain the potential for a temperate lifestyle (Breitbart et al. 2004). Clearly, there are more functions to be discovered considering the high prevalence of lysogeny, where most bacterial genomes harbor multiple prophage and at a maximum have been observed to comprise 20 percent of bacterial genomic sequence space (Canchaya et al. 2003; Casjens 2005).

The role of prophages in disease

In 1951, the first report of phage-mediated virulence was described in the bacterium *Corynebacterium diphtheriae*, the disease-causing agent of diphtheria. When non-virulent strains of *C. diphtheriae* were challenged with phages, the next generation of bacterial

progeny presented a virulent phenotype (Freeman 1951). Since this first discovery of prophage-mediated bacterial fitness enhancements, it has been revealed that a large portion of strain-to-strain differences are due to phage-mediated horizontal gene transfer (Lawrence 2002).

Bacterial strains exhibiting pathogenicity have been shown to contain a higher proportion of phage genes compared to non-pathogenic strains, and currently twelve prophages encoding virulence genes have been discovered among seven relevant bacterial pathogens including *C. diptheriae, E. coli, S. enterica, P. aeruginosa, S. mitis, C. jejuni,* and *V. cholerae* (Busby et al. 2013; Davies et al. 2016). Temperate phages can produce a variety of exotoxins such as cholera, Shiga toxin, and botulism, and these types of prophage-mediated functions are extremely relevant in the case of marine disease pathogenesis. Since the first phage-mediated phenotype was observed in diphtheria, the *E. coli* prophage system has been studied extensively. This *E. coli* prophage encodes Shiga toxin (Stx) whose production is independent of phage lytic activity. Conversely, in the case of *C. diphtheriae*, the production and secretion of the toxin does not require lysis of the lysogen (Holmes 2000).

Some bacterial toxins, many of which contribute to pathogenicity, likely evolved to evade predation from other microorganisms, such as protists **(Figure 1.1D)**. An example of this survival strategy is the aforementioned Shiga toxin, which confers *E. coli* anti-predatorial defense against the bacteriovore *Tetrahymena thermophila* (Lainhart et al. 2009). In the marine environment, a study on *Serratia marcescens* challenged a population of this bacterium against two bacterivorous predators with different feeding mechanism—*Acanthamoeba castellanii*, a surface feeder, and *Tetrahymena thermophila*, a particle feeder—and observed that the *S. marcescens* population became more resistant to the infection by lytic

phages, presumably due to the acquisition of a prophage in their genome (Örmälä-Odegrip et al. 2015). These findings may suggest that predation pressure by bacterivores selects for bacteria carrying prophage in their genomes. These prophages potentially encode for proteins that either are directly toxic to bacteriovores or indirectly deter them from predating on the bacterial host. These phenomena are relevant when considering the ability of pathogens to evade protist predation as well as infection by lytic phages in the marine environment.

Prophages in marine diseases

Understanding the distribution and role of temperate phages in marine bacterial pathogens is of high relevance considering their ability to exhibit superinfection exclusion to other phages targeting the same host range. This is important to consider because it allows for pathogenic lysogens to prevent lytic control (i.e., bacterial death via cell lysis) by other phages naturally present or introduced to the community as phage therapeutics. These dynamics are likely at large in instances of bacterially mediated marine disease and pathogenesis. To test this hypothesis, we searched for predicted temperate phages in publicly available marine bacterial pathogen and non-pathogen genomes from host-associated marine environments on a global scale. This meta-analysis utilized bioinformatic tools to demonstrate significantly higher proportion of prophages in pathogenic than in non-pathogenic marine host-associated bacteria **(Figure 1.2)**.

The results mentioned above were not unexpected since virus-like particles have been identified as playing a role in ecologically relevant marine diseases. For example, putative phage hyperparasites have been described to be associated with the bacterium *Candidatus* Xenohaliotis californiensis (WS-RLO), which causes withering syndrome in abalone

(Friedman and Crosson 2012). These virus-like particles have similar morphology to that of the *Siphoviridae* family of phages, although their genomes have not been sequenced (Cruz-Flores et al. 2016). While the function of these intracellular viruses in pathogenesis is not yet understood, due to their 50-nm size and the pleomorphic traits conferred to bacteria, it is possible that they constitute generalized transducing agents. Furthermore, this system may be an example of how lytic viruses play a role in bacterial pathogenesis.

Evolutionary implications of prophages in marine diseases

Temperate phages are present in 40–50 percent of all known microbial genomes and in twenty-one of thirty known bacterial phyla (Canchaya et al. 2003; Touchon et al. 2016). In addition, lysogen abundance is more prevalent in pathogens and negatively correlated with spacer acquisition across CRISPR-Cas systems in respective host microbial genomes (Touchon et al. 2016). The contribution of phages in structuring microbial communities through predation and lysogeny is apparent, but the need for understanding functional roles in microbial disease ecology is often overlooked. Researchers have only begun to understand the roles of lysogens in nature in either pathogenic or ecological contexts. In this chapter we seek to shed light on what is known about phage-encoded function in bacterial pathogenesis and dysbiosis in marine disease.

While bacteriovore anti-predation conferred by horizontal gene transfer or phage infection has yet to be assessed in marine host-associated pathogenic bacteria, it is likely to explain the selection of systems that can lead to microbial disease in macro-organismal hosts. This process would require receptor-mediated endocytosis (RME), via cell-surface receptors of the eukaryotic host, where the exo-toxins against bacteriovore predation have a secondary

effect on the macro-organismal host. These dynamics should be considered as marine disease ecologists investigate and attempt to address problems and develop or apply therapies in aquaculture and the environment. Here, prophages, the microbiological Trojan Horse, are clearly an important overlooked component of disease etiologies. Opportunely, genomics and bioinformatics advance rapidly and allow for the evaluation of the contribution of prophage-encoded traits to the global pool of marine diseases (Frasca et al. Chapter 11, this volume).

The events associated with the acquisition of a potentially mutualistic phage require lytic-to-temperate switching during an active infection event and this is defined as lysogeny. Lysogeny can introduce novel phenotypes to the bacterial host via gene expression (Brüssow et al. 2004). Recent metagenomic-based studies on lytic-to-temperate switching dynamics in marine ecosystems demonstrate that at high microbial abundances and within certain environments, there is a higher presence of integrase and excisionase genes, suggesting an ecological lytic-to-temperate switch in these viral communities (Knowles et al. 2016). Integrases are phage-encoded proteins that enable viruses to incorporate their genome into bacterial chromosomes, while excisionases allow prophages to exit the chromosome during induction to the lytic cycle. Therefore, the relative level of integrase and excisionase genes in viral communities suggests the functional potential for lysogeny (Knowles et al. 2016). Mathematical modeling further demonstrates that temperate phage lifestyles can be more prevalent under environmental conditions that favor bacterial growth (Maslov and Sneppen 2015). Although marine bacterial pathogeneses are currently increasing at unprecedented rates, these are relatively isolated phenomena when considering the spatial scale at which organisms exist in any given ecosystem.

The life history of temperate phages can shed light on the understanding of how prophages are distributed across bacterial phyla. The abundance of bacterial mechanisms that protect against phage infection, such as CRISPR-Cas systems, has been negatively correlated with the presence of prophages in a global dataset of bacterial genomes (Touchon et al. 2016). In this study, the bacteria with minimal doubling time, meaning those with genomes with the fastest growing capability, were strongly correlated with the occurrence of lysogeny (Touchon et al. 2016). This finding adds further support to the observed increases of lysogeny in certain environments, as described in the Piggyback-the-Winner (PtW) model, where lysogen occurrence is correlated with higher microbial abundance (Knowles et al. 2016). In contrast, an alternative scenario can take place when virion numbers increase with bacterial abundance via lytic activity, and these dynamics are explained by the "Kill-the-Winner" model (Thingstad 2000). In either case, bet-hedging strategies of allocating bacterial resources to viral lytic production and others to lysogeny greatly influence ecological dynamics where phage impact bacterial growth and function (Morton et al. Chapter 3, this volume).

Meta-analysis of prophage-encoded functions

Prophage-encoded functions have yet to be assessed in pathogenic and dysbiotic host-associated system dynamics. This meta-analysis covers a total of eighty-nine publicly available complete marine genomes of host-associated bacteria, with sequences coming from thirty-two non-pathogenic and fifty-seven pathogenic isolates found in algae, invertebrates, and vertebrates (Klemetsen et al. 2018). The reference database used is the most extensive marine bacterial genome resource to date and includes comprehensive metadata on the source of the isolates, organismal pathology at the species level, bacterial genome length, data on

encoded proteins per genome, and many other useful metadata (Klemetsen et al. 2018). The results of our analysis revealed a significantly higher abundance of prophage in marine pathogens when compared to non-pathogens **(Figure 1.2)**.

A comparison of the number of hits to prophage-encoded genes between pathogenic and non-pathogenic host-associated marine bacteria revealed a significantly higher level of prophages per genome across two different normalization methods **(Figure 1.2)**. The log abundance of prophage hits was divided by either the respective bacterial genome length **(Figure 1.2A)** or the predicted number of bacterial-encoded proteins in the respective genomes **(Figure 1.2B)**. Both normalization methods revealed a significantly higher number of prophage genetic elements in the pathogenic host-associated bacteria **(Figure 1.2)**.

To understand the functional relevance of these predicted prophage-encoded elements, we analyzed the results with a computer program (PhiSpy) designed to find prophages in bacterial genomes. The predicted prophage-encoded gene hits were annotated against the SEED project subsystems database, which integrates the data generated by the Rapid Annotation of microbial genomes using Subsystems Technology (RAST), to gain insight into what known functions may be prophage-encoded in these ecologically relevant marine pathogens. The SEED database uses a system that organizes families of functional genes into categories. To determine which functions were positively enriched in prophage-encoded genes carried by pathogenic bacteria, the relative abundance of subsystems was averaged, and the averaged abundance of non-pathogenic bacteria was subtracted from that of pathogenic bacteria **(Figure 1.3)**. The three highest positively enriched subsystems in pathogens were carbohydrate utilization, membrane transport, and virulence. The observation of carbohydrate utilization and membrane transport suggests that prophages within pathogenic marine bacteria

encode other functions, beyond virulence factors, that are ecologically interesting (e.g., fitness enhancement), and that have a direct or indirect effect on host disease. Interestingly, ~ 50 percent of the prophage hits did not match to any known function in the SEED subsystems database, which confirms that there is still much more to learn about these ecologically relevant prophages and prophage-encoded elements. The results suggest PtW dynamics, where the functional genes acquired via lysogeny would enable these pathogenic strains to outcompete the natural holobiont bacterial community members and ultimately lead to diseased states.

The tripartite eukaryote–microbe–phage dynamics discussed in this chapter are essential for marine biologists to consider when trying to understand the etiology and ecology of marine infectious diseases. While this chapter considers all fully sequenced host-associated marine bacterial pathogens at a more general level across global scales, it offers a baseline for future targeted studies on marine bacterial pathogenesis and prophage-encoded phenotypes. Future approaches include determining the specific protein or function that these genes encode to predict their role in fitness, pathogenicity, metabolism, competition, and microbial as well as ecological dynamics. This knowledge could inform researchers and clinicians when designing strategies to enrich desired bacterial traits, for instance to reverse dysbiosis in different ecosystems or directly beat pathogenesis.

Case study: The role of prophage in a coral pathogen

Vibrio coralliilyticus is a bacterial pathogen implicated in coral and oyster disease. All five documented strains of *V. coralliilyticus* harbor at least one or multiple prophages and are implicated in diseases of the corals *Pocillopora damicornis*, *Montipora aequituberculata*, and

Acropora cytherea, and of the Pacific oyster *Crassotrea gigas*. These bacterial strains are associated with different pathogenic phenotypes across coral species, such as bleaching and white syndrome (WS) (Kimes et al. 2012; Ushijima et al. 2014). Traditionally, 16S rDNA is used as taxonomic marker gene for bacterial species-level identification; however, the field of metagenomics and whole-genome sequencing is expanding the ability to analyze these genomes and understand the functional capacity of horizontal gene transfer between prophages and bacteria. Bioinformatic analyses show that *V. coralliilyticus* can carry integrated in its genome a complete prophage that codes for a zonal occludens toxin (ZOT) (Rohwer and Thurber 2009; Weynberg et al. 2015). This exotoxin gene has striking homology to that of *Vibrio cholerae* prophage-encoded cholera toxin (CTX) gene. *Vibrio cholerae* infection in humans causes cholera, an acute diarrheal illness that results from the incorporation and expression of the prophage CTXφ in the bacterial genome (Waldor and Makalanos 1996). This homology suggests that ZOT could play a role in coral and oyster disease through a mechanism similar to that of CTX **(Figure 1.4)**.

Summary

1. Phages, the viruses that infect bacteria, can confer functions to the bacterial host that contribute to pathogenicity and dysbiosis through the lysogenic lifestyle.
2. Bacterial pathogens in the marine environment contain higher abundances of prophages in their genome than non-pathogenic bacteria.
3. A comparison of prophage genetic content between marine pathogens and non-pathogens revealed that pathogen associated prophages are enriched in genes encoding for carbohydrate metabolism, membrane transport, nitrogen metabolism, virulence, and others.

4. Horizontally acquired prophage-encoded DNA regions may play a large role in the ecology and evolution of marine diseases, due to the functions they confer.
5. Future studies on the bacteria that are associated with diseases should examine the regions of prophages in the genome for insights into the etiology and ecology of pathogenicity.
6. These approaches are relevant to non-genome associated datasets (e.g., metagenomic data), and much is to be discovered about the ecology and evolution of prophages in diseases as these types of analyses become better explored and implemented

Figures

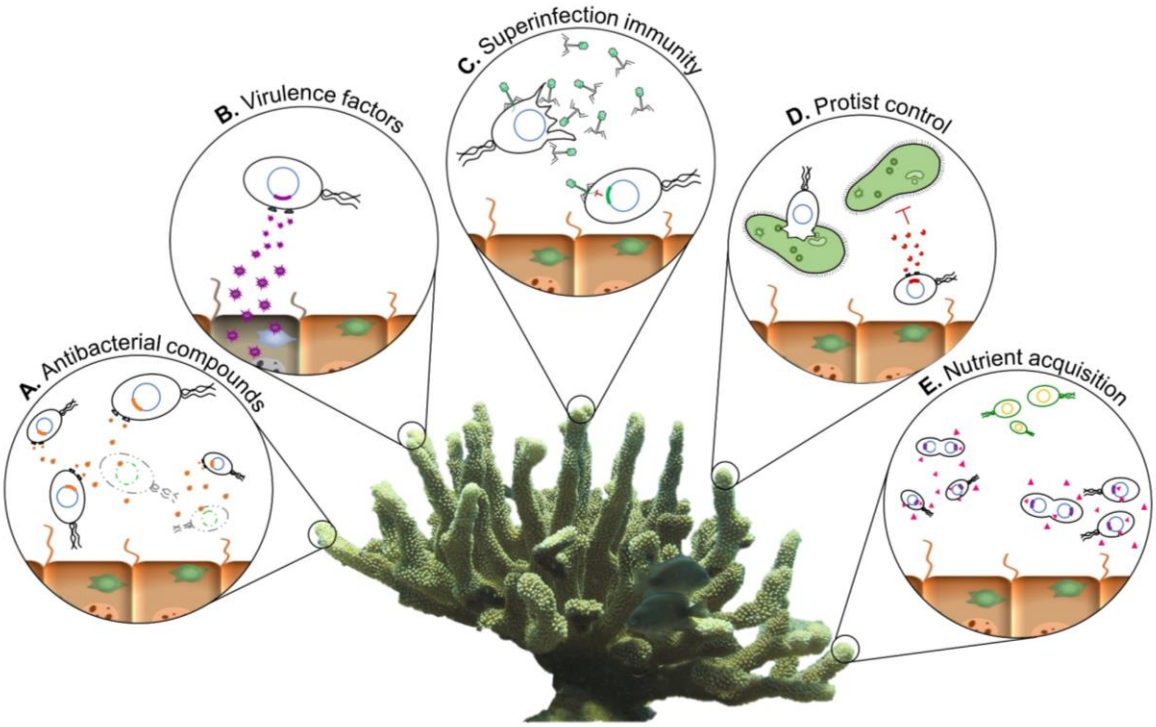

Figure 1.1 Conceptual overview of genes encoded by temperate phages that enhance lysogen fitness in ecologically relevant marine holobionts. **(A)** Prophage-encoded proteins that inhibit competing bacteria and enable expansion of the lysogen's niche space. **(B)** Prophage-encoded virulence factors that directly affect the host. **(C)** Superinfection immunity prevents lytic control of lysogens by lytic phages. On mucosal metazoan epithelium the progeny of spontaneous prophage inductions can cause lytic infection in competing commensal or pathogenic non-lysogens (Barr et al 2013; Silveira and Rohwer 2016). **(D)** Prophage-encoded proteins inhibit predation by unicellular protists that could have a negative secondary effect on the multicellular host. **(E)** Prophage-encoded genes that allow bacteria to expand their metabolic repertoire and niche.

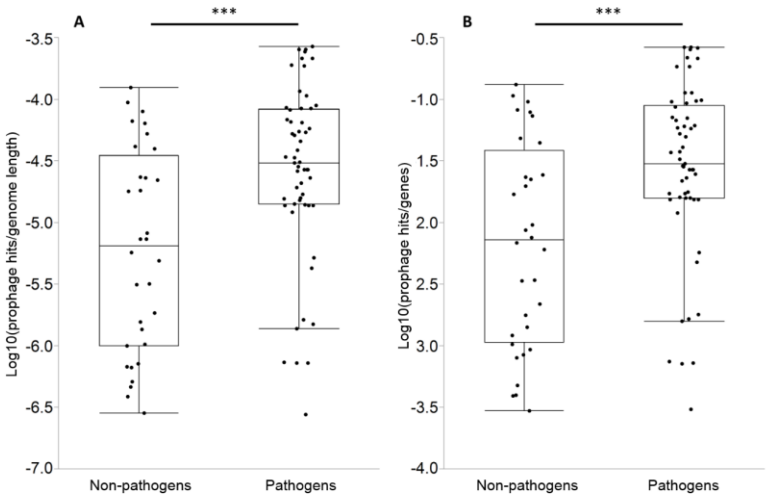

Figure 1.2 Prophage gene abundance in publicly available host-associated bacterial genomes in marine ecosystems. **(A)** Comparison of number of hits to predicted prophage-encoded genes in marine bacterial genomes normalized by the respective genome length for host-associated non-pathogens and pathogens. **(B)** Comparison of number of hits to predicted prophage-encoded genes in marine bacterial genomes normalized by total number of bacterial genes in the respective non-pathogen or pathogen classification. Data were accessed from public databases in March 2018. *** $p \leq 0.001$; two-sample Wilcoxon test.

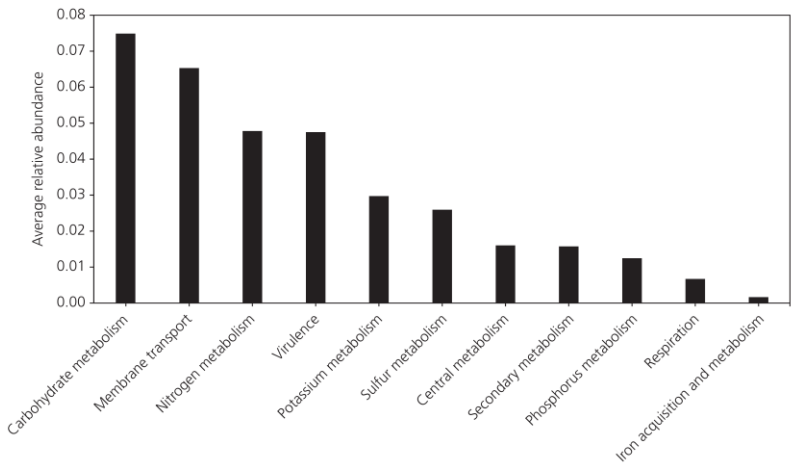

Figure 1.3 Subsystems level 1 functional profiles of prophage-encoded functions in publicly available host-associated bacterial genomes between non-pathogens and pathogens. Average abundance of subsystem level 1 genes of pathogens relative to the corresponding genes in non-pathogenic bacteria. Data were accessed from public databases in March 2018.

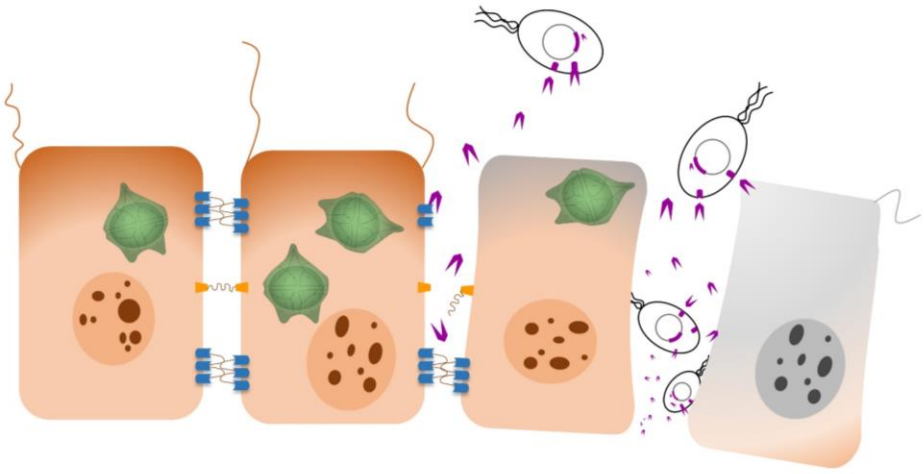

Figure 1.4 Prophage-encoded ZOT toxin of *Vibrio coralliilyticus* disrupting intercellular occluding junctions (tight junctions) that maintain integrity of epithelium during coral infection and disease.

Acknowledgements

Chapter 1, in full, is published in Marine Disease Ecology (First Edit.), 2020. Oxford Press. Mark Little*, Maria I. Rojas*, Forest Rohwer. The dissertation author was the co-primary investigator and co-first author of this paper.

I would like to thank Taylor O'Connell for developing the bioinformatic pipeline used to assess the genomes of interest for this chapter. This research was sponsored by the GBMF Investigator Award 3781.

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Chapter 2 : The stringent response as a regulator of lysogenic-to-lytic switch

Abstract

The human gut microbiome is composed of a variety of bacteria many of which perform essential metabolic functions. One such bacterium is *Bacteroides cellulosilyticus* WH2, a commensal symbiont of the human gut necessary for the breakdown of otherwise indigestible polysaccharides of the human diet. Here, prophage induction experiments were performed *in vitro* in the lysogen *Bacteroides cellulosilyticus* WH2. An INSeq library was screen by treatment with a known prophage inducer to search for candidate genes with regulatory function in the lysogenic-to-lytic switch. The results showed that mutations in the hydrolysis domain of the stringent response gene *spoT* are associated with a lower rate of prophage induction. SpoT encodes for a bifunctional protein that synthetizes ppGpp and pppGpp from GDP and GTP, respectively. To confirm that mutations in *spoT* increase the frequency of lysogenization, prophage induction experiments were conducted in *spoT* mutants of a *Salmonella* Thyphimurium LT2, a study organism for the stringent response. A relative quantification of metabolites involved in the response showed that ATP may be an important driver of the differences in prophage induction observed in the wild type versus the mutant. Lastly, the synthesis, hydrolysis, and regulation domains of *spoT* gene were searched for in a large database of publicly available predicted-complete-prophage genomes. This work aims to better understand the mechanism of prophage induction in *B. cellulosilyticus WH2* and *Salmonella* Typhimurium LT2, and the presence of stringent response domains within prophage genomes of bacterial isolates.

Introduction

Phages are the most abundant biological entity in the planet. Temperate can switch between two lifestyles, the lytic and the lysogenic cycle. In the lytic cycle, after infecting the host phage replicate, package their genome, and release their progeny, often killing the bacterial cell. In the lysogenic cycle, phage introduce and maintain their genome into the bacterial chromosome and replicate with their host. Prophage can shape bacterial populations by conferring fitness advantages to the bacteria or by directly depleting a fraction of the host population. The mechanism regulating the switch has mainly been described through the bacterial response to DNA damage. There is evidence that the products or other physiological responses play a regulatory role on the switch. One such metabolite is ppGpp, an alarmone synthetized in the stringent response to amino acid, fatty acid, and carbon starvation. Little is known about the rules that govern the switch regulation. The lack of knowledge about these factors is a significant barrier to understanding how phage shape bacterial population dynamics.

The mechanism of prophage induction has mainly been studied *in vitro* using Mitomycin-C (MMC), an antibiotic and intercalating agent that triggers the SOS response in bacteria (Matson et al. 2005; Stanton et al. 2008). When the bacterial protein RecA recognizes ssDNA it triggers the autoproteolytic cleavage of the cI phage repressor, which has similar structure to LexA, the SOS response repressor (Shearwin, Brumby, and Egan 1998; Walker 1984). Cleavage of the cI repressor in turn activates the lytic cycle of λ-like prophages by allowing the RNA polymerase to access promoters of phage replication and structural genes (Roberts and Devoret 1983; Walker 1984). In phage λ, integrase gene *int* and the excisionase gene *xis* are expressed during prophage induction and necessary for the excision of the

prophage sequence from the host genome (Ptashne 2004). Like MMC, Carbadox (CX), a mutagenic antibiotic, is a strong prophage-inducing agent (Köhler, Karch, and Schmidt 2000; Stanton et al. 2008). CX has antimicrobial activity against gram-negative bacilli and it is commonly used in animal feed pellets to increase animal growth efficiency (Q. Chen et al. 2008; Looft et al. 2014; Stanton et al. 2008). In *Salmonella* Typhimirium LT2 and *Escherichia coli* respectively, CX has been shown to induce prophage and prophage-encoded genes, like the Shiga toxin (Bearson et al. 2014).

The stringent response was first defined in *E. coli* as the arrest of protein and rRNA biosynthesis in response to depravation of exogenous amino acid required for optimal growth (Stent and Brenner 1961; Gros and Gros 1958; Pardee and Prestidge 1955). Supplementation of a small amount of the required amino acid restores the rate of rRNA synthesis even in the presence of a protein synthesis inhibitor, suggesting a catalytic role of amino acids in RNA synthesis (Gros and Gros 1958; Pardee and Prestidge 1955; Stent and Brenner 1961). Shortly after starvation, there is an increase in basal levels of the alarmones ppGpp and (p)ppGpp (Irr and Gallant 1969; Cashel 1969). The GDP/GTP pyrophosphokinase (RelA), in limiting amino acid conditions, and Guanosine-3',-5'-bis(diphosphate)3'-pyrophosphohydrolase (SpoT), in response to other environmental cues as phosphate, carbon, iron, and fatty acid depletion, and osmotic shock, catalyze the conversion of GDP and GTP into ppGpp and (p)ppGpp, respectively (Cashel and Rudd 1987). To arrest transcription from promoters of proteins non-essential for survival in starvation, ppGpp lowers ribosome abundance and binds to initiation factors to stall RNA polymerase (RNAP) and suppress transcription initiation (Rasouly, Pani, and Nudler 2017; Potrykus, Wegrzyn, and Hernandez 2002). Thermodynamic characterization shows that ppGpp also regulates protein synthesis by exhibiting higher

affinity for initiation factor IF2 and elongation factor EF-G than the GTPases that compete for binding during translation (Mitkevich et al. 2010). Inversely, ppGpp influences the interaction of RNAP with alternative sigma factors to activate transcription of stress response genes such as antibiotic genes (Inaoka et al. 2002), and virulence factors (Aberg, Shingler, and Balsalobre 2006).

Intracellular levels of ppGpp/(p)ppGpp have been shown to play a role in phage development. Studies have observed that levels higher than basal of ppGpp in rich media favor lysogeny (Potrykus, Wegrzyn, and Hernandez 2004; 2002), and below-detection or high levels of ppGpp favor lysis (Slomińska, Neubauer, and Wegrzyn 1999; Potrykus, Wegrzyn, and Hernandez 2002; Potrykus and Cashel 2008; Potrykus, Wegrzyn, and Hernandez 2004). ppGpp can regulate expression of prophage genes by inhibiting or activating promoters necessary to enter the lytic cycle or to establish and maintain lysogeny. In phage lambda (λ) ppGpp inhibits transcription initiation of replication and structural genes by reducing the stability of the open complex at promoter P_R, for expression of early products of lytic growth (Potrykus, Wegrzyn, and Hernandez 2002). Although ppGpp also decreases the half-life of open complexes at promoter P_{AQ}, it activates transcription of lysogeny genes by increasing the rate of production of competent open complexes (Potrykus, Wegrzyn, and Hernandez 2004).

A critical representative of the Bacteroidetes phylum is *Bacteroides cellulosilyticus* WH2 which is a human gut symbiont. Its genome has been sequenced, and contains a high number of enzymes necessary for the metabolism of a plethora of polysaccharides in the intestine otherwise indigestible by the human host (McNulty et al. 2013). *B. cellulosilyticus* WH2 harbors at least one prophage inducible *in vitro* and *in vivo* under anaerobic conditions (Reyes et al. 2013; Wu et al. 2015). Elucidating the regulation of prophage induction in *B.*

cellulosilyticus WH2 poses an opportunity to clarify the population dynamics of these integral human gut symbionts and the role of prophage in health and disease.

The *S.* Typhimurium *LT2* genome harbors 4 prophages, Gifsy-1, Gifsy-2, Fels-1, and Fels-2, that follow a similar replication mechanism than phage λ (Gunderson et al. 2009; Gemski, Baron, and Yamamoto 1972; Bunny, Liu, and Roth 2002). Gifsy-1 and Gifsy-2 have similar size and genomes, and are present in other *Salmonella* strains; while Fels-1 and Fels-2 are specific to LT2 (Figueroa-Bossi et al. 1997). All 4 prophages are inducible *in vitro* when the host is treated with DNA damaging agents such as MMC and hydrogen peroxide (H_20_2) (Frye et al. 2005). Low concentrations of CX can induce prophage in wild type *S.* Typhimurium *LT2* (Bearson et al. 2014), although CX causes DNA damage (Yoshimura et al. 1981), the mechanism of prophage induction has not been characterized.

This work focuses on understanding the lysogenic-to-lytic switch in temperate phages of the human gut. Here, *B. cellulosilyticus* WH2 and *S.* Thyphimurium LT2 are used to study how the physiological state of the host regulates the switch in non-model systems.

Results

Prophage induction and bacterial growth kinetics in Bacteroides WH2

Bacteria growth curves showed a distinctive decline in the OD_{600} readings for both WT and the transposon (Tn) INSeq library after treatment with CX, while in the control the bacteria reached maximum growth followed by stationary phase (data not shown). To determine the change in abundance of induced prophage, VLPs were stained with SYBR Gold stained VLPs under an epi fluorescence microscope. The morphology of the induced phage was observed by transmission electron microscopy (TEM) **(Figure 2.1)**. In the control, the

counts show that 10^6 (VLP/mL) prophages were spontaneously induced as a result of the growth conditions, while in the CX treated population the induced prophages reached their highest count of 10^8 (VLP/mL) as the population reached its lowest abundance. This shows that CX was effective at inducing the phage in the population of isogenic mutants associated with the decline in bacterial abundance and suggests that the mutants recovered were potentially enriched as a result of the CX treatment.

Candidate genes for regulation of the lysogenic-to-lytic switch in Bacteroides WH2

To identify genes involved in the stabilization of lysogeny, a pool of isogenic transposon mutants was treated with CX, a known prophage inducer. The statistical analysis of significant increase in the abundance of reads for each of the 5,476 recovered genes in the treatment with CX yielded a significant increase in over 1,200 genes. The second most abundant mutant had a Tn insertion in the stringent response gene *spoT* **(Figure 2.2)**. It was picked because it was the most CX-enriched mutant that had a lead to follow in previous literature, its expression levels had shown to affect lysis/lysogeny switching (Potrykus and Cashel 2008).

Wilcoxon Exact test was run using R Studio software to determine which genes were significantly enriched after CX treatment, since relative abundances of the recovered genes follow a non-normal distribution. This also allowed to include "0" values into the statistical analysis. The population before CX-treatment was named "INput" populations and the population recovered after treatment at the equivalent time point in the control was named "OUTput" populations, experiments were performed in four biological replicated. The normalized read abundance of the INput population was subtracted from each of the replicates

of the corresponding OUTput replicate. A one-sided Wilcoxon Exact test was applied to the median of these four values to obtain p-values for a significant increase in gene abundance in the treatment versus the control. Lastly, a false discovery rate (FDR) was applied to correct the *p-values* (*q-value*). The locations of the Tn insertions were mapped against the *spoT*. Tn insertions were higher in the hydrolysis (HD) domain and the region that regulates the bifunctionality of the gene (TGS) **(Figure 2.3)**. Pathways and modules, when available, for the WH2 annotated genes and their corresponding sequences were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

SpoT as a regulator of prophage induction in Salmonella

The stringent response study organism, *S. enterica* serovar Typhimurium LT2 was used to test induction in wild type and the isogenic *spoT*- mutants. Two wild type-mutant pairs were used, a point mutant with a R515H mutation that maps to the HD region expressed at all temperatures, *spoT1*, and a temperature sensitive point mutant with a G833D mutant that maps to the TGS region, *spoT23*(42°C). CX-induced phages were quantified by plaque assay **(Figure 2.4)** and qPCR **(Figure 2.5)**. The ratio of plaque-forming units (PFU) per mililiter in both wild type strains was higher than in the isogenic mutants after CX treatment **(Figure 2.4)**. Fold change of prophage replication of Gifsy-1 and Gifsy-2 was also higher in the wild type strains relative to the corresponding mutants **(Figure 2.5)**. Additionally, bacterial cell quantification was performed by counting colony forming units (CFU) per milliliter over time. A slight difference was observed between the growth rate of spoT+ and spoT1 specially after T=4hr and the beginning of stationary phase, in both the treatment and control groups **(Figure 2.6)**.

Thin-layer chromatography of spoT+ and spoT-

To understand the fluctuation of the products of the spoT gene during induction in the *spoT+* and *spoT1* isogenic pair, intracellular metabolites were extracted at T=10hr using cold formic acid followed by thin-layer chromatography **(Figure 2.7)**. The ratios of the intracellular concentrations of phosphorylated metabolites relative to GTP were calculated. The biggest difference between the wild type and mutant strain was observed in the relative abundance of ATP (Figure 2.8) where ATP was higher in the SpoT+ strain in the treatment group, and higher in the SpoT- strain in the control **(Figure 2.8)**.

Stringent response genes in predicted-prophage genomes

To analyze if the HD and SYNTH domains *spoT* are carried by prophage genomes, a database of 36,488 predicted-prophage genomes from 11,941 bacterial genomes was used. The sequence of the Salmonella LT2 *spoT* gene was mapped against the database using tBLASTx. The search found 79 prophages carrying *spoT,* 40 carried both HD and SYNTH domains and 39 carried only the HD domain **(Table 2.1)**. The bacterial hosts belonged to 7 different orders that included environmental isolates from varied ecosystems, suggesting that the presence of *spoT* in prophage genomes is widely distributed **(Table 1)**.

Discussion

The present work sheds light onto the mechanism that regulated how temperate phage switch from the lysogenic to the lytic cycle in bacteria from the human gut. Here, a gene from the central metabolism *spoT,* was identified as being associated with stability of lysogeny in prophages of *B. cellulosilyticus WH2*. Mutants that survived the induction treatment were

assumed to have a mutation that rendered them unable to induce the prophage or resistant to other effects of CX. An enrichment in mutant with a Tn insertion in the HD domain of *spoT* after treatment with a known prophage inducer, CX, suggested that an accumulation of the (p)ppGpp alarmones could be involved in the switch regulation.

To further study the effect of *spoT* in prophage induction regulation, a *Salmonella* LT2 harboring a point mutation in the HD domain and its isogenic wild type strain were treated with CX. Consistently, the wild type showed higher prophage induction than the mutant measured by plaque assays, that recovered the four phage present in *Salmonella* LT2 (data not shown), and qPCR for Gifsy-1 and Gifsy-2. However, slight differences in the growth rates of the isogenic strains may explain these observations and a mechanistic mathematical model is needed to address this concern.

A deeper look into the effects of the *spoT1* mutation in the central metabolism of the cell led to the quantification of intracellular. The (p)ppGpp alarmones were expected to be higher in the mutant since the mutation maps to the HD domain and this mutants with a Tn in this region were enriched in the *Bacteroides* system as well. Nevertheless, the differences in ppGpp and ppGpp between wild type and mutant were small and it was ATP that was observed to be significantly higher in the wild type after prophage induction. The system presented here, potentially captured the competition for intracellular GTP and ATP where, cleavage of the CI repressor requires ATP (Ptashne 2004), while the stringent response requires GTP to synthesize ppGpp in an equally ATP-dependent matter. Hence, we hypothesize that it is ATP that has the highest effect on the stability of lysogeny in the mutant and to deconvolute the kinetics of reactions where these metabolites are involved, future

efforts to repeat these experiments with sampling time points indicated by the mechanistic model are needed.

Lastly, the presence of *spoT* domains in predicted-prophages across bacterial orders from a wide variety of ecosystems, is not surprising given the ubiquity of the stringent response domains across the three domains of life (Atkinson, Tenson, and Hauryliuk 2011). Whether prophages carry these to control their lysogenic-to-lytic switch remains unknown.

Methods

Bacteria cultures

The Bacteroides cellulosilyticus WH2 wild type strain and INSeq Tn mutant library were obtained from the Laboratory of Dr. Jeffrey Gordon at the Center for Genome Sciences & Systems Biology (CGS) at Washington University in Saint Louis (WUSTL). The library contains >90,000 isogenic mutants, each with only one Tn insertion per genome (Reyes et al. 2013; Wu et al. 2015). The wild type and the INSeq library stocks were subcultured at a 1:4 dilution in TYG media overnight at 37ºC inside an anaerobic chamber. Each of these seed cultures were subcultured at 1:500 dilution into two new cultures, one for the treatment and the other for the control. Once these two daughter cultures reached mid-exponential phase ($OD_{600}\approx0.4$), they were diluted 1:10 and dispensed into 25mL crimp tubes. After cultures were dispensed, they were taken out of the anaerobic chamber to continue bacteria growth in an incubator at 37°C. At each corresponding sampling point, crimp tubes from each control (n=4) and treatment (n=4) batches were processed and served as biological replicates.

Prophage induction experiments

The prophage induction assays were performed with CX, an antibacterial and mutagen/carcinogen (Allen et al. 2011; Looft et al. 2014; Stanton et al. 2008). The inducer was tested at a sublethal concentration (4 ug/mL). DNase-free sterile water was used as a control. CX or water was anaerobically added to the corresponding culture tubes at mid-exponential phase (OD_{600}~0.4). When bacterial growth reached stationary phase in the first induction experiment, each of the remaining culture tubes were anaerobically subcultured to fresh media at a 1:10 dilution. The second induction experiment was performed at the mid-exponential growth phase (OD_{600}~0.4). At the final sampling point of each of the induction assays, four additional replicates of each control and treatment, were saved in 15% glycerol at -80°C for future studies. The bacteria samples were centrifuged at 4000x for 20 minutes. Bacteria pellets from wild type samples were stored at -80°C for future experiments. Bacterial pellets from INSeq library samples were stored at -20°C for downstream DNA extraction. Supernatant was saved for phage processing.

Prophage induction in stringent response mutants

Induction experiments were performed in *Salmonella* Thyphimurium LT2 isogenic mutants for *spoT*, *relA*, and *gppA* (Rudd et al. 1985) using different prophage and stringent response inducing agents: 4 μg/mL of CX, an antibiotic and genotoxic and other unknown modes of action); 1 μg/mL of serine hydroxamate (SHX), a serine analogue, and ppGpp/(p)ppGpp inducer; 0.5% H_2O_2 (oxidative stress); 2 μg/mL of MMC, as positive control for genotoxic stress; and H_2O, as control for spontaneous induction.

Induced Prophage Quantification

Supernatant from centrifuged samples were filtered twice through a 0.2µm EMD Millipore Steriex™ pore diameter filter, followed by two SM washes on an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-100 membrane, to clean phage lysate from any remaining bacteria or bacterial debris. Viral-like particles (VLPs) were stained with SYBR Gold and counted in an epifluorescence microscope. Plaque lysates used cured hosts to determine proportion of induced Gifsy-1, Gifsy-2, Fels-1, Fels-2. Relative quantification of *attB* and *attR* sites in lysates after treatment was performed previously reported primers and qPCR conditions Gunderson, 2009.

INSeq library sequencing data analysis

Library Preparation of the samples followed the protocol for multi-taxon INSeq libraries, sequencing was performed after digestion with MmeI of the fragment containing the Tn sequence at 16-17bp of flanking chromosomal DNA, followed by sample-specific adaptor ligation and final enrichment of the target sequence with linear PCR (Wu et al. 2015). Sequencing was performed on an Illumina HISeq platform at the Genome Technology Access Center at Washington University in St. Louis. Tn insertions were trimmed-out of raw reads, reads were separated by samples bar code, only reads mapping to the first 80% of the inserted gene were kept and were normalized by a scale factor of 1 million/(total filtered reads in a given sample) **(Figures 1, S1)**. The change in abundance of isogenic mutants as a result of the CX treatment was calculated comparing the "INput" population, defined as the sampling point before induction, and the "OUTput" population, defined as the time point after the population

growth curve recovered. A Wilcoxon Exact statistical analysis was performed to identify the isogenic mutants that were significantly enriched after the CX treatment.

Relative quantification of stringent response metabolites

To determine relative abundance of (p)ppGpp, ppGpp, GTP, and ATP, one-dimension thin layer chromatography was performed according to previously described methods (Bochner and Ames 1982; Rudd et al. 1985; Orchard, Rostron, and Segall 2012; Sivapragasam and Grove 2016; Schneider, Murray, and Gourse 2003) with wild type and stringent response mutants of *S. Typhimurium* LT2 and *Bacteroides* WH2. Low phosphate modified-MOPS media was prepared according to Bochner, 1982, and Davis, 1980. Cultures were labeled with 30 μCi/mL of ^{32}P. Inducers were be added at early exponential phase, at the following concentrations: 4 μg/mL of CX, 1 μg/mL of SHX, 2 μg/mL of MMC. A phosphorimager was used to visualize and compare the intensity of ^{32}P spots in the different strains and treatments.

Predicted prophage-encoded genes in bacterial genomes

In a previous study (Kang et al. 2017) 11,941 bacterial genomes were downloaded from the SEED database. The prophage-predicting software PhiSpy recovered 36,488 predicted prophages. tBLASTx was used to search for stringent response and associated genes in the predicted prophages **(Figure S2)**. Initially, the *S. Typhimurium LT2 spoT*, *relA*, *gppA*, *cya*, and *dksA* were used as query. A query was run as a single database to avoid redundancy in hits to *spoT* and *relA*, because these genes have highly conserved HD and SYTH domains.

Figures and Tables

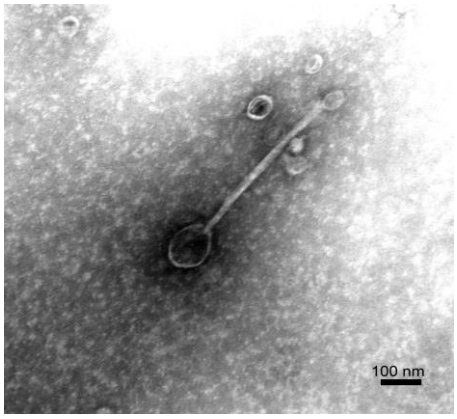

Figure 2.1 Image of Carbadox-induced *B. cellulosilyticus* WH2 prophage.Image captured in a transmission electron microscope (TEM). Morphology resembles that of phage λ

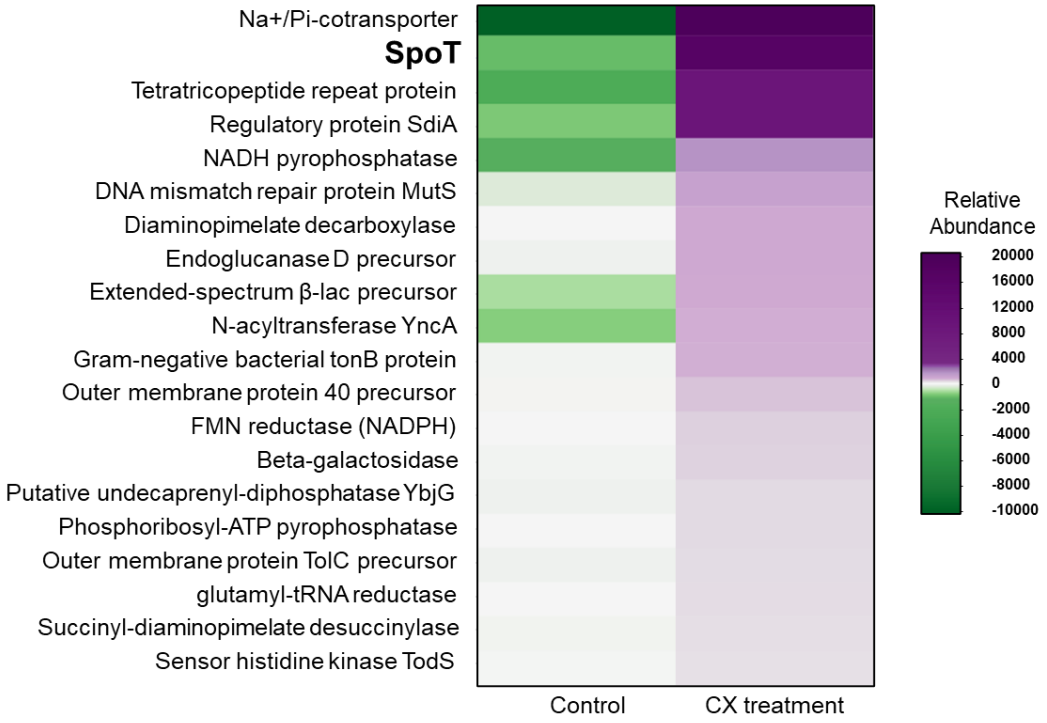

Figure 2.2 Relative abundance of mutants after CX treatment.Genes with Tn insertion in enriched mutants (purple) and depleted mutants (green). Arrow indicates one of the most CX-enriched Tn insertion in the stringent response gene *spoT* of *B. cellulosilyticus* WH2. Reads were normalized by a scale factor of 1million/(total filtered reads in sample) and analyzed with a one-sided (treatment-control) Wilcoxon Exact tests, FDR, q-value<0.05.

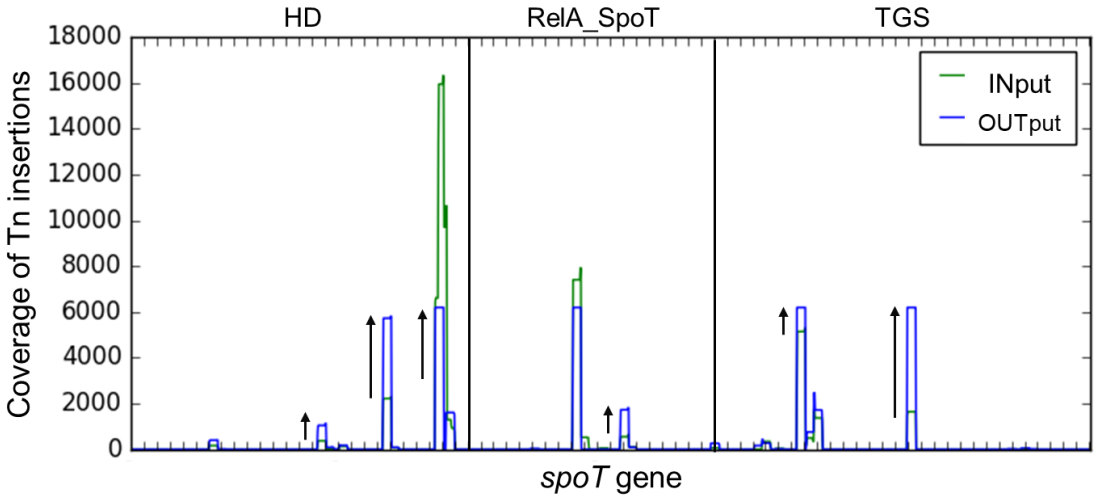

Figure 2.3 Location of Tn insertions along the *spoT*.Coverage plot of region flanking the Tn insertion in mutants before CX-induction (INput) and after cell growth recovered (OUTput). Hydrolysis domain (HD), synthesis domain (RelA_SpoT), and regulation regulatory region (TGS).

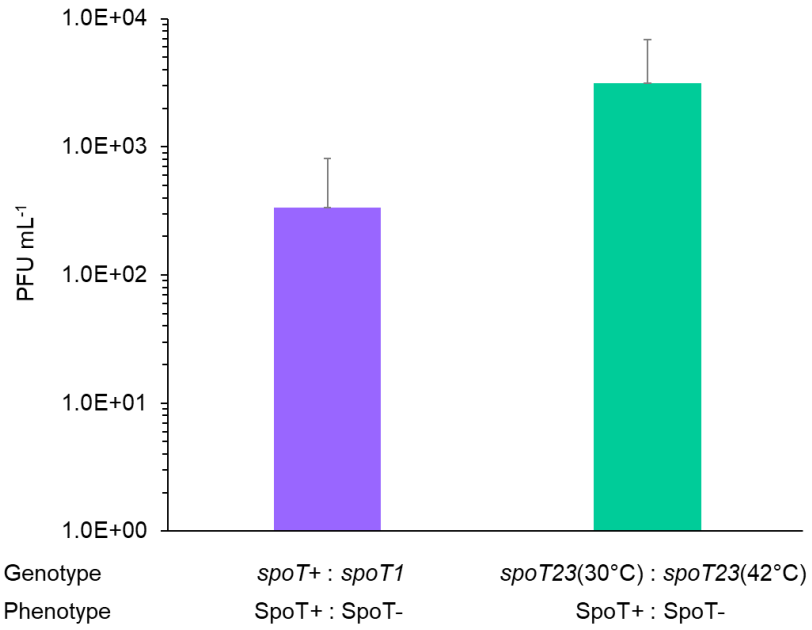

Figure 2.4 Ratios of induced prophage in wild type relative to *spoT* mutant. Counts from plaque assays of induced prophage after treatment with CX (4μg/mL) in wild type LT2 and various stringent response isogenic mutants.

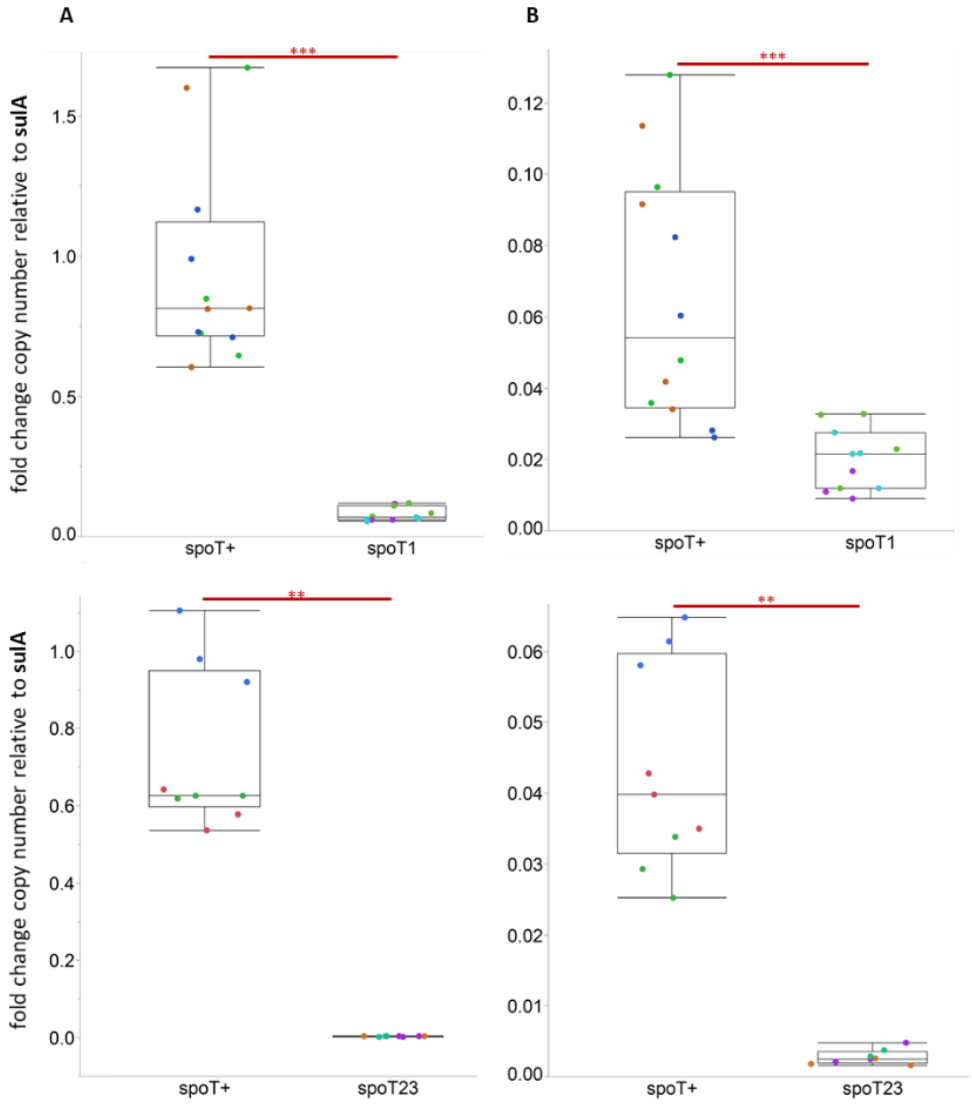

Figure 2.5 Fold change of prophage gene replication.Counts of Gifsy-2 **(A)** and Gifsy-1 **(B)** *attB* sites in isogenic *spoT* mutants (*spoT*1, *spoT*23) and wild type strains (*spoT*+) normalized by a single-copy-number gene (*sulA*). Experiment performed with 5 biological replicates. *p-value* <0.001, measured by Wilcoxon exact.

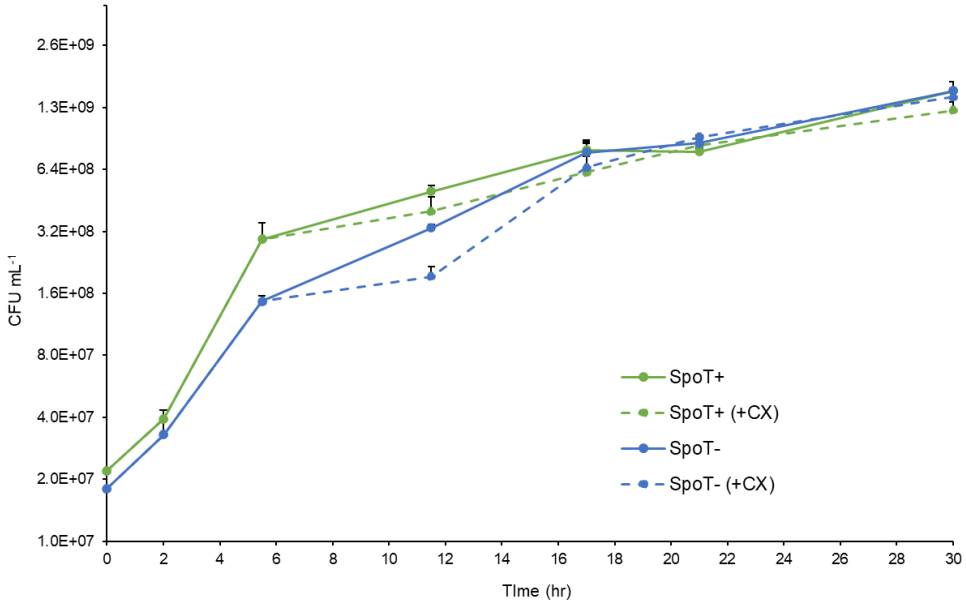

Figure 2.6 Cell growth of *Salmonella* LT2. Isogenic pair *spoT*+ (green) and *spoT1* (blue). Carbadox treatment (dotted lines) applied at T=5.5hr. Curves are average of three biological replicates.

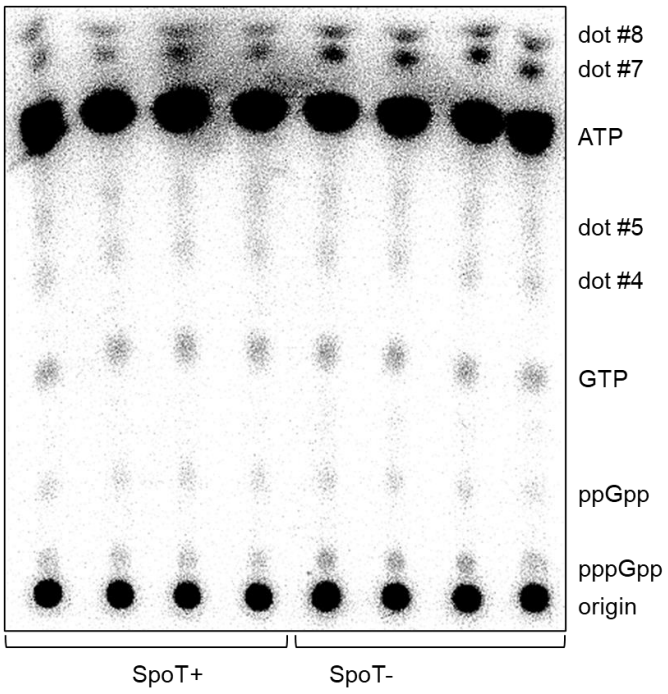

Figure 2.7 Thin-layer chromatography membrane of ^{32}P labeled metabolites.Metabolites extracted from four biological replicates of *Salmonella* LT2 *spoT*+ and *spoT*- at T=10hr. Metabolites labeled "dot" are species that appeared in the TLC membrane image but have not known identity.

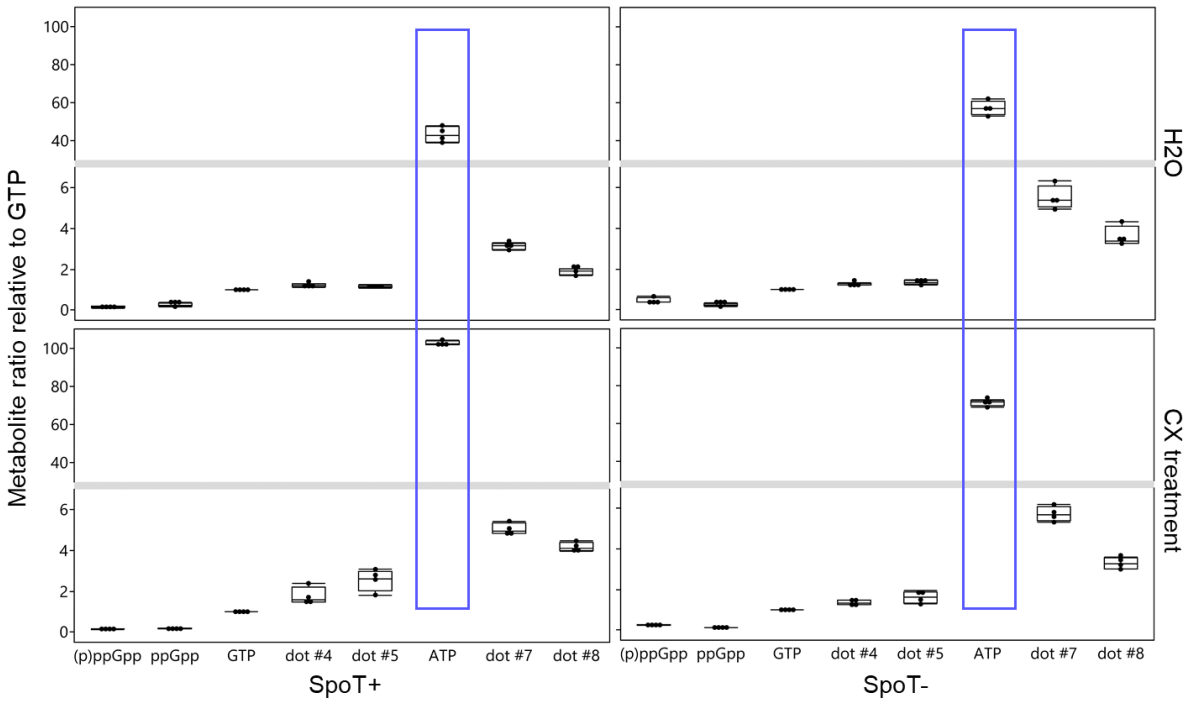

Figure 2.8 Relative quantification of stringent response metabolites.Metabolite abundances relative to GTP at T=10hr. Blue boxes highlight changes in ATP ratios. Metabolites labeled "dot" are species that appeared in the TLC membrane image but have not known identity.

Table 2.1 Hosts of predicted prophage genomes that carry stringent response domains. Hydrolysis (HD) and synthesis (SYNTH) domains from the *spoT* gene of *S. Typhimurium LT2*. % ID≥40; e-value: $\leq 10^{-10}$.

order	genus	HD	HD-SYTH	respiration	environment
Alteromonadales	*Alishewanella*	1		facultative	sediment
Alteromonadales	*Shewanella*	1		facultative	marine
Burkorderiales	*Acidovorax*		1	facultative	fruit
Burkorderiales	*Alicycliphilus*		1	facultative	wastewater
Burkorderiales	*Burkholderia*		7	aerobic	soil/groundwater
Burkorderiales	*Polaromonas*		1	aerobic	Ice/sediment
Clostridiales	*Acetivibrio*	1		anaerobic	soil/gut
Clostridiales	*Clostridium*	14		anaerobic	soil/gut
Enterobacterales	*Escherichia*		28	facultative	gut
Enterobacterales	*Shigella*		2	facultative	gut
Lactobacillales	*Enterococcus*	18		facultative	gut
Lactobacillales	*Lactobacillus*	1		facultative	gut/plants
Rhizobiales	*Ancylobacter*	1		aerobic	roots
Rhizobiales	*Nitrobacter*	1		aerobic	sewage
Vibrionales	*Vibrio*	1		facultative	marine

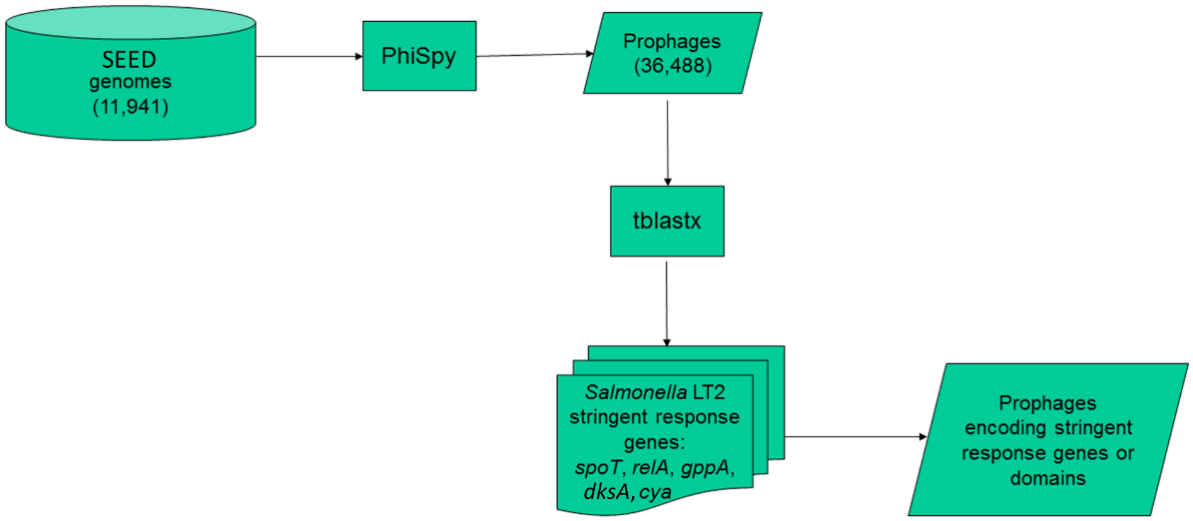

Figure S2. Bioinformatic pipeline for search of prophage-encoding stringent response domains in predicted prophage genomes.

Acknowledgements

Chapter 2 is coauthored with Maria I. Rojas, Hamza Hajama, Ashley R. Wolf, Adrian V. Cantu, Ana G. Cobián-Güemes, Mark Little, Michael L. Patnode, Matthew C. Hibberd, Daniel A. Cuevas, Janaki L. Guruge, Barbara Bailey, Jeffrey I. Gordon, Anca Segall, Forest Rohwer. The dissertation author was the primary investigator and author of this paper.

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Chapter 3 : Swabbing the urban environment—a pipeline for sampling and detection of SARS-CoV-2 from environmental reservoirs

Abstract

To control community transmission of SARS-CoV-2 during the 2020 global pandemic, most countries implemented strategies based on direct human testing, face covering, and surface disinfection. Under the assumption that the main route of transmission is aerosols and respiratory droplets, efforts to detect SARS-CoV-2 in fomites have focused on locations suspected of high prevalence (e.g., hospital wards, cruise ships, and mass transportation systems). To investigate SARS-CoV-2 on surfaces in the urban environment that are rarely cleaned and seldomly disinfected, we enlisted 350 citizens from the greater San Diego County. In total, theses citizen scientists collected 4,080 samples. An online platform was developed to monitor sampling kit delivery and pickup, as well as to collect sample data. The sampling kits were mostly built from supplies available in pandemic-stressed stores. Samples were processed using reagents that were easy to access despite the recurrent supply shortage. The methods used were highly sensitive and resistant to inhibitors which are commonly present in environmental samples. The proposed experimental design and processing methods were successful at engaging numerous citizen scientists that effectively gathered samples from diverse surface areas. These workflow and methods are relevant to survey the urban environment for other viruses which are of public health concern and pose a threat to future pandemics.

Introduction

The severe acute respiratory syndrome coronavirus (SARS-CoV-2)is thought to be transmitted mainly via the inhalation of contaminated aerosols and droplets from direct contact with infected individuals (Alsved et al. 2020; Morawska and Cao 2020; Stadnytskyi et al. 2020; Yu et al. 2004). However, during the initial phases of the global COVID-19 pandemic, efforts to control transmission of SARS-CoV-2 focused strongly on disinfecting surfaces, handwashing, and sanitization. By the end of 2020 transmission guidelines from the World Health Organization (WHO)(World Health Organization 2020) and the U.S. Centers for Disease Control and Prevention (CDC)(Centers for Disease Control and Prevention 2020b) deemed airborne transmission a hazard mainly when in close contact (< 2 m) with an infected person or in the presence of aerosol-generating medical procedures. Self-inoculation after contact with contaminated surfaces or inhalation of aerosolized fomites have not been ruled out as a route of transmission of SARS-CoV-2.

COVID-19 cases have been reported where airborne transmission seems unlikely (Moriarty et al. 2020; Cheng et al. 2020). SARS-CoV-2 virions remain infectious on copper for up to 8 h, cardboard and stainless steel for up to 24 h, and on plastic for up to 48 h (Van Doremalen et al. 2020). In cruise ship cabins, SARS-CoV-2 RNA was detected 17 days after passengers departed (Moriarty et al. 2020). Air and surface samples from hospitals and mass-transit systems have tested positive for SARS-CoV-2 and other Coronaviruses (Liu et al. 2020; Cheng et al. 2020; Butler et al. 2020; Döhla et al. 2020; Ikonen et al. 2018; Chia et al. 2020). A study performed on the outer packaging of Halloween candy handled by asymptomatic and moderate/mildly symptomatic COVID-19 patients concluded that the

combination of hand washing by the handler and hand soap treatment of the candy reduced the SARS-CoV-2 RNA below threshold levels (Salido et al. 2020).

Several methods for SARS-CoV-2 diagnostics have been published based on real-time RT- PCR (RT-qPCR) (Chan et al. 2020; Dao Thi et al. 2020), reverse transcription Loop-Mediated Isothermal Amplification (RT-LAMP) (Butler et al. 2020; Rauch et al. 2020; F. Zhang et al. 2020; Y. Zhang, Odiwuor, et al. 2020; Y. Zhang, Ren, et al. 2020a), and CRISPR-Cas (Broughton et al. 2020; Lucia, Federico, and Alejandra 2020; Rauch et al. 2020; F. Zhang et al. 2020). Most require RNA extraction kits that are often in short supply during significant global demand and very few have been used for environmental screening of the virus (Danko et al. 2020). The detection of SARS-CoV-2 RNA using RT-LAMP has been demonstrated to be over 83% concordant to using RT-qPCR. Furthermore RT-LAMP had 25 % reduction in inconclusive results compared to RT-qPCR (Salido et al. 2020).

Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) is a simple technique that uses a reverse transcriptase to synthesize cDNA from an RNA template (Parida et al. 2005) followed by a DNA polymerase with strong strand-displacement activity that synthesizes DNA at constant temperature (i.e., isothermal amplification) (Notomi et al. 2000). Higher specificity of viral genome detection is achieved by using four or six primers that recognize six or eight regions of the target DNA. Amplification is initiated from an internal primer and yields a semi-dsDNA structure. The leading strand is then amplified by an outer primer. These amplifications are repeated for the reverse primers. Internal and outer primers on either end have an internal reverse self-complementarity site that forms a loop in the amplification product (Notomi et al. 2000; Nagamine, Hase, and Notomi 2002). In isothermal strand displacement, asynchronous DNA synthesis generates high quantities of

amplified product where the continuous polymerization amplifies the signal of as little as 10 copies per reaction (Butler et al. 2020; Y. Zhang, Ren, et al. 2020b; Y. Zhang, Odiwuor, et al. 2020). The colorimetric RT-LAMP mix is weakly buffered and uses phenol red as a pH indicator. As the polymerase incorporates a nucleotide it releases a proton, and enough protons will change the pH of the solution as well as its color from pink to yellow (Butler et al. 2020; Y. Zhang, Ren, et al. 2020b; Y. Zhang, Odiwuor, et al. 2020; Tanner, Zhang, and Evans 2015).

RT-LAMP was developed for the detection of mosquito-borne diseases in peripheral health care facilities that lack fully equipped laboratories (Parida et al. 2005) and for the rapid detection of other RNA viruses like Human Immunodeficiency Virus (HIV) (Curtis, Rudolph, and Owen 2008). The most vulnerable populations in epidemic outbreaks—as per the WHO definition—often lack sufficient economic resources and the appropriate equipment to carry out detection (United Nations Global Public Health Agenda). In the current SARS-CoV-2 pandemic, supplies such as medical-grade swabs and reagents for RNA extraction kits have not been able to meet global demand, especially in non-manufacturing countries. The proposed protocol used a guanidinium thiocyanate (GITC)-based RNA crude extraction, which effectively preserved the RNA in a cold-chain independent manner and significantly reduced the persistence of inhibitors from the sample. Moreover, the GITC-chloroform extraction protocol is based on the separation of RNA from DNA and proteins, followed by the respective precipitation, allowing the recovery of most of the genetic material. These advantages outweigh the potential hazards of citizen scientists handling the chemical if proper measures are taken to appropriately inform them of the risks.

The proposed workflow uses materials and reagents that are of general use. They require equipment that is available in basic, often rural, laboratory settings. These methods are inexpensive, highly resistant to inhibitors often found in environmental samples or samples that cannot be processed with extractions kits and eliminate the need for a high precision thermocycler.

This study presents a pipeline for sampling and detection of SARS-CoV-2 from environmental reservoirs on commonly touched and rarely disinfected surfaces of households and the urban environment.

Results

Sample collection by citizen scientists

During an 8-month period (mid-March to the third week of November 2020) 482 citizens were approved to participate, of which 350 (73%) requested a kit. A total of 362 kits were delivered (i.e., some participants requested multiple kits) and 246 (70%) were returned **(Figures 3.1A, B)**. All 4,080 samples contained in these kits were processed. Collection sites were distributed across north coastal, north central, central, and south county, as well as a few at east county **(Figure 3.1A)**. These districts have the highest population density of San Diego County and the most COVID-19 documented cases, as reported by the San Diego Human Health Service Agency (County of San Diego Health and Human Services Agency 2020b).

Citizens requested pickup of most sampled kits (i.e., average success rate: 70.4 %). Each day, 1-16 kits were requested, and 0-14 kits were returned to the laboratory **(Figure 3.1B)**. A survey to citizen scientists showed that the collection of a complete kit (16 samples) took 1-3 h distributed throughout an average of 8 days **(Figure 3.1, Table 3.1)**. The great

majority of kits were complete (91.1 %), meaning they contained a swab inside all 16 sample tubes and the corresponding sampling data was uploaded to the LIMS **(Figure 3.1A, Table 3.1).**

Detection of SARS-CoV-2 using Trizol extraction and multiplex RT-LAMP

For the colorimetric RT-LAMP assay, two sets of primers (Butler et al. 2020; Y. Zhang, Odiwuor, et al. 2020; Y. Zhang, Ren, et al. 2020b) were used to target the nucleocapsid (N2) and the envelope (E1) genes **(Table 3.2)**. The sequences recognized by these primers are in the same region as the primers and probes approved by the CDC (Centers for Disease Control and Prevention 2020a) and the European Union (EU) (Corman et al. 2020) for human diagnosis of COVID-19 by RT-qPCR. These results corroborate what Zhang et al.(Y. Zhang, Ren, et al. 2020b) described, where adding 60 mM guanidine hydrochloride to the reaction increases the limit of detection (LOD) when run in multiplex. LOD at a frequency of 100 % was 500 copies per 25 µL reaction **(Figures 3.2A, B)**. In the colorimetric RT-LAMP, positive samples changed color from pink to yellow due to a pH shift from ~8 to 5.5 **(Figure 3.2B)**. When the reaction turned orange at low-copy numbers, samples were run in a 1.5 % agarose gel to confirm these were positive and resulted in a ladder-like pattern **(Figure 3.2C)**.

RT-LAMP was used to detect SARS-CoV-2 pooled RNA samples. To control for false negatives due to reaction inhibitors, each sample was tested in an additional reaction spiked with 500 copies of synthetic SARS-CoV-2. Positive pooled samples were dereplicated by isolating the RNA of each individual sample in the pool and run in a RT-LAMP reaction to determine the identity of the positive sample. The detection results were then uploaded to the

LIMS where the unique sample ID was paired with the information on date, time, GPS coordinates, site, and image of the sample.

Real-time and traditional RT-PCR methods

To select the best method suitable for the proposed detection pipeline, other RNA amplification methods were tested with environmental samples taken by a pilot cohort of citizen scientists. An example of the results of each of these methods are presented in **Figure 3.3** to depict their sensitivity to environmental inhibitors and background signal noise at low viral copy-number concentrations.

Six RT-qPCR formulations **(Table 3.3)** approved by the CDC and the WHO were tested for its detection on environmental samples. Protocols were followed according to manufacturer's instructions as well as CDC guidelines for the detection of SARS-CoV-2 in clinical settings(Centers for Disease Control and Prevention 2020a). Reactions containing different concentrations of synthetic SARS-CoV-2 RNA controls were spiked into swabbed-surface samples after crude RNA isolation. All master mixes were sensitive to inhibitors at LOD concentrations of the positive control **(Figures 3.3A)**.

To bypass inhibitors of the real-time technologies, a traditional RT-PCR system was tested. A one-step RT-PCR system **(Table 3.3)** was used to amplify the nucleocapsid gene using the primer sets N1, N2, and the envelope gene using the primer set E1 approved by the CDC (USA) and the ECDC (EU) respectively **(Table 3.2)**. Protocols were followed according to manufacturer's instructions as well as CDC guidelines for the detection of SARS-CoV-2 in clinical settings(Centers for Disease Control and Prevention 2020a). The primer sets N1 and N2 designed by the CDC yield a ~70 bp; however, low-copy number positives were not

distinguished from the amplification background noise of the negative control **(Figure 3.3B)**, which introduced false positives to the results. The product of the E1 primers had a weak signal at low-copy number **(Figure 3.3B)**, introducing false negatives to the results**.** Furthermore, the RT-PCR method tested was still sensitive to inhibitors present in the environmental samples (data not shown).

Other methods have been developed to detect very small quantities of target sequence. One of these methods is the Rolling Circle Amplification (RCA), whereupon recognition of the target sequence, RNA or DNA, by a specific linear probe, a ligase circularizes the template. Using primers designed to hybridize with the probe, a DNA polymerase with strand-displacement activity amplifies the probe in an isothermal reaction (Lizardi et al. 1998). The target sequence is not amplified, rather the probe that identified the target, which makes this method highly sensitive (Johne et al. 2009). Wang et al.(B. Wang et al. 2005) published an RCA protocol for the direct detection of SARS-CoV-1 RNA. The method was modified to use primers specific for SARS-CoV-2. Unfortunately, in the non-template control (NTC) the probe circularizes and yields product in the absence of RNA template, even when using a wide variety of ligases, including an SNP-sensitive ligase. In the absence of ligase, the NTC did not show amplification from the linearized probe **(Figure 3.3C)**.

Discussion

Citizen scientist engagement

Citizen scientists were recruited to swab the surfaces throughout San Diego County to sample and detect the presence of SARS-CoV-2 in the urban environment. The majority of delivered sampling kits (70 %) were returned to the lab, and of those, almost all samples were

complete (91 %) **(Figures 3.3A, 3B; Table 3.1)**. Volunteers could easily request kit delivery/pickup through the web-based platform, and the delivery route planning software notified citizen scientist of estimated times of arrival, both likely significant factors for this success. The average time from when the kit was delivered to the citizen scientist to when it was returned to the laboratory was 8 days, with a median of 3 days, and a range of 1-64 days **(Figure 3.3A; Table 3.1)**. More frequent reminders to volunteers would likely reduce this lag time.

The data collection platform was successfully used by a great majority of users (73 %). While we did not measure effort by the citizen scientists, field tests showed that the data collection platform significantly reduced the effort and time required to properly complete sample collection. Reducing the amount of bookkeeping should encourage citizen scientist engagement. The web-based platform intended to overcome demographic limitations by providing a multilingual neural machine translation service and by providing graphic and audiovisual protocols in English and Spanish. This was only partially successful since fewer samples were collected from both the South Bay and North County where most of the county's Hispanic/Latino population reside (County of San Diego Health and Human Services Agency 2020e). These areas also harbored 63% (1,700 cases per 100,000) of the total cases in San Diego County with the highest prevalence of the disease (County of San Diego Health and Human Services Agency 2020a) and rate of hospitalizations (62%) (County of San Diego Health and Human Services Agency 2020c; 2020d). Although most of the samples came from Central County, a representative number was collected from the most COVID-19 impacted districts and only a small fraction of the samples was positive, which suggests that surface reservoirs of SARS-CoV-2 in the urban environment are relatively rare.

Sample processing

Sampling swabs were wetted with SDS, which inactivated the virus by disrupting its envelope and stabilized the naked RNA by unfolding RNases (Tejaswi Naidu and Prakash Prabhu 2011). Conveniently, during collection, the detergent in the swab cleaned the sampled surface. Environmental samples often contain very small quantities of RNA. To maximize recovery, the RNA isolation was done using a GITC-based column-free crude extraction method. GITC, a strong chaotropic agent, disrupts the hydrogen bonds that maintain protein folding (i.e., hydrophobic effect). This action results in the inactivation of viral particles, and the RNA remains stable due to the inhibition of RNAses (Chomczynski and Sacchi 2006; Rio et al. 2010b; Hummon et al. 2007). The GITC solution maintained the sample RNA stable without strict cold-chain considerations which allowed the citizens to maintain the samples at room temperature if a freezer for the provided ice packs was not available. To reduce the potential hazard this reagent poses when direct skin or mucosal contact occurs, citizens were made aware of these hazards by a safety data sheet (MSDS) provided in the kit, and a warning seal was placed in the box containing the tubes.

The crude GITC-chloroform extraction method aided the recovery of traces of RNA from the swabs, and as shown by the amplification of spiked samples, inhibitors rarely persisted in the samples after extraction. Samples negative for SARS-CoV-2 that showed no RT-LAMP inhibition represented true negatives or had a lower copy number than the LOD at 100 % frequency. Conversely, detection of viral RNA on a surface does not directly imply risk of transmission through contact since the infectivity of the virus from positive samples needs to be tested. Prompt screening of the environment, not limited by availability of

sophisticated supplies or highly qualified personnel, is crucial to assess whether surfaces constitute a viral reservoir to better direct prevention and containment efforts.

RT-LAMP was selected to be the best method suitable for the proposed detection pipeline. It proved to be a rapid, inexpensive method, highly resistant to most of the remaining inhibitors, and as sensitive and specific as RT-qPCR methods. Because of their use in clinical settings during the SARS-CoV-2 pandemic, the availability of RT-qPCR kits was impacted by global demand. Moreover, RT-qPCR techniques, even those formulated to resist inhibitors, were sensitive to substances contained in the environmental samples taken by a pilot cohort of citizen scientist, even after the use of other common strategies to reduce inhibitor competition for enzyme binding (Schrader et al. 2012). Our findings are corroborated by a recent study that compared both methods to detect SARS-CoV-2 on swab samples from candy handled by COVID-19 patients and found over 83 % result concordance, with one fourth less inhibited samples analyzed by RT-LAMP (Salido et al. 2020). Furthermore, the GITC-chloroform crude extraction coupled with RT-LAMP reduced the cost of reagents and supplies in 42 % compared to RNA kit extraction and RT-qPCR **(Table 3.4)**.

The presented method allowed for high throughput analysis of thousands of surface swab samples. Up to 80 pools, representing 640 samples, were processed per day from RNA extraction to SARS-CoV-2 detection by RT-LAMP. The proposed protocol is semi-quantitative and is limited to the detection of viral RNA and does not indicate the presence of infective viral particles. Further analysis is required to assess the risk of transmission of SARS-CoV-2 from infected fomites present at the swabbed surfaces.

This study presents a protocol to quickly set up a testing strategy which includes an effective workflow when facing a health emergency with a communicable disease. The

proposed sampling protocol is simple and uses supplies commonly found in households, and the viral detection method is carried out on equipment available in basic laboratory settings, such as a water bath in lieu of a thermocycler. This study serves as a framework for the assessment of environmental viral reservoirs in future epidemic outbreaks and global pandemics.

Methods

See **Tables 3.3 and 3.4** for a detailed list of reagents and supplies, including catalog numbers, manufacturer, and corresponding costs.

1. Sampling the urban environment

1.1 Citizen scientist outreach

1.1.1 Recruit citizen scientists using a direct and clear call-to-action released via local and social media.

1.1.1.2 Create a social media handle (e.g., **#swab4corona**) to connect the topic across social media content.

1.1.1.3 Create an email account for direct communication between the laboratory and each citizen scientist, managed by a person fluent in the main languages of the region of interest (e.g., Spanish and English for San Diego County).

1.1.2 Build a secure web-based sample management platform (SMP) to serve as a database and a laboratory information management system (LIMS), and to communicate with citizen scientists.

NOTE: The SMP provides a centralized location where users request a kit, access sample collection protocols, submit sample metadata, and request a pickup for completed sample kits.

1.1.2.1 Create a link to the SMP (e.g., **https://demo.covidsample.org/**) **(Figure 3.4)** for individuals to apply to participate in the environmental sampling effort by answering biosafety-related questions specified in an online form.

1.1.2.1.1 Secure access to the SMP using an Authentication Application Programming Interface facilitated by a cloud computer service provider.

NOTES:

An Interface that uses the OAuth 2.0 protocol(Internet Engineering Task Force (IETF) 2012) for authentication and authorization, provides a frictionless sign-in process for citizen scientist volunteers. It also allows users to sign-in with an existing account, eliminating the need to create a custom sign-in solution and manage user credentials, which saves a significant amount of time and encourages participation.

According to recent reports, the free email service available for the chosen cloud computer service provider has approximately 1.5 billion monthly-active email users; requiring an email account from this service provider for participation is not considered a discouraging factor.

1.1.2.1.2 Give approved users access the SMP.

1.1.2.1.3 Explain the objective of the study and biosafety considerations to the citizen scientists at the SMP before they request the first kit.

1.1.2.1.4 Provide a multilingual plugin to enable navigation in any of the languages available from a multilingual neural machine translation service facilitated by a cloud computer service provider.

1.1.2.1.5 Include in the sampling section graphic and audiovisual protocols in English and Spanish.

1.1.2.1.6 Assign a unique identifier to each kit and design the user interface to utilize buttons linked to Sample ID to streamline the data entry process **(Figure 3.4A)**.

1.1.2.2 Use a delivery route planning software with a mobile-device application, to be used by drivers to optimize delivery/pickup routes and notify citizen scientists of accurate estimated times of arrival.

1.1.2.3 Build the LIMS platform on a PHP web service stack and host it on a commercial hosting platform (suggested operating system, web server software, and database software are specified in the **Table 3.3)**.

1.1.2.3.1 Provide a secure web-based application interface to enable lab personnel to manage data quickly and easily in the LIMS.

1.1.2.3.2 Provide data visualization using a Data Charting Application Programming Interface facilitated by a cloud computer service provider.

1.1.2.3.3 Visualize Geospatial Data using a Geospatial Application Programming Interface facilitated by a cloud computer service provider.

1.1.2.3.4 Store the data submitted to the LIMS through the SMP so it allows for: (1) centralized storage of project data; (2) tracking of sample/data

processing workflows; and (3) management of the logistics of sample kit distribution to citizen scientists.

1.1.2.3.4.1 Secure submitted metadata using best practices (e.g., **https://demo.covidsample.org/**).

1.1.2.3.4.2 Pre-load information such as Sample Kit ID, Sample ID, date, time, and GPS coordinates (automatically gathered from a picture of the site) to enable data-type compliance and minimize the submission of erroneous or missing data by the user **(Figure 3.3)**.

1.1.2.3.4.3 Include the following fields to be manually and swiftly (>1 min) filled by the citizen scientist: date and time of collection, a brief description of the location, and a picture of the sampling site.

1.1.2.3.4.4 Sanitize all submitted data and validate it for data type. For example, validate image data uploaded by users to select .jpg files and rename them with Sample ID for fast association with the sample; and store uploaded images in a separate secure location not accessible to users.

1.1.2.3.4.5 Activate the option to request kit delivery and pickup when all samples (16) have been completed. As well as the option to request a new kit to be delivered upon pickup of the previous one **(Figure 3.3A)**.

NOTE: For volunteers that prefer a non-web-based platform, and those worried about disclosing their GPS location (e.g., members of the community concerned about their migratory status), kits can be

delivered at an agreed meeting location and volunteers asked to record a written version of the data collection. For communication between the laboratory and each citizen scientist, have a bilingual member of the project available for telephone calls and texts.

1.2 Swab for Corona

1.2.1 Identify an epidemiologically relevant time window for the sampling effort.

1.2.2 Build a kit that contains all the sampling supplies, including the necessary personal protective equipment (i.e., mask, gloves), a sampling protocol, and biosafety relevant information (**Figure 3.5)**.

1.2.2.1 Pre-label each tube with the assigned unique identifier (Sample ID).

1.2.3 Swab rarely disinfected surfaces that are exposed to aerosolized fomites in households and the urban environment.

1.2.3.1 Wear the provided mask to protect yourself and others while in public. Wear a NEW pair of gloves for the collection of EACH sample to avoid cross-contamination. After finishing the sampling, clean your hands with the provided hand sanitizer.

1.2.3.2 Wet a 1 cm^2 polyester absorbent swab (e.g., mop pads) with a detergent (e.g., 0.5 % sodium dodecyl sulfate (SDS)). Detergents like SDS disrupt the envelope of the virus inactivating it and stabilizes the naked RNA by inducing unfolding of RNases (Tejaswi Naidu and Prakash Prabhu 2011).

1.2.3.3 Swab a surface of 10 cm^2.

1.2.3.4 Aided by a toothpick, completely submerge each sample swab in the corresponding pre-labeled tube containing 200 µL of guanidinium thiocyanate solution

(GITC) (**CAUTION! Toxic irritant; avoid contact with skin**). Store tubes at 4 °C until they are transported to the lab.

NOTES:

GITC solution is simple to prepare from common laboratory chemicals, for the recipe see (Chomczynski and Sacchi 1987; 2006).

GITC inactivates the virus and stabilizes RNA by denaturing RNases (Chomczynski and Sacchi 2006; Hummon et al. 2007; Rio et al. 2010b).

GITC stabilizes samples at room temperature. The kit however includes ice packs to keep the samples cold without the need to use household refrigerators for storage.

1.2.4 Once samples arrive to the lab, store at -80 °C.

2. SARS-CoV-2 Detection

2.1 Total RNA isolation

2.1.1 Disinfect surfaces, equipment, and pipettors with a solution of 2 mM copper sulfate and 3 % hydrogen peroxide; followed by a solution of 10 % bleach, 90 mM sodium bicarbonate, 5 % SDS, and 2.5 % NaOH. Wipe thoroughly with distilled water followed by 75 % EtOH.

NOTE: These solutions are an alternative to those commercially available.

2.1.2 Thaw samples on ice.

2.1.3 Vortex samples for 2 min at medium speed.

2.1.4 To increase the speed of the screening, process the samples in pools. If a pool is positive, extract the RNA of each sample independently to find the positive sample/s. Combine the samples from each sampling kit (16 total) into 2 pools of 8 samples.

NOTE: Pools of 8 samples means that only 2 pools need to be processed per kit. If a pool is positive, then the individual samples are re-processed for individual RT-LAMP analysis. This reduces time, costs, and reagents.

2.1.5 Pool 50 µL of each of 8 samples into a microcentrifuge tube (total volume 400 µL). Save the remaining sample at -80 °C.

2.1.6 Add 0.2 volumes (80 µL) of chloroform.

2.1.7 Vortex for 15 sec then incubate for 20 min at 4 °C.

2.1.8 Centrifuge at 13,000 x g for 20 min at 4 °C.

2.1.9 Transfer the aqueous (clear liquid) layer into a new microcentrifuge tube.

2.1.9.1 Store the remaining interface and pink liquid in the -80 °C freezer, these fractions contain DNA and proteins (Chomczynski and Sacchi 1987; Rio et al. 2010b).

2.1.10 Add an equal volume of isopropanol (~200 µL).

2.1.11 Add 2.6 µL of Glycogen coprecipitant (15 mg mL^{-1}) (Rio et al. 2010a).

2.1.12 Mix well and incubate at -20 °C for at least 1 h, followed by 4 °C for 10 min to precipitate RNA.

Safe stopping point – incubate samples at -20 °C overnight instead of 1 h.

2.1.13 Centrifuge at 13,000 x g for 20 min at 4 °C.

2.1.14 Remove supernatant. Do not disturb the pellet.

2.1.15 Resuspend the pellet in 50 µL of DPEC-treated water.

2.1.16 Add an equal volume (50 µL) of RNase-free 5 M Ammonium Acetate and 2.5 volumes (250 µl) of 100 % Ethanol (Rio et al. 2010a; Wallace 1987).

NOTES:

Ammonium ions inhibit polynucleotide kinase if used in a downstream process (Wallace 1987).

The mix precipitates RNA while leaving dNTPs and oligosaccharides in solution (Wallace 1987).

2.1.17 Mix well and incubate at -20 °C for at least 1 h to precipitate RNA, followed by 4 °C for 10 min to precipitate RNA.

Safe stopping point – incubate samples at -20 °C for overnight instead of 1 h.

2.1.18 Centrifuge at 13,000 x g for 20 min at 4 °C.

2.1.19 Wash pellet with 1 mL of cold (-20 °C) freshly made 75% Ethanol.

2.1.20 Centrifuge at 8,000 x g for 5 min at 4 °C.

2.1.21 Remove supernatant. Do not disturb the pellet.

2.1.22 Spin down quickly and remove the remaining Ethanol with a P10 pipette without disturbing the pellet.

2.1.23 Air dry for 10-15 min until there is NO remaining Ethanol.

2.1.24 Resuspend pellet in 50 µL of DPEC-treated water.

2.1.25 Add 5 µL of 10X DNase buffer + 1µL of DNase (2 Units μL^{-1}).

2.1.26 Incubate at 37 °C for 30 min.

2.1.27 Add 0.1 volumes (5.6 µL) of DNase inactivation reagent.

2.1.28 Incubate at room temperature for 5 min; mix gently every minute.

2.1.29 Centrifuge at 13,000 x g for 2 min.

2.1.30 Transfer supernatant to a new tube (~50 µL).

2.1.31 Place the tube on ice immediately while you prepare the RT-qPCR or RT-LAMP reactions, or store in a -20 °C freezer.

NOTE: Perform RNA Isolation in an amplicon-free room to avoid carry-over contamination!

2.2 Multiplex RT-LAMP

2.2.1 Prepare a 20X Primer Mix solution **(Table 3.5)** for each set of primers **(Table 3.2)**.

2.2.2 Prepare the RT-LAMP Reaction Mix **(Table 3.6)** at room temperature with 10% excess volume to account for pipetting loss.

NOTE: The colorimetric LAMP 2X Master Mix with Antarctic Thermolabile Uracil-DNA Glycosylase (UDG) prevents amplification of DNA contamination from previous reactions (Y. Zhang, Odiwuor, et al. 2020; Y. Zhang, Ren, et al. 2020b).

2.2.3 Vortex and spin down the mixture.

2.2.4 Dispense 20 μL of the mix into each reaction tube: “sample”, “spiked”, “positive control”, and “negative control”.

2.2.4.1 Let reaction sit in the tubes at room temperature for 10 min to allow the UDG to act on potential carry-over contamination.

2.2.5 Add 5 μL of RNA to the “sample” reaction, 5 μL of RNA + 2.5 μL (450 copies) of synthetic SARS-CoV-2 RNA to the “spiked” reaction, 2.5 μL (450 copies) of synthetic SARS-CoV-2 RNA to the “positive control” reaction, and 5 μL of H_2O to the “negative control” reaction. Mix well and spin down reaction. Thaw all RNA on ice.

2.2.6 While the thermocycler, or the water bath, is heated to 65 °C, allow reactions to rest at room temperature. Use thermocycler heated lid. The UDG is inactivated at > 50 °C.

2.2.7 Place reactions in the thermocycler and incubate at 65 °C for 50 min.

2.2.8 Let the reaction reach room temperature (~22 °C for 5 min) or cool on ice for 1 min.

2.2.9 Analyze results using the colorimetric feature (simple observation) or by running the products in an agarose gel.

NOTE: Although the limit-of-detection (LOD) is 10 copies per reaction, the frequency of detection increases as copy number approaches 500 copies per reaction **(Figure 3.2A)**.

2.2.9.1 Colorimetric: negative – PINK (pH = 8.8), positive – YELLOW (pH = 5) **(Figure 3.2B)**.

NOTE: The colorimetric option avoids opening the RT-LAMP tubes after amplification, which reduces RT-LAMP products in the working environment and carry-over contamination.

2.2.9.2 Gel electrophoresis:

2.2.9.2.1 Prepare a 1.5 % agarose gel with 1X DNA Gel Stain in 0.5 % TBE.

2.2.9.2.2 Load 25 μL of the reaction + 5 μL of 6X loading dye in each well. No ladder is needed.

2.2.9.2.3 Run reaction at 100 V for 60 min.

2.2.9.2.4 Positive samples show a ladder pattern **(Figures 3.2C)**.

Figure and Tables

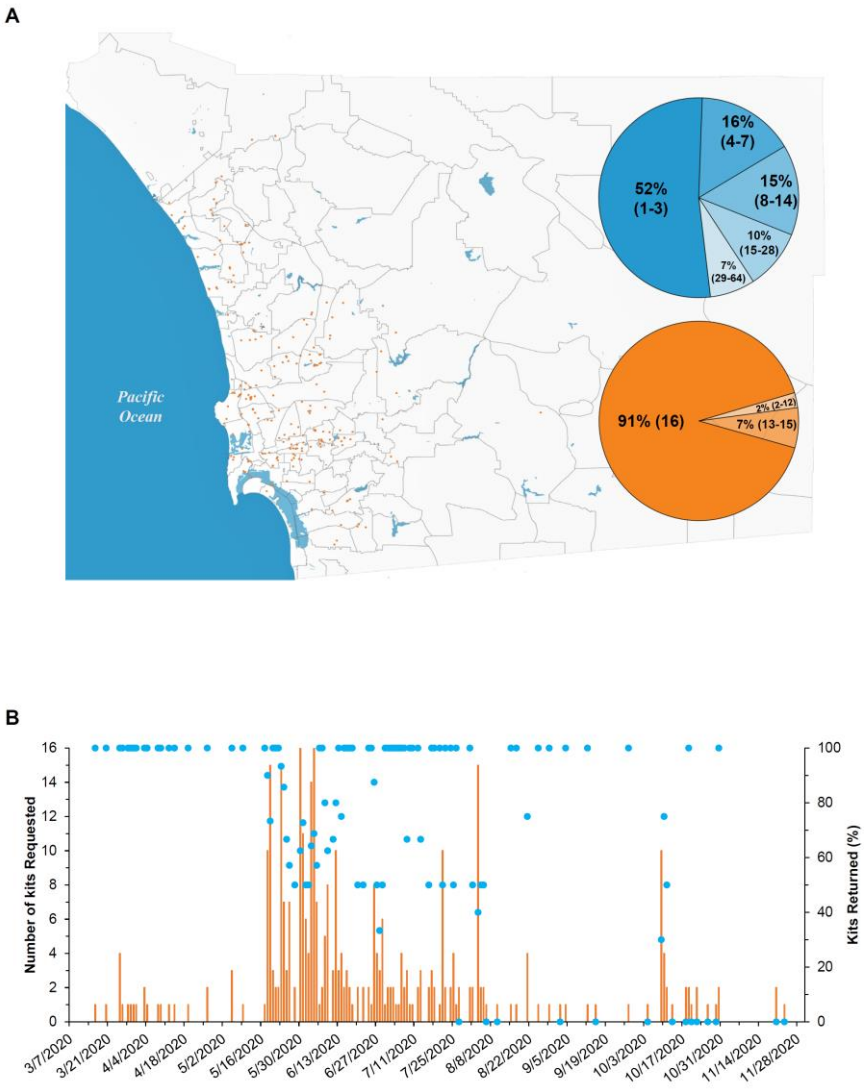

Figure 3.1 Location of citizen scientist sampling kits in San Diego County, and Success rate of requested kits.**(A)** Orange dots represent the location of 1 sampling kit which contains 16 samples. *(blue pie chart)* Percentage of kits that took various days from when they were delivered to the citizen scientists to when they were returned to the laboratory. Number of days in parentheses. *(orange pie chart)* Percentage of kits with different number of completed samples from a total of 16 samples. Number of completed samples containing a swab inside the sample tube and the corresponding sampling data uploaded to the LIMS in parentheses.**(B)** Percentage of kits that were returned to the laboratory (dots), and total number of requested kits (bars), relative to the date when the kit was requested.

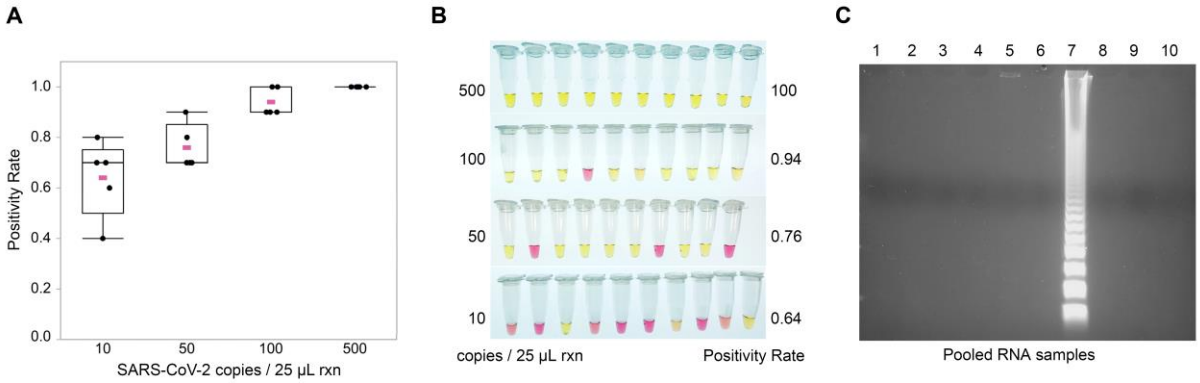

Figure 3.2 Multiplexed Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay.Multiplexed reaction using primers for SARS-CoV-2 nucleocapsid (N2) and envelope (E1) genes detect as little as 10 copies of the virus in the reaction. Synthetic SARS-CoV-2 RNA was used as positive control. **(A)** Frequency of detection in multiplex colorimetric RT-LAMP of SARS-CoV-2 at different genome copy numbers per reaction. Mean value of five replicates in pink. **(B)** Limit-of-detection (LOD) of SARS-CoV-2 in multiplex colorimetric RT-LAMP; yellow = positive (pH ~5); pink = negative (pH ~8). **(C)** Ladder pattern of positive SARS-CoV-2 RT-LAMP reactions in 1.5 % agarose gel electrophoresis.

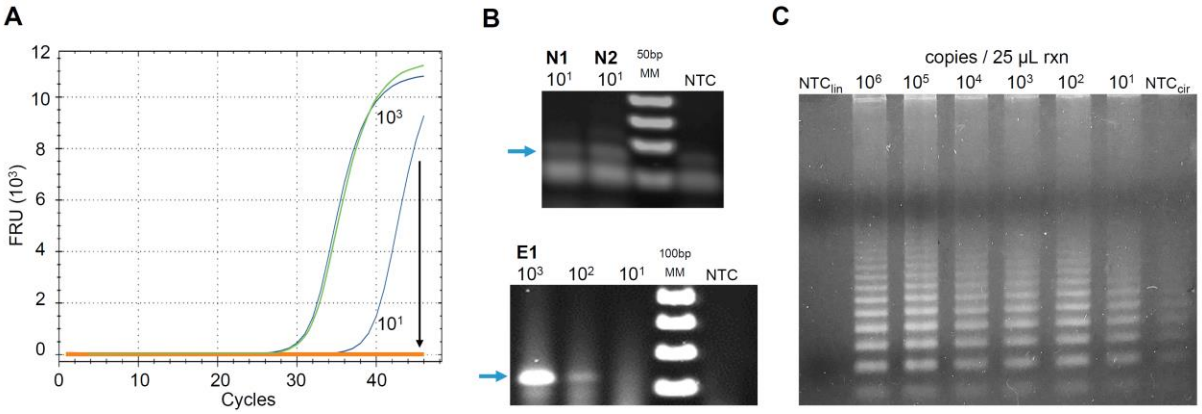

Figure 3.3 Alternative SARS-CoV-2 RNA detection methods.**(A)** RT-qPCR detection of SARS-CoV-2 nucleocapsid (N) gene using primer set N2. Pooled environmental samples spiked with 900 (green), or 9 (orange) copies of SARS-CoV-2. Positive controls of the same copy numbers in blue. Arrow indicates the decrease in fluorescence detection of low-copy number positive control when environmental sample is present. **(B)** Traditional RT-PCR detection of SARS-CoV-2 *(top)* RT-PCR products of the nucleocapsid gene using primer sets N1 and N2. A faint background signal is observed in the no-template control (NTC). *(bottom)* RT-PCR products of the envelope gene using primer set E1. Very low signal is observed at the LOD concentration. Blue arrows show expected positive product: *(top)* ~70 bp, and *(bottom)* 113 bp. 2 % agarose gel electrophoresis. **(C)** Rolling circle amplification of SARS-CoV-2 RNA**.** NTC_{cir}: linear probe in the presence of ligase and absence of RNA template; NTC_{lin}: linear probe in the absence of ligase and RNA template.

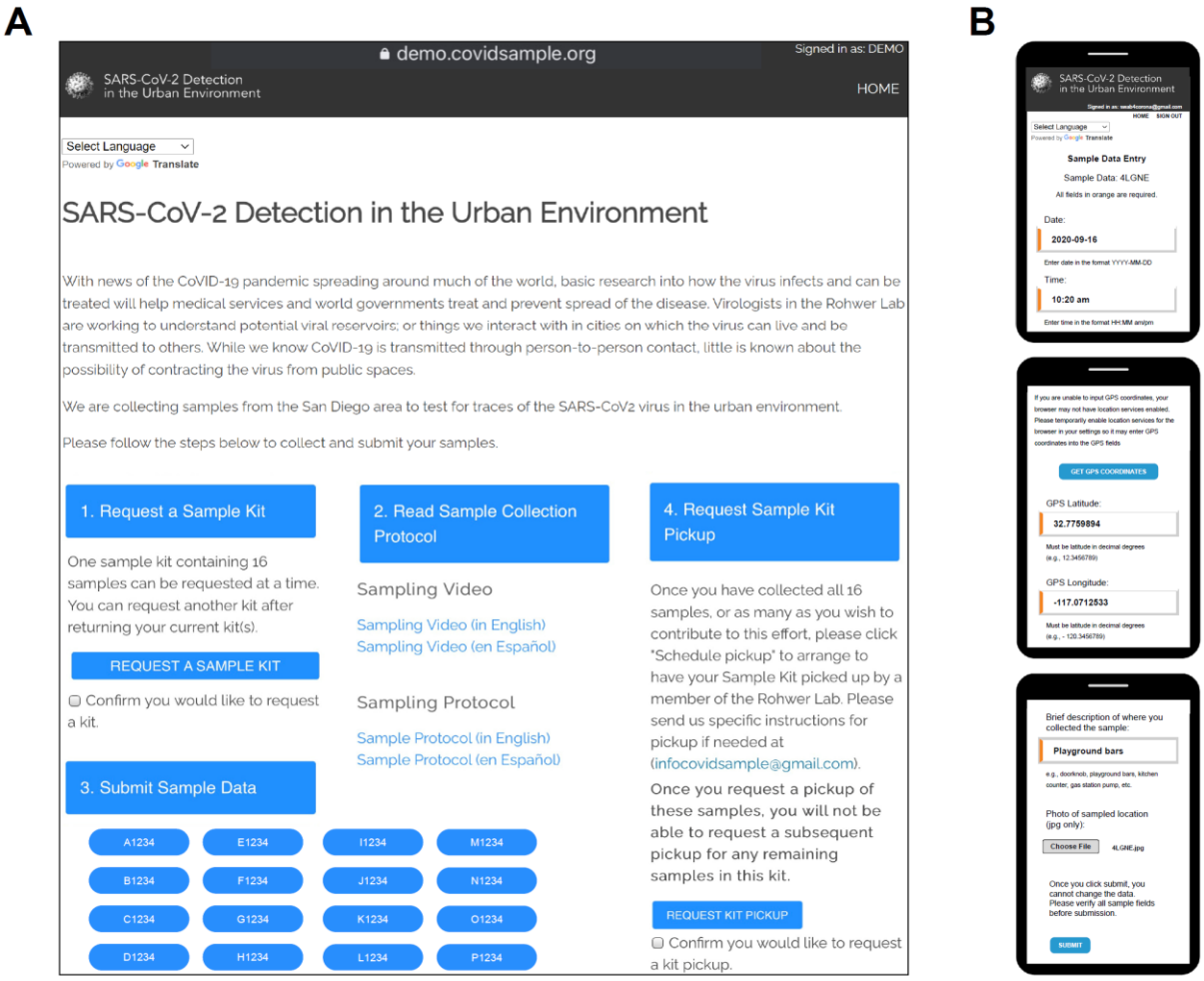

Figure 3.4 Web-based sampling platform with sample collection data interface for mobile devices.**(A)** A website was created to mediate the interaction between the laboratory and the citizen scientist, with a multilingual plugin. The platform was used for sample kit delivery/pickup request and sample data collection submission. Platform contained detailed English/Spanish graphic and audiovisual sampling protocols. **(B)** Mobile device view used to upload sample data: date, time, GPS coordinates, sample site description, and an image of the collection site.

Figure 3.5 Sample collection kit.Citizen scientists received a cooler containing two ice packs, a safety data sheet to inform volunteers about the hazards of GITC solution handling, a detailed sampling and mask-wearing protocol, an KN95 mask, a waste bag, a spray bottle with hand sanitizer, a spray bottle with 0.5 % SDS, 16 pairs of gloves, a small bag with 16 toothpicks and 16 polyester swabs, 16 pre-labeled microcentrifuge tubes containing 200 µL of GITC solution, a box containing the sampling tubes, and a bag used as secondary container for the tube box in the event of a spill.

Table 3.1 Swabbing for SARS-CoV-2 by the numbers. Outreach and sampling success rates.

Approved citizens	Citizens that requested a kit	Delivered kits	Returned sampled kits	Days dedicated to sampling		% Complete kits	% Incomplete kits	Processed samples
482	72.6 % (350/482)	362	70.4 % (255/362)	Mean	8	91.1 (224/246)	8.9 (22/246)	4,080
				Median	3			

Table 3.2 Primers used for RT-LAMP, RT-qPCR, and RT-PCR. Primer sequences, target gene, expected product size, and corresponding reference are detailed.

Primer	Sequence	Target	Product size
RT-LAMP			
E1-F3	TGAGTACGAACTTATGTACTCAT	E	ladder-like pattern
E1-B3	TTCAGATTTTTAACACGAGAGT		
E1-FIP	ACCACGAAAGCAAGAAAAAGAAGTTCGTTTCGGAAGAGACAG		
E1-BIP	TTGCTAGTTACACTAGCCATCCTTAGGTTTTACAAGACTCACGT		
E1-LoopB	GCGCTTCGATTGTGTGCGT		
E1-LoopF	CGCTATTAACTATTAACG		
N2-F3	ACCAGGAACTAATCAGACAAG	N	
N2-B3	GACTTGATCTTTGAAATTTGGATCT		
N2-FIP	TTCCGAAGAACGCTGAAGCGGAACTGATTACAAACATTGGCC		
N2-BIP	CGCATTGGCATGGAAGTCACAATTTGATGGCACCTGTGTA		
N2-LoopF	GGGGGCAAATTGTGCAATTTG		
N2-LoopB	CTTCGGGAACGTGGTTGACC		
qRT-PCR			
N1-F	GACCCCAAAATCAGCGAAAT	N	72 bp
N1-R	TCTGGTTACTGCCAGTTGAATCTG		
N1-probe	5’-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3’		
N2-F	TTACAAACATTGGCCGCAAA	N	67 bp
N2-R	GCGCGACATTCCGAAGAA		
N2-probe	5’-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3’		
RT-PCR			
E1_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	E	113 bp
E1_Sarbeco_R	ATATTGCAGCAGTACGCACACA		

Table 3.3 Details and costs of supplies, reagents, and equipment.

Name	Company	Catalog Number	Comments
SMP, LIMS, and community outreach:			
Authentication Application Programming Interface	Google	Google Sign-In	
Commercial hosting platform	GoDaddy		
Data Charting Application Programming Interface	Google	Google Charts	
Database software	MySQL		
Delivery route planning software	Circuit	Circuit for Teams	
Free email service	Google	Google Email	
Geospatial Application Programming Interface	Google	Google Maps API	
Multilingual neural machine translation service	Google	Google Translate	
Online form	Google	Google Form	
Operating system	Linux		
Web and database development	Big Rose Web Design		
Web server software	Apache		
Sampling kit:			
Coolers	Coleman (Amazon)	B00363X3F2	Cost (US$) per 100 rxns: 70
Gallon Ziploc bags	Solimo (Amazon)	B07BJ495GL	Cost (US$) per 100 rxns: 18
Glycerol (hand sanitizer)	FischerScientific	G33-4	Cost (US$) per 100 rxns: 9
Ice packs	Ice-Brix (Amazon)	B075GLD3X1	Cost (US$) per 100 rxns: 110
Isopropanol (hand sanitizer)	FischerScientific	AA36644K7	Cost (US$) per 100 rxns: 43
KN95 masks	Echo-Sigma	Echo-Sigma	Cost (US$) per 100 rxns: 400
Paper for Protocols and Trizol Safety Sheet	Office Depot	348037	Cost (US$) per 100 rxns: 36
30 mL spray bottles (SDS and hand sanitizer)	Anyumocz (Amazon)	B07T64FHXR	Cost (US$) per 100 rxns: 80
RNase, DNase, DNA & PCR inhibitors free Microcentrifuge tubes	Genesee Scientific	22-281	Cost (US$) per 100 rxns: 83
Sample ID solvent resistant labels	LABTAG	XST-10C1-1WH	Cost (US$) per 100 rxns: 68
Swiffer WetJet pads (swabs)	Swiffer (Amazon)	B001F0RBT2	Cost (US$) per 100 rxns: 8
Toothpicks	Kitchen Essential (Amazon)	B00PBK4NG6	Cost (US$) per 100 rxns: 8
Trizol Reagent (guanidinium isothiocyanate solution - GITC), not LS	Invitrogen	15596018	Cost (US$) per 100 rxns: 40
Tube boxes	Genesee Scientific	21-119	Cost (US$) per 100 rxns: 180
Small Ziploc bags	Ziploc (Amazon)	B01LRKEI9K	Cost (US$) per 100 rxns: 8
Zebra Thermal Transfer Desktop Printer	Zebra	GK420t	
			Total Sampling kit Cost (US$) per 100 rxns: 1,160
Trizol RNA extraction:			
Ammonium Acetate RNase-free	Invitrogen	AM9070G	Cost (US$) per 100 rxns: 2
Chloroform	FisherScientific	C298-500	Cost (US$) per 100 rxns: 2
GlycoBlue (glycogen 15 mg/mL)	Invitrogen	AM9515	Cost (US$) per 100 rxns: 80
Molecular-grade absolute (200 proof) Ethanol	FisherScientific	BP2818500	Cost (US$) per 100 rxns: 30
Molecular-grade Isopropanol	FisherScientific	BP2618500	Cost (US$) per 100 rxns: 3
TURBO DNA-free Kit	Invitrogen	AM1907	Cost (US$) per 100 rxns: 110
Multiplexed colorimetric RT-LAMP:			
Guanidine Hydrochloride	Alfa Aesar	AAJ6548522	Cost (US$) per 100 rxns: 1
RT-LAMP E1-Primers	IDT	n/a	Cost (US$) per 100 rxns: 7
RT-LAMP N2-Primers	IDT	n/a	Cost (US$) per 100 rxns: 7
Synthetic SARS-CoV-2 RNA	ATCC	VR-3276SD	Cost (US$) per 100 rxns: 14
WarmStart Colorimetric LAMP 2X Master Mix with UDG	NEB	M1800S	Cost (US$) per 100 rxns: 210
Eppendorf Mastercycler Pro Thermal Cycler	Eppendorf	950030010	
			Total Trizol RNA extraction + LAMP Cost (US$) per 100 rxns: 470
Kit for RNA extraction:			
QIAamp DSP Viral RNA Mini Kit	Qiagen	61904	Cost (US$) per 100 rxns: 570
RT-qPCR:			
Synthetic SARS-CoV-2 RNA	ATCC	VR-3276SD	Cost (US$) per 100 rxns: 14
TaqMan Fast Virus 1-Step Master Mix	Applied Biosystems	4444432	Cost (US$) per 100 rxns: 180
SARS-CoV-2 (2019-nCoV) N1,N2 Primers and Probes	IDT	10006713	Cost (US$) per 100 rxns: 20
qScript XLT 1-Step RT-qPCR ToughMix	Quantabio	95132-100	
QuantiNova Pathogen	Qiagen	208652	
QuantiNova Probe	Qiagen	208352	
UltraPlex 1-Step ToughMix	Quantabio	95166-100	
CFX96 Touch Real-Time PCR Detection System	BioRad	1855196	
			Kit for RNA extraction + RT-qPCR Cost (US$) per 100 rxns: 790
RT-PCR:			
SuperScript IV One-Step RT-PCR	Invitrogen	12594025	
Lab cleanup:			
DNAZap	Invitrogen	AM9890	
RNAZap	Invitrogen	AM9780	

Table 3.4 Supplies for sampling kits, and comparison of materials and reagents required for multiplexed colorimetric RT-LAMP and RT-qPCR.Contents of sampling kits are found in home-essential stores. Costs of RT-LAMP reagents for 100 reactions are significantly lower than those needed for RT-qPCR and less susceptible to global high demand scenarios.

	Reagent/Supply	Cost (US$) per 100 rxns	Catalog	Manufacturer
Sampling kit	KN95 masks	400	Echo-Sigma	Echo-Sigma
	Trizol Reagent	40	15596018	Invitrogen
	Isopropanol (hand sanitizer)	43	AA36644K7	FischerScientific
	Glycerol (hand sanitizer)	9	G33-4	FischerScientific
	Paper for Protocols and Trizol Safety Sheet	36	348037	Office Depot
	30 mL Spray bottles (SDS and hand sanitizer)	80	B07T64FHXR	Amazon
	Toothpicks	8	B00PBK4NG6	Amazon
	Tube boxes	184	21-119	Genesee Scientific
	RNase, DNase, DNA & PCR inhibitors free Microcentrifuge tubes	83	22-281	Genesee Scientific
	Gallon Ziploc bags	18	B07BJ495GL	Amazon
	Small Ziploc bags	8	B01LRKEI9K	Amazon
	Swiffer WetJet pads (swabs)	8	B08MT43J1Z	Amazon
	Sample ID solvent resistant labels	68	XST-10C1-1WH	LABTAG
	Coolers	1,200	B00363X3F2	Amazon
	Ice packs	108	B075GLD3X1	Amazon
	Total:	**1,893**		
Multiplexed colorimetric RT-LAMP	WarmStart Colorimetric LAMP 2X Master Mix with UDG	208	M1800S	NEB
	RT-LAMP N2-Primers	7	n/a	IDT
	RT-LAMP E1-Primers	7	n/a	IDT
	Guanidine Hydrochloride	0	AAJ6548522	Alfa Aesar
	Synthetic SARS-CoV-2 RNA	14	VR-3276SD	ATCC
Trizol RNA extraction	Chloroform	2	C298-500	FisherScientific
	Molecular-grade absolute (200 proof) Ethanol	30	BP2818500	FisherScientific
	GlycoBlue	80	AM9515	Invitrogen
	Molecular-grade Isopropanol	3	BP2618500	FisherScientific
	Ammonium Acetate RNase-free	2	AM9070G	Invitrogen
	TURBO DNA-free Kit	106	AM1907	Invitrogen
	Trizol RNA extraction + LAMP SARS-CoV-2 detection:	**458**		
Viral RNA extraction kit	QIAamp DSP Viral RNA Mini Kit	572	61904	Qiagen
RT-qPCR	TaqMan Fast Virus 1-Step Master Mix	185	4444432	Applied Biosystems
	SARS-CoV-2 (2019-nCoV) N1,N2 Primers and Probes	20	10006713	IDT
	Synthetic SARS-CoV-2 RNA	14	VR-3276SD	ATCC
	Kit RNA extraction + RT-qPCR SARS-CoV-2 detection:	**790**		

Table 3.5 Formulation for 20X RT-LAMP Primer Mix. In the RT-LAMP reaction, 6 primers recognize 8 regions of the targeted DNA.

Primer	20X Concentration (µM)	1X Concentration (µM)
FIP	32	1.6
BIP	32	1.6
F3	4	0.2
B3	4	0.2
LoopF	8	0.4
LoopB	8	0.4

Table 3.6 Reaction Master Mix for multiplex colorimetric RT-LAMP.(*) Guanidine Hydrochloride has been shown to increase the sensitivity and speed of the reaction by an uncharacterized mechanism (Y. Zhang, Ren, et al. 2020b).

Reagent	Volume rxn^{-1} (µl)
WarmStart Colorimetric LAMP 2X Master Mix with UDG	12.5
N2 Primer Mix (20X)	1.25
E1 Primer Mix (20X)	1.25
Guanidine Hydrochloride (600 mM)*	2.5
Target RNA	5
Nuclease-free H2O	2.5
Total volume:	**25**

Acknowledgements

Chapter 3, in full, is published in Jove, 2021. Maria I. Rojas, Giles SS, Little M, Baron R, Livingston I, Dagenais RT, Baer J, Cobián-Güemes AG, White B, Rohwer F 2020. The dissertation author was the primary investigator and author of this paper.

I would like to thank the Viral Information Institute (VII) investigators, Dr. Anca M. Segall, Willow Segall, Patricia L. Rohwer, Gary Rohwer, Cary L. Rohwer, Magda Silvia Pinetta, Elizabeth Cruz Cano, Dr. Gregory Peters, Dr. Stuart A. Sandin, and Dr. Jennifer Smith for taking the time to collect numerous samples. Dr. Rob Knight, Dr. Jack Gilbert, Dr. Pedro Balda-Ferre, and Dr. Sarah Allard from the department of Pediatrics at the School of Medicine University of San Diego California (UCSD) for facilitating positive controls and useful feedback. Stacey Carota (SDSU College of Sciences) and Gina Spidel (SDSU) for the logistical support. Juan Rodríguez for the art and graphic design of the sampling protocol. To all for the commitment and dedication to this project during very difficult times. This work was supported by a generous donation from Dr. Jo Ann Lane (SDSU College of Sciences), and the National Science Foundation RAPID: Environmental Reservoirs of SARS-CoV-2 grant (Award Number:2030479).

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Chapter 4 : Viruses in the urban environment and evidence for low risk of SARS-CoV-2 fomite transmission amidst the COVID-19 pandemic

Abstract

During the 2020 SARS-CoV-2 global pandemic, data concerning the risk of transmission by inhalation or direct contact with infected fomites primarily targeted mass-transit systems, hospitals, and laboratory-controlled environments. To identify environmental reservoirs for SARS-CoV-2 and other RNA viruses and to estimate the risk of fomite transmission, a large unbiased *in situ* screening of public and private locations of San Diego County was performed by citizen scientists. An unprecedented dataset of samples from commonly touched and rarely disinfected surfaces was generated to better understand the presence of SARS-CoV-2 in the urban environment. A surface positivity rate of 0.6% was found over a total of 3,282 samples collected at the main populated ZIP Codes of San Diego County, confirmed by RT-LAMP and RNA sequencing. Additionally, the viral RNA community was greatly composed of fecal-associated viruses. Mathematical modeling, using official case reports from the initial phase of the pandemic and experimental data, predicted new daily COVID-19 infections in the community caused by fomite transmission under the studied conditions. This study presents the largest spatiotemporal study to date that quantifies the presence of an airborne human virus on surfaces amidst a global pandemic.

Introduction

The severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic began in December 2019 with dozens of cases of viral pneumonia in China and henceforth quickly spread across the world. To date, over 130M COVID-19 cases and 2.8M COVID-19 associated deaths have been confirmed by the World Health Organization. During the first year of the pandemic, public health efforts aimed at preventing the spread of the virus primarily focused on surface disinfection and mandatory mask wearing in public spaces. In the United States, the global pandemic was declared a national emergency on March 13th, 2020. Several months later, the amalgamation of global efforts to control community transmission failed to flatten the curve of rising COVID-19 cases. Meanwhile, environmental reservoirs of SARS-CoV-2 on inanimate surfaces, with which humans commonly interact, remained an underexplored aspect of the ongoing pandemic.

Enveloped RNA viruses, and specifically closely related Coronaviruses such as MERS and SARS-CoV-1, are known to persist in the environment. Based on that evidence, studies have tested the presence of SARS-CoV-2 in the urban environment by targeting mass-transit environments (Danko et al. 2020) and risk assessment of transmission through contaminated fomites has focused on routinely disinfected surfaces at hospital wards or rooms harboring COVID-19 patients (Colaneri, Seminari, Novati, et al. 2020; Colaneri, Seminari, Piralla, et al. 2020; Ong et al. 2020; Döhla et al. 2020; Santarpia et al. 2020; Chia et al. 2020). The stability of viable SARS-CoV-2 on surfaces has been reported to be up to 72 hours with a decay rate dependent on the size of the shed inoculum (Van Doremalen et al. 2020; Pastorino et al. 2020). At high titer inoculum, the virus remains viable in the environment for a minimum of 3 days at 30°C (nasal cavity temperature) and 24 h at 40°C (body fever temperature), with 50%

humidity on non-porous surfaces (Riddell et al. 2020). Outdoors, UV light contributes to viral decay (Lytle and Sagripanti 2005) and simulated sunlight (UVB) has been seen to inactivate small droplets of SARS-CoV-2 within minutes (Ratnesar-Shumate et al. 2020). Similarly, detergents that disrupt the viral envelope (e.g., 0.6 % SDS), and other disinfectants that disrupt the lipidic membrane, denature proteins, or cause oxidative stress (e.g., 0.6 % sodium hypochlorite, 0.5% hydrogen peroxide, and 70 % ethanol) successfully deactivate enveloped viruses (Linley et al. 2012; Kampf 2018; Mcdonnell and Russell 1999; Kampf et al. 2020; Salido et al. 2020). Although extensive research on the persistence of viruses in the environment and the efficiency of disinfectants is available, the prevalence of these viruses in the environment, the probability of encounter with humans, and their ability to cause infection, remain virtually unknown.

The transmission of SARS-CoV-2 is predominantly airborne(Tang et al. 2021; Miller et al. 2020; Stadnytskyi et al. 2020; Prather et al. 2020; Morawska and Milton 2020), however, infection by other respiratory viruses, such as Influenza A, has been seen to occur, although rarely, through contaminated fomites (Asadi et al. 2020), and recently this has been considered for SARS-CoV-2 (Ferretti et al. 2020; Eslami and Jalili 2020; Al Huraimel et al. 2020). Evidence for viral transmission through inhalation of or direct contact with infected fomites remains anecdotal (Liu et al. 2020; Xu et al. 2020), although an environmental isolate of SARS-CoV-2 has been shown to replicate in the upper respiratory tract of an animal model (Shi et al. 2020). Large-scale *in situ* screenings outside of laboratory-controlled conditions are necessary to determine environmental reservoirs of the virus and to estimate the risk of transmission through contaminated fomites.

This study presents a large-scale transcriptomic survey of the urban environment during the initial phase of the COVID-19 pandemic. Here, volunteers were recruited to swab surfaces they often interact with across San Diego County. Samples collected by citizen scientists were processed and analyzed to determine the presence of SARS-CoV-2 using RT-LAMP. Additionally, total RNA sequencing was used to characterize the microbial and viral communities associated with urban surfaces. Lastly, mathematical modeling that combines official case reports and the experimental data collected in this study, was used to predict the potential contribution of fomite transmission to daily new COVID-19 infections in the community. To the best of our knowledge, this is the first *in situ* study to combine community building and molecular techniques to conduct a large-scale unbiased assessment of SARS-CoV-2 on surfaces in households and public spaces amidst the ongoing pandemic.

Results

Sample collection across San Diego County

Over a period of 6 months in 2020, from mid-March to mid-September, 225 citizens from the main populated districts of San Diego County volunteered to swab the urban environment. Citizen scientists were provided a collection kit for 16 samples and encouraged to sample an area of 10 cm^2 on surfaces that are seldomly disinfected and relevant for their everyday routines. Cumulatively, volunteers spent over 675 hours performing the sampling, and the total sampled area was equivalent to approximately 328,200 fingertips (1 cm^2 each) **(Figure 4.1)**.

The samples were collected from 74 of the 113 ZIP Codes in the county which harbor 87% of the total cases (37,349/42,737) and 90% of the San Diego population. There was no

correlation between the number of collected samples and the COVID-19 cases on the day of sampling at the ZIP Code where the surface was swabbed **(Figures 4.1, 4.2)**. However, despite efforts to engage the areas with higher Hispanic/LatinX population, these ZIP Codes were underrepresented in the sampling.

Persistence of SARS-CoV-2 RNA on frequently touched surfaces

To establish the limit of detection (LOD) of the multiplexed colorimetric RT-LAMP (NEB) assay, different concentrations of synthetic SARS-CoV-2 RNA (ATCC) were tested in replicates. The LOD of the reaction was 300 copies per sample (sampling area = 10 cm^2) with a beta error (probability of a false negative) of 36% **(Figure 4.3)**. At 3,000 viral copies per sample, the positivity rate was >90%. To rule out false negatives due to inhibitors persisting after RNA extraction, all samples were run in an additional reaction spiked with synthetic SARS-CoV-2.

A total of 3,708 samples were collected; 424 samples were excluded from the analysis: 126 (3.4%) had no collection data and 298 (8%) had an inconclusive RT-PCR due to the presence of inhibitors. Only samples that amplified the spiked control template were used for further analysis. From the remaining 3,282 samples, 3,262 (99.4%) were negative and 20 (0.6%) were positive for SARS-CoV-2 nucleocapsid or envelope genes **(Figures 4.1)**. Ten citizen scientists collected more than one positive sample on the same day at neighboring sampling sites, which increased the number of positive samples to 39. Because this was likely a result of cross contamination by the sampler, only one positive sample per collection effort was counted, which reduced the number to 20 positive samples. Interviews with these volunteers found that 2 had a negative RT-qPCR test close to the time of sampling; 2 were

negative for antibody tests 3 months after sampling; and 6 were not aware of being sick with COVID-19 or having been in contact with anyone with symptoms.

Surfaces were manually curated into 20 categories based on sampling sites location; each category contained indoor and outdoor sites and surfaces of different materials (i.e., metal, plastic, cardboard, cement). To estimate the approximate number of virions on each sample, representative surfaces of the sampling site categories were analyzed by microscopy. The number of virions ranged from $5.9x10^4$ to $4.0x10^5$ per surface area sampled (10 cm^2), with an average of $1.3x10^5$. A total of $4.4x10^{10}$ virions were collected, of which $6x10^3$ were estimated to be SARS-CoV-2 virions. The most sampled sites were doorknob/handle/railing ($9.2x10^9$ total virions) and home/car ($7.6x10^9$ total virions) and the highest proportion of positive samples were found at playground/park/bench (20%) and doorknob/handle/railing (15%) **(Table 4.1)**.

RNA community on swabbed surfaces

To characterize the RNA community present on the sampled surfaces, 20 SARS-CoV-2 positive samples and 18 negative samples –collected at similar sampling sites– were sequenced **(Figure 4.4)**. From a total of 1,017,213,518 raw reads, 191,532,435 passed quality filtering and dereplication. Samples were mainly composed of eukaryotic (64%) and bacterial (35%) sequences, with the remaining 1% corresponded to viral, archaeal, and plasmid-associated sequences **(Figure 4.5A, Table 4.2)**. Analysis of the viral RNA assigned taxa reveled that all samples were rich in fecal-associated viruses **(Figures 4.5B, Table 4.3)**. Relative abundances between the two groups were analyzed with two approaches: (1) Shannon diversity, Pielou's evenness, and richness analyses showed higher overall diversity in

the negative group, determined by a Wilcoxon exact test (p-value 0.015). (2) A classification Random Forest for two groups, positive and negative samples, was used to perform a dimension reduction from 77 to 12 taxa selected from a variable importance plot. An additional Random Forest was run on the 12 taxa, which showed an out of bag miss-classification error of 24%. A permutation test applied to the Random Forest variable importance estimated a significant (unscaled) mean decrease accuracy for Pepper Mild Mottle Virus (PMMV, NC_003630.1) (p-value = 0.01), Ryegrass Mottle Virus (RMV, NC_003747.2) (p-value = 0.01), Black Queen Cell Virus (BQCV, NC_003784.1) (p-value = 0.04), and Sinapis Alba Cryptic Virus 1 (NC_030243.1) (p-value = 0.05). A different permutation test was performed on the 4 significant taxa which identified PMMV (p-value 0.001) and RMV (p-value 0.02) as drivers of variation between the two groups **(Figure 4.6)**.

One of the SARS-CoV-2 positive samples contained enough copies of the viral genome to be detected by sequencing **(Figure 4.5B)**. Reads mapped against the SARS-CoV-2 reference genome (NC_045512.2) were distributed along the genome indicating that the signal does not correspond to amplicon contamination **(Figure 4.5C)** and showed 32% genome recovery.

COVID-19 cases predicted from fomite transmission

To examine the accuracy with which the sample size captured the surface positivity rate of SARS-CoV-2 (0.6%), a statistical power analysis of several hypothesis tests was performed based on the variation of cumulative number of COVID-19 cases per person distributed across all ZIP Codes in San Diego County. At 80% power, the uncertainty analysis confirmed that the SARS-CoV-2 positivity rate with a sample size of 3,282 falls between

0.566% and 0.653% **(Figure 4.7)**. If the number of samples were increased to 10,000, the positivity rate falls between 0.585% and 0.634% **(Figure 4.7)**.

To test whether a 0.6% surface positivity rate significantly contributes to the COVID-19 cases in the community, the number of new COVID-19 cases per day in San Diego County due to fomite transmission was estimated as the product of the probability of infection per virion and the number of contacts with the virus per day. To predict the probability of infection per virion from a surface, a model was built to describe SARS-CoV-2 transmission dynamics from COVID-19 case data in San Diego County **(Figure 4.8)**. The model estimated the probability of infection per SARS-CoV-2 virion to be $8.3x10^{-8}$. In a scenario with a 0.6% surface positivity rate, using 300 LOD of SARS-CoV-2 virions present per positive sample, and assuming a mean of 2,000 touches per hour, up to 7 new COVID-19 infections per 100,000 people in San Diego are explained by fomite transmission per day **(Figure 4.9)**.

Discussion

Here, a large-scale unbiased screening was performed on surfaces of the urban environment across San Diego County to identify environmental reservoirs of SARS-CoV-2. This study investigates fomite-mediated transmission of SARS-like viruses outside healthcare facilities and on surfaces that are rarely cleaned or disinfected, distinctly from other reports (Y. C. Chen et al. 2004). An unprecedented dataset containing thousands of samples showed the presence of SARS-CoV-2 RNA on 0.6% of swabbed surfaces. Additionally, metatranscriptomic analysis evidenced that the viral RNA community at these surfaces were mainly comprised of fecal-associated viruses. Mathematical modeling using local COVID-19 case rates and experimental data estimated that a small fraction of daily new COVID-19

infections was caused by fomite transmission. These findings are relevant to accurately design and implement prevention measures and strategies that reduce community transmission.

The sampling experimental design aimed to capture, through the location of the collection sites, the exposure realities of a diverse community over the course of the pandemic. To achieve this, volunteers swabbed surfaces the community interacts with on regular bases at households and public places, while avoiding putting themselves at risk or deviate from their daily routines when sampling for the virus. The cumulative sampled surface area was equivalent to 328,200 fingertips across San Diego County. In addition to recruiting citizen scientists to collect samples, the community outreach provided them with real-time updates of the results which maintained engagement. Surface types were diverse and positive samples were found throughout surface materials showing no evidence of a specific surface reservoir for the virus in the urban environment.

Positive samples occurred throughout the entire sampling period and across ZIP Codes with varied local infection rates. In fact, only one positive sample was collected at a ZIP Code with the third highest infection rate in the county **(Figure 4.1)**. These findings assert that the sampling was unbiased and not correlated with infection rates both spatially and temporally. Furthermore, given the distribution of COVID-19 cases across San Diego County, the uncertainty analysis confirmed that the sample size of the study was sufficient to predict the surface positivity rate.

While the average UV index for Southern California during the sampling period was moderate to high (United States Environmental Protection Agency), no significant differences were found in the proportions of positive samples that were collected outside or indoors. Since studies have shown that simulated sunlight reduces viral viability within minutes on

non-porous surfaces (Ratnesar-Shumate et al. 2020), the positivity rate of 0.6% and RNA-based detection are likely factors that limit this observation. Future studies focused on SARS-CoV-2 viability in non-laboratory settings are necessary to better assess the survival of the virus in the environment, and accurately predict the probability of infection by contact with contaminated fomites.

The reported number of positive samples accounted for potential cross-contamination during sampling, thus the positivity rate comprises single shedding events. Interviews with volunteers who collected more than one positive sample revealed that three were collected from outside and inside a delivery package; three were collected in different areas of the same park after being crowded during Memorial Day weekend; and six were collected inside a home of people whose occupation is housekeeping. The possibility that positive samples captured an event of direct shedding by the sampler or by transport of contaminated fomites from a viral hot spot such as a COVID-19 hospital ward is low since no interviewed volunteers expressed having a COVID-19 case in their household during the sampling time. Additionally, citizen scientists were instructed to wear an N95 mask while sampling, to change gloves between samples, and to apply hand sanitizer when finishing sampling. In view of these confounding effects, the results of this study represent a realistic surface positivity rate of SARS-CoV-2 in the urban environment under the experimental conditions.

In addition to understanding the prevalence of SARS-CoV-2 on urban surfaces, other human-associated viruses and microbes were characterized in a representative subset of samples using metatranscriptomic analyses. The viral RNA community present on various surfaces was largely comprised of viruses commonly found in the gastrointestinal tract. Statistical analysis revealed that Pepper Mild Mottle Virus (PMMV), a human fecal-

associated plant pathogen, is one of the largest drivers of the differences between SARS-CoV-2 positive and negative samples. The ubiquity of PMMV virus on the swabbed surfaces emphasizes the ecological and agricultural relevance of humans as transmission vectors (Naqvi 2004; T. Zhang et al. 2006). Furthermore, the high prevalence of fecal-associated viruses on all surfaces highlights the importance of urban environmental reservoirs in the dispersal of gastrointestinal viruses. Surface reservoirs are indicators of microbial and viral movement through air, where they are shed by exhalation, and through surfaces, where they are deposited by touch or urban runoff. This is particularly relevant for frequently touched and rarely disinfected surfaces that interconnect human populations throughout their daily routines, where surveillance before, during, and after an outbreak informs prediction and strategies to reduce risk of infection.

SARS-CoV-2 is mainly transmitted through air droplets and aerosols (Tang et al. 2021; Miller et al. 2020; Stadnytskyi et al. 2020; Prather et al. 2020; Morawska and Milton 2020), however, virions are potentially brought into contact with facial mucosa by unwashed hands that have touched a contaminated surface. Virus viability in the environment over time is often correlated with the size of the shed inoculum, and risk of encounter with contaminated surfaces highly depends on individual human behavior. In this study, frequencies of surface touching were assumed for a wide range of occupations assuming 2,000 touches per hour as the mean interaction intensity; in scenarios like office work, a person has been seen to touch between 0 and 300 surfaces per hour (N. Zhang, Li, and Huang 2018; N. Zhang and Li 2018; Kraay et al. 2018; Li et al. 2009). Considering this alternative infection route, and assuming SARS-CoV-2 persistence of viability in the environment, the use of common disinfectants at the correct concentration efficiently deactivates the virus after

only one minute exposure (Kampf et al. 2020; Salido et al. 2020; Cook et al. 2015). For example, SDS-containing soaps and sodium hypochlorite solutions, which are found in households, disrupt the viral envelope, and denature proteins that form the viral capsid (Linley et al. 2012; Kampf 2018; Mcdonnell and Russell 1999; Kampf et al. 2020; Salido et al. 2020). In this study, 0.5 % SDS and TRIzol Reagent were used to ensure viral inactivation to protect citizen scientist, and to preserve the RNA at room temperature (Mcdonnell and Russell 1999; Rio et al. 2010b). Other models have assessed the risk of SARS-CoV-2 fomite transmission under laboratory conditions and subsequently emphasized the importance of surface disinfection, hand washing, and household structural design (Kraay et al. 2020; Pitol and Julian 2021; Wilson et al. 2020; Wijaya et al. 2020). Nevertheless, the probability of fomite transmission fluctuates across viral variants or strains, environmental conditions, and social factors.

Analyses combining experimental data and COVID-19 cases in San Diego County predicted that with the frequency at which SARS-CoV-2 was found on surfaces, fomite transmission in the community is rare. The estimated risk of infection per virion was obtained from fitting a transmission dynamics model to the number of COVID-19 cases during the initial phase of the pandemic. While confirming findings from other studies that address the survival of the virus on surfaces, here, the presence of viral RNA outside laboratory settings under real-life conditions demonstrated a 0.6% surface positivity rate. From these results, 7 daily new infections per 100,000 people are predicted to occur in San Diego County from contact with contaminated fomites. This proportion represents an even smaller fraction of the total daily infections since reported cases lack accounts for untested symptomatic individuals (i.e., mild infections that do not warrant hospitalization) and asymptomatic individuals that

are unaware of the disease. This study provides a range of values for a sensitivity analysis, where the numbers are estimates under the conditions of the uncertainties dealt with during experimentation (e.g. temporal variation of the surface positivity rate, human behavior through surface touches per hour). Furthermore, as the virus mutates and becomes better at spreading from person to person, selection of attenuated variants that persist better and longer on surfaces will follow.

This work contains the largest study yet to monitor a virus of global epidemiological concern and to characterize microbial and viral communities on surface reservoirs in urban human ecosystems, while engaging the local community for education, participation, and public health awareness. Future studies testing survival and infectivity of these viruses under uncontrolled environmental conditions are the next step. Experimental data combined with mathematical modeling found strong support for low risk of transmission of SARS-CoV-2 by contact with contaminated fomites, even when detection was limited to viral RNA. These findings serve as a baseline for urban environmental surveillance during the ongoing SARS-CoV-2 pandemic; similar approaches implemented by the public health sector will inform accurate prevention and contention efforts in future outbreaks.

Methods

Community engagement

A call-to-action was released by local media outlets to invite citizens to participate in the sampling effort. Volunteers were approved based on their willingness to handle the material contained in the kit and their commitment to exclude human samples from the collection. Citizen scientists were asked to avoid actively searching for the virus but rather

sample sites that were part of their daily routines. A website was created to serve as communication interface between the lab and the citizen scientists and for users to record sample collection details: date, time, sampling site description, and a picture of the sampling site with embedded GPS coordinates. The website also contained a detailed protocol and a video with sampling instructions in English and Spanish. Sample collection kits were delivered to each volunteer and the website was used to request pickup of the samples once all data was recorded. A laboratory information management system (LIMS) was built to facilitate sample organization and processing, and to serve as a database for participant and sample information. (Rojas et al. 2021)

Sample collection

Sampling kits contained materials to collect 16 samples. Each kit consisted of gloves, 1 x 1 cm polyester swabs, prelabeled microcentrifuge tubes containing 150 µL of Trizol inside a cardboard box protected by a plastic bag, a spray bottle with 0.5 % SDS, a Safety Data Sheet for TRIzol Reagent, a detailed sampling protocol, a waste bag, ice packs, a cooler, and hand sanitizer. A swab was soaked in 0.5 % SDS to inactivate the virus. An area of 10 x 10 cm was swabbed. The swab was immediately inserted in a microcentrifuge tube containing Trizol to preserve the RNA. The tube was placed in a cooler with an ice pack to maintain the samples cold until pickup. A different pair of gloves was used for each sample to avoid cross contamination. (Rojas et al. 2021)

RNA isolation

A crude extraction (Chomczynski and Sacchi 2006; 1987) was performed on pooled samples. Briefly, 50 µL of each of 8 samples were pooled, 0.2 vol. of chloroform were added and mixed well. Pools were incubated at 4 °C for 20 min and centrifuged at 13,000 x g for 20 min at 4 °C. The aqueous layer was recovered, an equal volume of isopropanol and 2.6 µL of GlycoBlue (Invitrogen) were added, and the mix was incubated at -20 °C for 1 h. The pellet was recovered by centrifuging at 13,000 x g for 20 min at 4 °C, then resuspended in DPEC-treated water. An equal volume of 5M Ammonium Acetate and 2.5 vol. of 100 % ethanol was added, and the mix was incubated at -20 °C overnight. After centrifugation at 13,000 x g for 20 min at 4 °C, the pellet was washed twice with cold freshly made 75 % ethanol. The pellet was resuspended in DPEC-treated water and the RNA was treated with Turbo DNase (Invitrogen). (Rojas et al. 2021)

Multiplexed RT-LAMP

WarmStart® Colorimetric LAMP 2X Master Mix with UDG (NEB) was used following manufacturer's instructions with minor modifications: 60 mM guanidine hydrochloride was used instead of 40 mM; incubation at 65 °C was performed for 40 min instead of 30 min; reactions were run in multiplex with primers N2 and E1 from Zhang et al. 2020; 5 µL of sample RNA were loaded per reaction; if the pH of the sample changed the color of the reaction prior to incubation, an agarose gel was run to confirm all results. To discard false negatives, all samples were tested simultaneously in a reaction spiked with 450 copies of synthetic SARS-CoV-2 RNA (ATCC). (Rojas et al. 2021)

Estimation of viral count per surface sample

Surface samples were taken from the different sampling site categories (**Table 4.1**). Replicates were collected by repetitively pipetting 200 µL of SM buffer over an area of 1 cm^2 for each sample. Microbial and viral counts were taken using previously described methods (Haas et al. 2014). Briefly, immediately after collection, samples were fixed with paraformaldehyde (2% final concentration) for 30 min at room temperature. SYBR Gold (Invitrogen) was then added to a final concentration of 2X; after mixing vigorously, samples were incubated for 30 min at room temperature in the dark. Samples were concentrated on a 0.02 µm Whatman Anodisc 25 Filter, then mounted on a microscope slide. Virus-like particles (VLPs) were manually counted using an epifluorescence microscope using a threshold size of 0.02 µm; the reported counts are the average of 10 fields of view per sample. The number of virions on a surface sample was estimated from the average of 20 sample replicates.

Sequencing and metatranscriptomic analysis

The Swift RNA Library Kit (Cat# R1096) was used to construct the sequencing libraries according to the manufacturer's manual (Swift RNA Library Kit v3.0) with the following specifications: RNA Fragmentation off bead Step 3: 65 °C – 5 min; post RT SPRI Clean-up Step 2: SPRI ratio 1.2X, bead volume 60 µL; indexing PCR: Step 1: Unique Dual Indexing (Swift #X9096), Step 3: 18 cycles; post indexing second 0.85X SPRI Clean-up. Pre-amplification steps of the library preparation were performed in an amplicon-free room. Libraries ran in 2 lanes of an Illumina NovaSeq 6000 Sequencing System with a read length of paired end 100bp (PE100). PRINSEQ++ was used to quality filter reads as follows: "-

ns_max 0 -derep -lc_entropy=0.5 -trim_qual_right=15 -trim_qual_left=15 -trim_qual_type mean -trim_qual_rule lt -trim_qual_window 2 -min_len 30 -min_qual_mean 20 -rm_header" (Schmieder and Edwards 2011; Cantu, Sadural, and Edwards 2019). Quality-filtered R1 sequences were mapped against the NCBI Viral RefSeq and the NCBI SARS-CoV-2 databases with the Fragment Recruitment Assembly Purification algorithm (FRAP) (Cobián Güemes et al. 2016) that uses SMALT (Ponsting and Ning 2010) as a sequence aligner at 100% identity. A recruitment plot was generated by mapping SARS-CoV-2 hits against the NCBI SARS-CoV-2 genome reference sequence (NC_045512) using FRAP-tools. FRAP and FRAP-tools are located at the GitHub repository https://github.com/yinacobian/frap. In parallel, all quality-filtered R1 sequences were uploaded to MG-RAST (Meyer et al. 2008) for phylogenetic analysis. The MG-RAST web application uses BLAT (Kent 2002) to align sequences against various protein and rRNA databases with the following cut-off parameters: e-value $\geq 10^{-5}$, percent identity ≥ 60.

Diversity of viral RNA taxa in surface samples

Relative abundances of reads that mapped to the NCBI Viral RefSeq of 20 SARS-CoV-2 positive and 18 negatives were compared by two approaches. The Shannon diversity index, Pielou's evenness, and richness were calculated using the *diversity* function in the RStudio package *vegan* (Oksanen et al. 2020). In parallel, all 38 samples were analyzed by Random Forest (Breiman 2001) (trees = 5000) and a dimension reduction was performed. The top 12 taxa shown by a Variable Importance Plot was subjected to a new Random Forest (trees = 5000). The estimate permutation *p-values* for importance metrics was performed using the RStudio package *rfPermute* (Archer 2020). A two-sample permutation test was

performed on the taxa with significant *p-values* (<0.05) using the RStudio package *exactRankTests* (Hothorn and Hornik 2019) with a two-sided *perm.test*; taxa with significant *p-values* were plotted **(Figure S4)**.

Uncertainty analysis of sample size

To quantify the confidence level of the 0.6% SARS-CoV-2 surface positivity rate, an uncertainty analysis was performed using a hypothesis test that identifies the level of power achieved for the sample size (*n = 3,282*). To estimate the true proportion of surface positivity rate in San Diego County, the true proportion of SARS-CoV-2 positive surface (p_0), was compared to the experimental positivity rate (p_1). Specifically, the statistical power was computed as a function of the sample size that asserts $p_1 > p_0$ and $p_1 < p_0$. The analysis was performed for a wide range of p_0, and for each sample size, p_l and p_u were estimated using an 80% power (generally accepted threshold) to assert $p_1 > p_0$ and $p_1 < p_0$, respectively. p_l and p_u provide the lower and the upper limits of the confidence interval for each sample size. Because the distribution of potential positive surface samples across the county is correlated to the spatial distribution of COVID-19 cases in the county, the standard deviation ($\sigma = 0.01$) was calculated from the reported cumulative COVID-19 cases per person in all 113 ZIP Codes in San Diego County. The σ in the 74 sampled ZIP Codes was also 0.01.

Probability of new COVID-19 cases by fomite transmission

To predict the number of new COVID-19 cases per day in San Diego County that are likely due to fomite transmission, the number of times an individual touches a surface

contaminated with SARS-CoV-2 per day and the risk of infection per SARS-CoV-2 virion were calculated:

New COVID-19 cases in San Diego from fomites day^{-1} = *Total SARS-CoV-2 virions touched day*$^{-1}$ (1) • *Probability of infection SARS-CoV-2 virion*$^{-1}$ (2)

(1) The total SARS-CoV-2 virions touched per day in San Diego County was calculated by the following equation:

$$SARS2\ touched\ per\ day = Individuals\ \cdot T_{ave} \cdot V_{ave}$$

Where:

$$T_{ave} = \frac{SARS2\ positive\ samples}{total\ sampled\ area} \cdot \frac{area}{touch} \cdot \frac{touches}{hour} \cdot \frac{24hours}{day}$$

$$V_{ave} = \frac{SARS2}{sample}$$

Assuming: Individuals = 100 or 100,000

Positive SARS-CoV-2 samples = 20

Total sampled surface area = 328,200 cm^2

Surface area $touch^{-1}$ = 1 cm^2

Surface touches per person $hour^{-1}$ = 0 – 4,000 (mean = 2,000)

SARS-CoV-2 virions $sample^{-1}$ = 0 – 10^6 (LOD = 300)

(2) The probability of infection per SARS-CoV-2 virion (*p*) was estimated by a COVID-19 transmission dynamics model **(Figure 4.8A)**. To estimate the parameter *p* along with other uncertain parameters, the model was fitted to the new COVID-19 case data for San Diego County **(Figure 4.8B)**. In the model, the amount of virus in the environment (*V*) was incorporated, and the total population was composed of the following classes: susceptible

individuals (*S*), pre-infectious individuals (*E*), symptomatic infected individuals (I_s), asymptomatic infected individuals (I_a), and recovered or dead individuals (*R*). Susceptible individuals become infected directly from $E, I_s,$ and I_a classes at infectious rates of $\beta_e, \beta_s,$ and $\beta_a,$ respectively, per individual per day. Susceptible individuals become infected indirectly from virus in the environment, $V,$ assumed to be dispersed across San Diego County.

In this case, the average virus per sample area is $\frac{V}{p_1 r_a}$, where p_1 is the positivity rate (0.6%), and r_a is the ratio between the San Diego area and the sampled area. Following the formulation above, the daily new infections due to fomite transmission is $\frac{T_{ave}}{p_1 r_a} pVS$. Due to the behavior of the data and policy implementation, the model includes three time phases with different infectious rates per phase ($\beta_{s1}, \beta_{s2}, \beta_{s3}, \beta_{a1}, \beta_{a2}, \beta_{a3}$). Pre-infectious individuals transition to one of the infectious classes at a rate of λ per day. θ represents the fraction of infected individuals who show symptoms, I_s, and $(1-\theta)$ the fraction of individuals who are asymptomatic, I_a. Infected individuals either recover or die and move into the R class at a rate of γ per day. E, I_s and I_a shed virus into the environment at rates of ρ_e, ρ_s, and ρ_a respectively, where the virus remains for $\frac{1}{\Omega}$ days.

The schematic of the transmission dynamics model (**Figure S5A**) is described by the following system of equations:

$$\dot{S} = -\left(\beta_e E + \beta_s I_s + \beta_a I_a + \frac{T_{ave}}{p_1 r_a} pV\right) S$$
$$\dot{E} = \left(\beta_e E + \beta_s I_s + \beta_a I_a + \frac{T_{ave}}{p_1 r_a} pV\right) S - \lambda E$$
$$\dot{I}_s = \theta\lambda E - \gamma I_s$$
$$\dot{I}_a = (1-\theta)\lambda E - \gamma I_a$$
$$\dot{R} = \gamma(I_s + I_a)$$
$$\dot{V} = \rho_e E + \rho_s I_s + \rho_a I_a - \Omega V$$

All computations were performed in MATLAB.

Figures and Tables

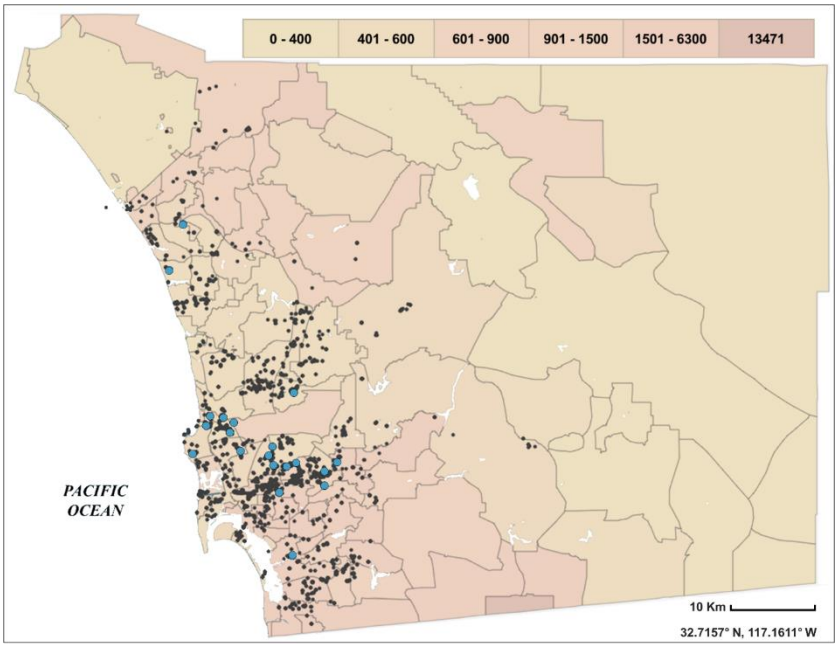

Figure 4.1 Distribution of sampling sites in San Diego County. A total of 3,282 samples were collected by 225 citizen scientists along the main populated ZIP Codes of San Diego County. The percentage of swabbed surfaces that are often touched and rarely disinfected that contained SARS-CoV-2 RNA was 0.6% (20/3,282). The total sampled surface area was equivalent to 328,200 fingertips (10 cm^2 per sample). The volunteers dedicated over 675 hours to sample collection. Black dots mark negative samples. Blue dots mark positive samples. The shades of orange correspond to the cumulative COVID-19 cases per 100,000 people at a given ZIP Code on the last day of sampling. (data retrieved from Rojas et al. 2021).

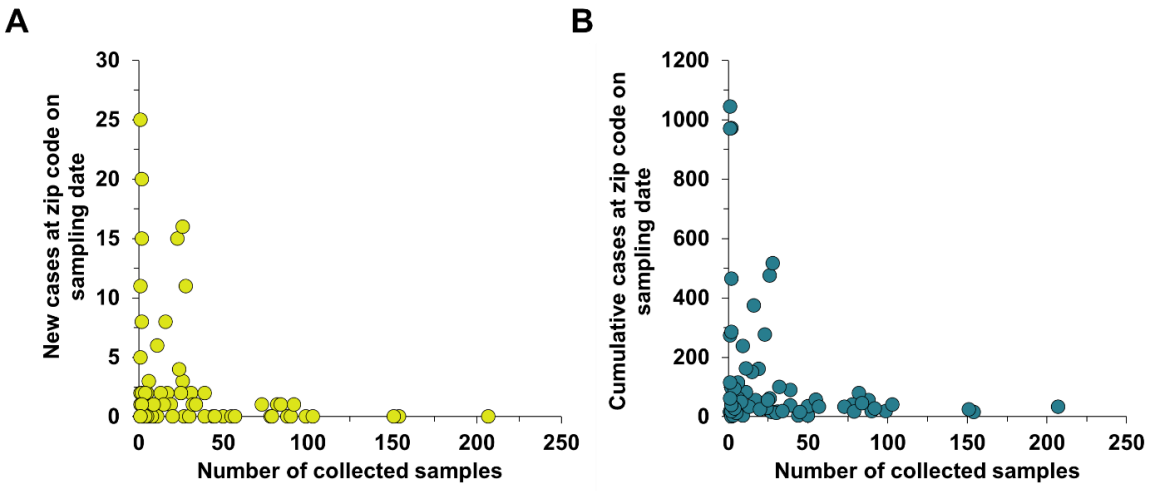

Figure 4.2 Reported COVID-19 cases at sampling ZIP Codes on the respective sampling day. There was no correlation between collected samples and **(A)** new cases or **(B)** cumulative COVID-19 cases at sampling ZIP Codes on the day that each sample was collected. Surfaces were swabbed in 74 ZIP Codes of San Diego County that harbor 90% of the population and 87% of the COVID-19 cases reported to date (SDHHSA).

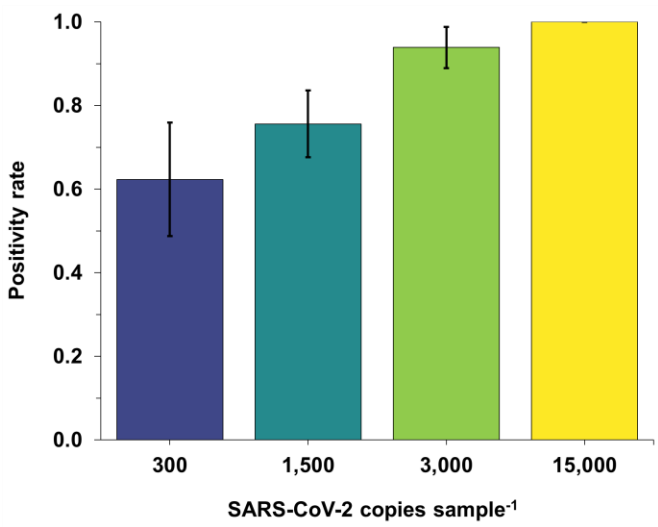

Figure 4.3 Limit-of-detection of RT-LAMP at different RNA template concentrations. The LOD of the assay is 300 copies of SARS-CoV-2 per 150 µl sample (sampling area = 10 cm^2). Positivity rate at different copy numbers per sample (10 replicates each): 300 copies – 64%; 1,500 copies – 76%; 3,000 copies – 94%; 15,000 copies – 100% (data retrieved from Rojas et al. 2021).

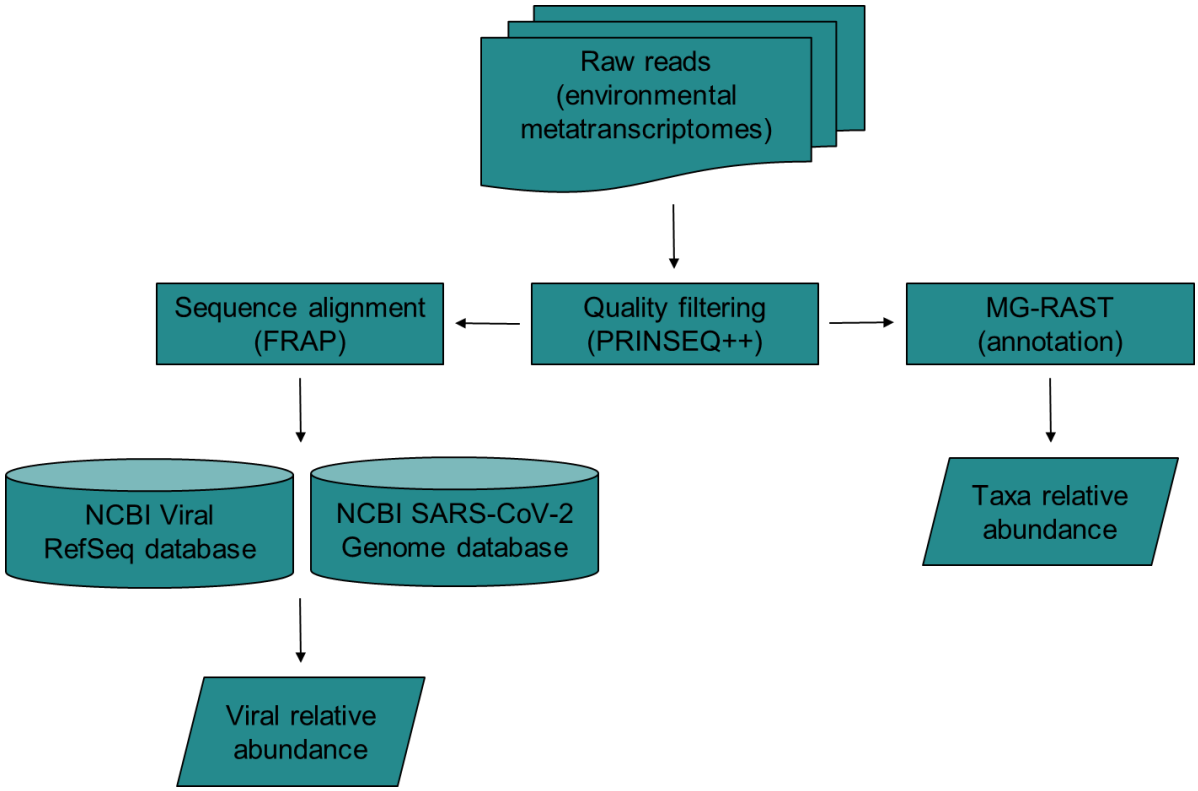

Figure 4.4 Bioinformatic pipeline for metatranscriptomic analyses. Quality-filtered reads were mapped against the NCBI Viral RefSeq and SARS-CoV-2 Genome databases using the SMALT aligner at 100% sequence identity. R1 sequences were uploaded to MG-RAST for taxonomic annotation. Relative abundances were calculated by dividing the number of hits by the total number of quality-filtered reads in a metatranscriptome.

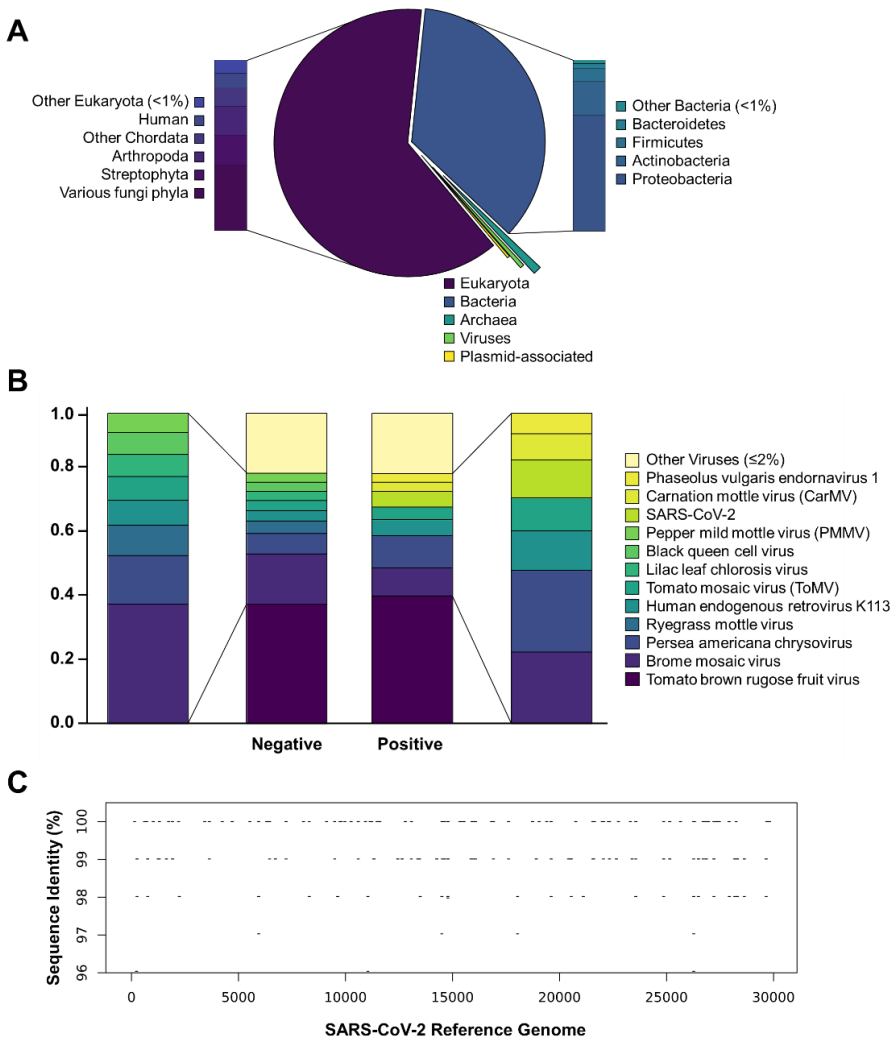

Figure 4.5 RNA community present at swabbed surfaces. **(A)** Phylogenetic analysis of RNA community. Proportion of reads assigned to Eukaryota, Bacteria, Archaea, Virus, and plasmid-associated sequences **(Table S2)**. **(B)** Viral RNA community present at surfaces. Averaged relative abundance of top 12 viral RNA taxa in 20 SARS-CoV-2 positive samples and 18 negative samples collected at similar surfaces. Samples are largely composed of fecal-associated viruses **(Table S3)**. Shannon diversity between the two groups was significantly higher in the negative group compared to the positive group as determined by a Wilcoxon exact test (p-value 0.015). Random Forest and a permutation test confirmed that this difference is driven by a significant increase in Pepper Mild Mottle Virus (p-value 0.001) and Ryegrass Mottle Virus (p-value 0.02) in the negative samples **(Figure S4)**. **(C)** Fragment recruitment plot of reads from a positive sample mapped against the SARS-CoV-2 reference genome (NC_045512.2).

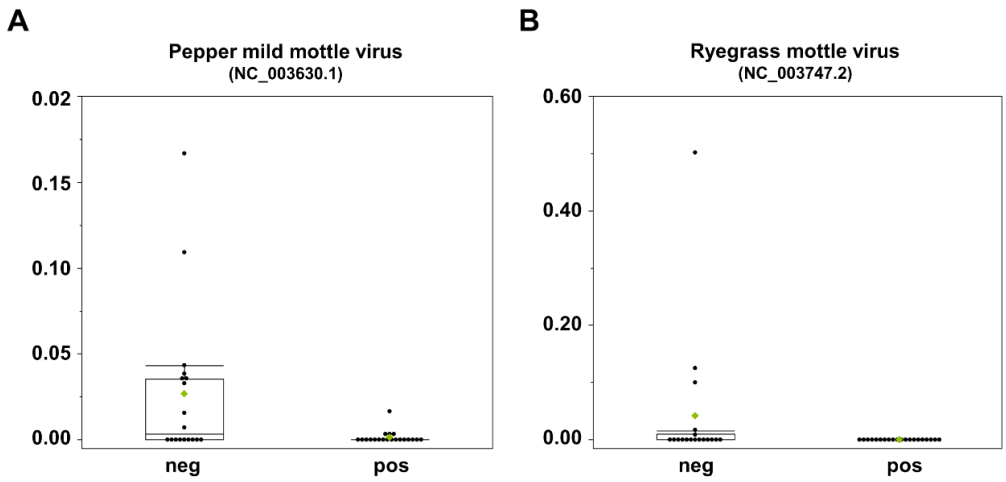

Figure 4.6 Results of a two-sample permutation test. **(A)** Pepper Mild Mottle Virus (p-value 0.001) and **(B)** Ryegrass Mottle Virus (p-value 0.02) are significantly higher in negative samples.

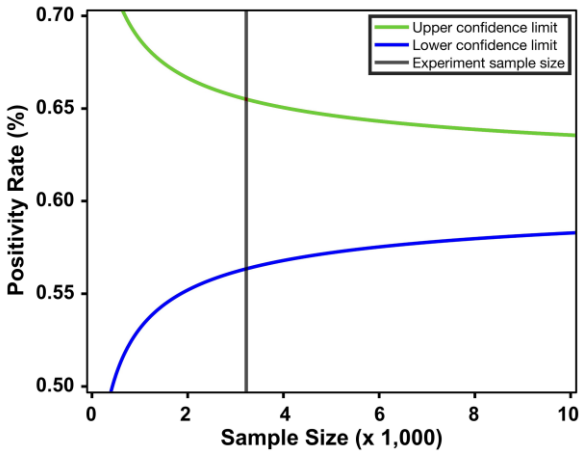

Figure 4.7 Uncertainty analysis of sample size. To determine the confidence interval that contains the true proportion of SARS-CoV-2 positive surfaces in San Diego County, several hypotheses were tested on the experimental surface positivity rate. The curves represent the upper (green) and lower (blue) bounds of the confidence interval for the 0.6% positivity rate at several sample sizes. To achieve 80% power, the surface positivity rate is between 0.566% and 0.653% based on the variation of cumulative number of cases by ZIP Codes per person in San Diego County. With a larger sample size of 10,000 samples, the confidence interval is smaller, and the proportion of expected positives falls between 0.585% and 0.634%. The back vertical line represents the 3,282-sample size used in the study.

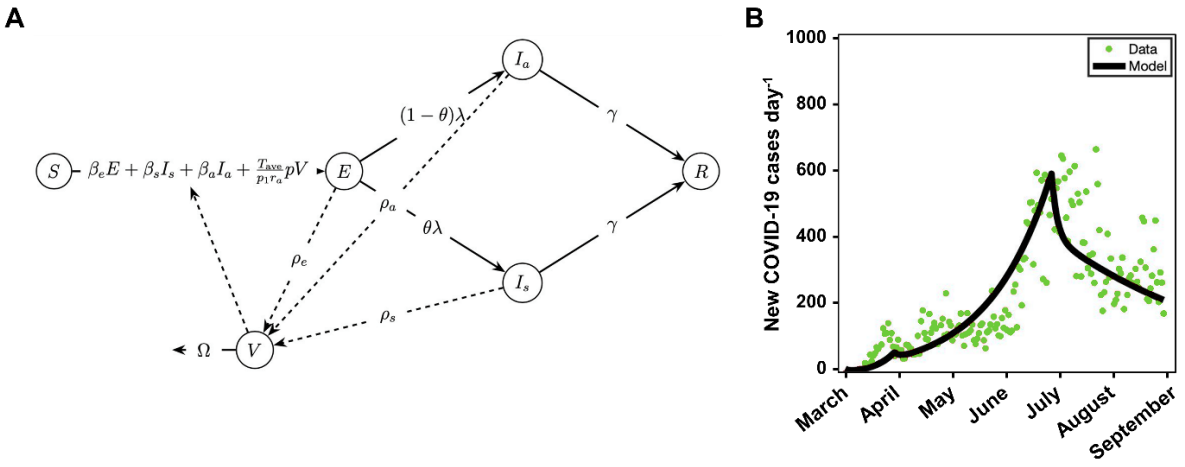

Figure 4.8 SARS-CoV-2 transmission dynamics model. **(A)** Schematic diagram of transmission dynamics in a population composed of the following classes: susceptible individuals (S), pre-infectious individuals (E), symptomatic infected individuals (I_s), asymptomatic infected individuals (I_a), and recovered or dead individuals (R). The model assumes that individuals become directly infected by direct contact with infected individuals (E, I_s, I_a) and from virus in the environment (V) at rates $\beta_e, \beta_s, \beta_a$ and $\frac{T_{ave}}{p_1 r_a} p$, respectively. Pre-infectious individuals become infectious at a rate of λ per day. θ is the fraction of infectious individuals that become symptomatic (I_s) and 1- θ is the fraction of infectious individuals that remain asymptomatic (I_a). Infected individuals recover or die and enter the R class at a rate γ per day. E, I_s, and I_a shed virus into the environment at daily rates ρ_e, ρ_s, and ρ_a, respectively, where the virus remains for $\frac{1}{\Omega}$ days. **(B)** SARS-CoV-2 transmission model fitted to San Diego County publicly available data on new COVID-19 cases per day during sampling months.

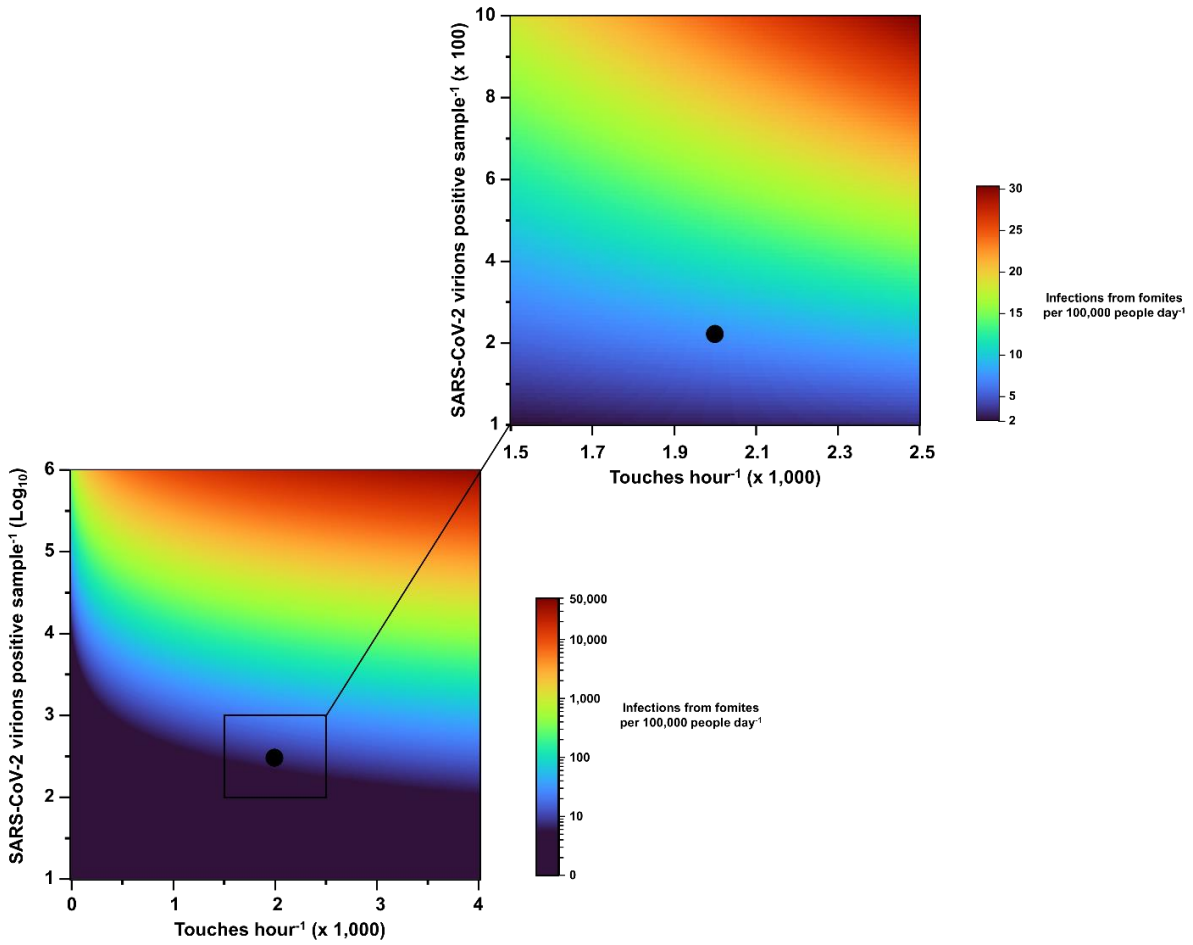

Figure 4.9 Proportion of new COVID-19 cases from fomite transmission. For a 0.6% surface positivity rate, a limit of detection of 300 SARS-CoV-2 virions per sample, and assuming a mean surface touch of 2,000 per hour, the transmission dynamics model **(Figure 4.8)** estimated the risk of infection per SARS-CoV-2 virion to be 8.3×10^{-8}. In this scenario (inset), up to 7 new COVID-19 infections per 100,000 people in San Diego county are explained by fomite transmission per day (black dot).

Table 4.1 Numbers of virions collected at different sampling sites. The number of virions per surface sample was calculated from the average of 20 swabbed surfaces, each of an area of 10 cm^2. The average number of virions per sample was $1.3x10^5$ ($5.9x10^4$-$4.0x10^5$) and the estimated total number of virions collected was $4.4x10^{10}$. Assuming the LOD of the assay of $3.0x10^2$ SARS-CoV-2 virions per sample, a total of $6.0x10^3$ SARS-CoV-2 virions were collected. The proportion of positive samples collected at each sampling site is specified and in parenthesis the number of positive samples at the corresponding sampling site category. Sampling surfaces were located at indoor and outdoor sites.

Sampling site category	**Total virions collected (x 10^7)**	**Total SARS-CoV-2 virions collected (x 10^2)**	**Proportion of positive samples (%)**
ATM	210	3.0	5 (1/20)
church	20	0.0	
crosswalk	190	3.0	5 (1/20)
delivery/takeout	240	6.0	10 (2/20)
doorknob/handle/railing	920	9.0	15 (3/20)
elevator button	60	0.0	
face mask	9.4	0.0	
gas station/car wash	230	3.0	5 (1/20)
home/car	760	6.0	10 (2/20)
keypad/touch screen	60	0.0	
mailbox	180	0.0	
other	370	3.0	5 (1/20)
phone/computer	130	3.0	5 (1/20)
playground/park/beach	310	12	20 (4/20)
public transport	90	6.0	10 (2/20)
restroom	230	0.0	
scooter	30	0.0	
shoe	40	0.0	
shopping cart	190	3.0	5 (1/20)
trashcan/dumpster	170	3.0	5 (1/20)
	4,400	60.	

Table 4.2 Taxonomic annotation of quality-filtered reads. R1 sequences aligned against protein and rRNA databases using MG-RAST.

Domain	Relative abundance (%)	Eukarya	Relative abundance (%)	Bacteria	Relative abundance (%)
Eukaryota	64	Various fungi phyla	91	Proteobacteria	68
Bacteria	35	Streptophyta	2.4	Actinobacteria	20
Viruses	0.2	Arthropoda	2.4	Firmicutes	7.9
Archaea	0.03	Chordata	1.5	Bacteroidetes	2.5
other	0.002	Human	1.2	Other (<1%)	2.0
(plasmid-associated)		Other (<1%)	1.1		

Table 4.3 Viral RNA community. Average of relative abundance of viral taxa in 18 negative and 20 positive samples.

Virus	Accession #	Negative	Positive
Alfalfa mosaic virus	NC_002025.1	1.6	0.11
Alternaria alternata chrysovirus 1	NC_040737.1	0	0.63
Avian myelocytomatosis virus	NC_001866.1	0	1.5
Bell pepper endornavirus	NC_039216.1	2.0	0.81
Bermuda grass latent virus	NC_032405.1	0.0092	0
Bipolaris maydis botybirnavirus	NC_040395.1	0.20	1.0
Black queen cell virus	NC_003784.1	3.0	0
Blueberry shock virus	NC_022252.1	1.2	0
Botrytis cinerea mitovirus	NC_011372.2	0.18	0
Botrytis cinerea mitovirus	NC_028471.1	0	0.18
Brome mosaic virus	NC_002028.2	16	9.0
Carnation mottle virus (CarMV)	NC_001265.2	0	3.3
Columbid alphaherpesvirus	NC_034266.1	0	0.00025
Cucumber green mottle mosaic virus (CGMMV)	NC_001801.1	0.24	0.89
Cucumber mosaic virus (CMV)	NC_002034.1	0	0.16
Cucumis melo endornavirus	NC_029064.1	0.053	0
Cucurbit yellow stunting disorder virus	NC_004810.1	0.35	0
Deformed wing virus	NC_004830.2	0.32	0
Enterobacteria phage GA	NC_001426.1	0.055	0
Enterobacteria phage Hgal1	NC_019922.1	0	1.3
Erysiphe necator mitovirus 1	NC_037054.1	0	0.38
Escherichia virus Lambda_1H12	NC_049947.1	0.95	1.9
Feline papillomavirus 2	NC_038520.1	0	0.12
Garlic virus A	NC_003375.1	0.055	0.0012
Grapevine associated narnavirus 1	NC_028473.1	0.26	0.21
Grapevine yellow speckle viroid 2	NC_003612.1	0.064	0
Helianthus annuus alphaendornavirus	NC_040799.1	0	0.0042
Hibiscus latent Fort Pierce virus	NC_025381.1	0.69	0
Homalodisca vitripennis reovirus	NC_012536.1	0	0.049
Hop latent viroid	NC_003611.1	0.092	0
Hot pepper endornavirus	NC_027920.1	0.19	0
Hubei picorna-like virus 51	NC_033226.1	0.46	0
Hubei tombus-like virus 2	NC_032965.1	0.69	0
Human endogenous retrovirus	NC_022518.1	3.4	5.1
Human herpesvirus 1	NC_001806.2	0.46	1.1
Human papillomavirus 41	NC_001354.1	0.096	0.36
Kashmir bee virus	NC_004807.1	1.6	0
Lilac leaf chlorosis virus	NC_025481.1	3.0	2.0
Medicago sativa alphapartitivirus 1	NC_040457.1	0.18	0
Merkel cell polyomavirus	NC_010277.2	0	0.041
Ostreid herpesvirus 1	NC_005881.2	0	0.00030
Paprika mild mottle virus	NC_004106.1	0	0.00089
Peanut stunt virus	NC_002038.1	0	0.11

Acknowledgements

Chapter 4, in full, is being prepared for submission for publication of the material. Maria I. Rojas, Little M, Giles SS, Baron R, Bloomquist A, Sanchez-Quinto A, Cantú VA, Van der Greer A, Cobián-Güemes AG, Burkeen G, Livingston I, Aquino J, Jon R, Baer J, Dagenais RT, Bailey BA, Vaidya N, Rohwer F. The dissertation author was the primary investigator and author of this paper.

I would like to thank the Viral Information Institute (VII) investigators, Dr. Anca M. Segall, Willow Segall, Patricia L. Rohwer, Gary Rohwer, Cary L. Rohwer, Magda Silvia Pinetta, Elizabeth Cruz Cano, Dr. Gregory Peters, Dr. Stuart A. Sandin, and Dr. Jennifer Smith for taking the time to collect numerous samples. I also thank Dr. Rob Knight, Dr. Jack Gilbert, Dr. Pedro Balda-Ferre, and Dr. Sarah Allard from the department of Pediatrics at the School of Medicine University of San Diego California (UCSD) for facilitating positive controls and useful feedback. We thank Stacey Carota (SDSU College of Sciences) and Gina Spidel (SDSU) for the logistical support and Juan Rodríguez for the art and graphic design of the sampling protocol. And the citizen scientist and co-authors for the commitment and dedication to this project during very difficult times. This work was supported by a generous donation from Dr. Jo Ann Lane (SDSU College of Sciences), and the National Science Foundation RAPID: Environmental Reservoirs of SARS-CoV-2 grant (Award Number:2030479). This publication includes data generated at the UC San Diego IGM Genomics Center utilizing an Illumina NovaSeq 6000 that was purchased with funding from a National Institutes of Health SIG grant (#S10 OD026929)

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Chapter 5 : A Distinct Contractile Injection System Gene Cluster Found in a Majority of Healthy Adult Human Microbiomes

Abstract

Many commensal bacteria antagonize each other or their host by producing syringe-like secretion systems called Contractile Injection Systems (CIS). Members of the Bacteroidales family have been shown to produce only one type of CIS—a contact-dependent Type 6 Secretion System that mediates bacteria-bacteria interactions. Here we show that a second distinct cluster of genes from Bacteroidales bacteria from the human microbiome may encode a yet uncharacterized injection system that we term Bacteroidales Injection System (BIS). We found that BIS genes are present in the gut microbiome of 99% of individuals from the United States and Europe, and that BIS genes are more prevalent in the gut microbiomes of healthy individuals than in those individuals suffering from inflammatory bowel disease. Gene clusters similar to that of BIS mediate interactions between bacteria and diverse eukaryotes like amoeba, insects and tubeworms. Our findings highlight the ubiquity of the BIS gene cluster in the human gut and emphasize the relevance of the gut microbiome to the human host. These results warrant investigations into the structure and function of BIS and how they might mediate interactions between Bacteroidales bacteria and the human host, or microbiome.

Introduction

Many bacteria produce syringe-like secretion systems called Contractile Injection Systems (CIS) that are related to the contractile tails of bacteriophage (bacterial viruses) (Cianfanelli, Monlezun, and Coulthurst 2016; Salmond and Fineran 2015). CIS are composed of conserved structural elements including a rigid inner tube surrounded by a baseplate complex and contractile sheath. Contraction of the sheath propels the inner tube through cell membranes often delivering protein effectors to target cells (Basler et al. 2012; Ge et al. 2020). Most CIS characterized to date, termed Type 6 Secretion Systems (T6SS), are produced and act from within an intact bacterial cell (**Figure 5.1A**). In contrast, extracellular CIS (eCIS) are released by bacterial cell lysis, paralleling the mechanism used by tailed phages to escape their bacterial host (N. J. Shikuma et al. 2014; Nakayama et al. 2000; Desfosses et al. 2019) (**Figure 5.1A**). Prominent producers of CIS are members of the Bacteroidetes phylum (*Bacteroides* and *Parabacteroides*), which constitute 20-80% of the total microbial composition (Huttenhower et al. 2012). To date, *Bacteroides* from the human gut have been shown to produce one type of CIS, a Subtype-3 T6SS that mediates bacteria-bacteria interactions and helps them colonize the human gut (Russell et al. 2014; Chatzidaki-Livanis et al. 2016; Verster et al. 2017; Coyne, Roelofs, and Comstock 2016).

Distinct from Bacteroidales Subtype-3 T6SS is a different class of CIS that may have evolved independently (Böck et al. 2017). Intriguingly, all previously described examples of these distinct CIS mediate bacteria-eukaryote interactions. Three of these CIS are classified as eCIS and include: (1) MACs (Metamorphosis Associated Contractile Structures) that stimulate the metamorphosis of tubeworms (Nicholas J Shikuma et al. 2016; Ericson et al. 2019; N. J. Shikuma et al. 2014), (2) PVCs (*Photorhabdus* Virulence Cassettes) that mediate

virulence in grass grubs (Yang et al. 2006), and (3) Afp (Anti-Feeding Prophage) that cause cessation of feeding and death of grass grub larvae (Hurst et al. 2007; Heymann et al. 2013; Hurst et al. 2018; Hurst, Glare, and Jackson 2004; Jiang et al. 2019). A fourth CIS from *Amoebophilus asiaticus* (Bacteroidetes phylum) (Böck et al. 2017) promotes intracellular survival in amoeba and defines the Subtype-4 T6SS group. Examples of this Subtype-4 T6SS have been functionally confirmed in *A. asiaticus* (Böck et al. 2017), and identified *in silico* in a few other Bacteroidetes genomes (Penz et al., 2012; Sarris et al., 2014). Recently, a genome-wide identification of bacterial extracellular contractile injection systems predicted more than 50 Bacteroidetes eCIS gene clusters, including Bacteroidales from the human gut (L. Chen et al. 2019).

In this study, we extend the previously known diversity of Bacteroidales species that encode a distinct CIS within their genome, which we term Bacteroidales Injection Systems (BIS). BIS are related to eCIS and T6SS that mediate tubeworm, insect, and amoeba interactions (MACs, PVCs, Afp, Subtype-4 T6SS). Here, we show that BIS genes are present within the gut microbiomes of over 99% of healthy human adult individuals from Western countries (Europe and the United States) and are expressed *in vivo*. We further find that individuals suffering from inflammatory bowel disease (IBD) possess fewer BIS gene counts in their gut microbiomes when compared to the gut microbiomes of healthy individuals. Our results reveal that genes encoding a putative Contractile Injection System are encoded within the microbiomes of nearly all healthy adults from Western countries and may be correlated with host health.

Results

A distinct Contractile Injection System in Bacteroidales from the human gut

Using PSI-BLAST to compare previously identified eCIS and Subtype-4 T6SS proteins to the non-redundant (nr) protein sequence database, we identified CIS structural proteins (baseplate, sheath, and tube) that matched with proteins from various human Bacteroidales isolates, including a bacterial isolate from the human gut, *Bacteroides cellulosilyticus* WH2 (McNulty et al. 2013), *Bacteroides fragilis* BE1, and *Parabacteroides distasonis* D25 (**Table 5.1**). To determine the relatedness of these distinct CIS with all known CIS subtypes, we performed phylogenetic analyses of CIS proteins that are key structural components of known CIS—the CIS sheath and tube. Multiple methods of phylogenic analyses (maximum likelihood, neighbor joining, maximum parsimony, UPGMA, and minimum evolution) showed that Bacteroidales sheath and tube proteins consistently formed a monophyletic group with other eCIS and Subtype-4 T6SS sheath and tube proteins (**Figures 5.1B, 5.2, Table 5.2**). Moreover, the BIS sheath and tube were clearly distinct when compared to previously characterized T6SS Subtype 1, 2, and 3, including *Bacteroides* Subtype-3 T6SS (**Figures 5.1B, 5.2**) (Chatzidaki-Livanis et al. 2016; Russell et al. 2014). Based on these data and results below, we name this distinct CIS Bacteroidales Injection Systems (BIS).

BIS gene cluster forms three different genetic arrangements

To identify Bacteroidales species that possess a bonafide BIS gene cluster, we performed a comprehensive search of 759 sequenced *Bacteroides* and *Parabacteroides* genomes from the Refseq database. Our sequence-profile search revealed 66 genomes from

Bacteroides and *Parabacteroides* species that harbor complete BIS gene clusters **(Table 5.3)** in three conserved gene arrangements (**Figure 5.3A**). The first architecture is exemplified by *B. cellulosilyticus* WH2, which harbors two sheath proteins, two tube proteins, and a protein with unknown function intervening between putative genes encoding the baseplate (gp25, gp27 and gp6). The second architecture is exemplified by *B. fragilis* BE1. This architecture has a single sheath protein and lacks the hypothetical proteins observed in architecture 1 between gp25 and gp27, and between Tube2 and LysM. The third architecture defined by *P. distasonis* D25 is the most compact, lacks four hypothetical proteins found in architectures 1 and 2. Additionally, gp27 and gp6 proteins are shorter, and the genes FtsH/ATPase and DUF4157 are inverted. Importantly, all three genetic architectures have genes with significant sequence similarity (e-value <0.001) to MAC, Afp and PVC genes (**Table 5.1**) shown previously to produce a functional CIS, including baseplate proteins (gp25, gp27, and gp6), sheath, tube, and FtsH/ATPase (**Figure 5.3B**). These genes were also independently identified in the dbeCIS (database of extracellular Contractile Injection Systems) (L. Chen et al. 2019).

BIS genes are present in human gut, mouth, and nose microbiomes

To determine the prevalence and distribution of BIS genes in human microbiomes, we searched shotgun DNA sequencing data from 11,219 microbiomes from the Human Microbiome Project database, taken from several locations on the human body of 232 individuals (Levi et al. 2018; Huttenhower et al. 2012). We sampled these metagenomes for the presence of 18 predicted BIS proteins **(Table 5.4)**. Across all HMP metagenomes, 8,320 (74%) showed hits to at least one of the 18 BIS proteins. Hits were distributed across metagenomes from various mucosal tissues, and were more abundant in the gut and in the

mouth, where Bacteroidales are commonly found (Huttenhower et al. 2012). The dataset included stool (1,851, 99.6% of total stool metagenomes), oral (4,739, 79.2% of total oral metagenomes), nasal (630, 41.8% of total nasal metagenomes), and vaginal (232, 27.7% of total vaginal metagenomes) samples (**Figure 5.4A**).

To determine how often any of the 18 genes co-occurred within the same metagenome sample, we constructed a co-occurrence network (**Figure 5.4B**). Ten genes appeared together at high frequencies including Sheath1, Sheath2, FtsH/ATPase, Baseplate (gp25, gp27, and gp6), LysM, Spike, and two hypothetical proteins (**Figure 5.4B**). The gene with the highest hit abundance encodes for an ATPase homologous to the *Escherichia coli* FtsH, known to be involved in cleavage of the lambda prophage repressor, followed by a hypothetical protein, and Sheath1. The remaining genes, including Tube1, Tube2, Tip, DUF4255 domain-containing protein, DUF4157 domain-containing protein, and three hypothetical proteins, were detected together less often within the microbiome samples.

BIS genes are expressed in vivo and in vitro

To determine whether BIS genes are transcribed inside the gut or under laboratory growth conditions, we searched for the 18 major BIS proteins in publicly available RNA sequencing data from *in vivo* metatranscriptomes of humanized mice (Ridaura et al. 2013) (gnotobiotic mice colonized with human microbiome bacteria) and *in vitro B. cellulosilyticus* WH2 pure cultures (McNulty et al. 2013). We inspected 59 metatranscriptomes from a previously published *in vivo* study (Ridaura et al. 2013) for the presence of the 18 major BIS proteins, where gnotobiotic mice were inoculated with human gut microbiome cultures. In 48 out of 59 metatranscriptomes (81.4%), we found expression of at least 15 BIS proteins

(Figure 5.5). Similarly, when *B. cellulosilyticus* WH2 was cultured in minimum media (MM) supplemented with 31 different simple and complex sugars (McNulty et al. 2013), all 18 genes were expressed at least once in at least two of the three replicate cultures (**Figure 5.6**). The highest expression was seen under growth in N-Acetyl-D-Galactosamine (GalNAc) and N-Acetyl-Glucosamine (GlcNAc), amino sugars that are common components of the bacterial peptidoglycan, in high abundance in the human colon, and implicated in many metabolic diseases (Myslicki, Belke, and Shearer 2014; Baudoin and Issad 2014). Our analyses of metatranscriptomes show that BIS genes are transcribed by Bacteroidales bacteria under laboratory growth conditions and within humanized mouse microbiomes *in vivo*.

BIS genes are present in the microbiomes of nearly all adult individuals

To determine the prevalence of BIS genes within the microbiomes of human populations, we analyzed 2,123 fecal metagenomes from 339 individuals; 124 Individuals from Europe from the MetaHIT (Metagenomics of the Human Intestinal Tract) study (Qin et al. 2010), and 214 individuals from North America from the Human Microbiome Project (HMP) study (Huttenhower et al. 2012). In both MetaHIT and HMP studies the cohort of individuals were sequenced more than once, to account for this the BIS prevalence analysis was normalized by individual donor. We found that all individuals possessed at least 1 of the 18 BIS genes within their gut microbiome (**Figure 5.7**). Most individuals carried at least 9 BIS proteins (83.0% HMP, 90.3% MetaHIT). A lower number possessed all 18 BIS proteins (8.96% HMP, 6.45% MetaHIT).

BIS genes are more abundant in healthy gut microbiomes compared to IBD

Individuals suffering from Inflammatory Bowel Disease (IBD), and prediabetes have been shown to possess an altered gut microbiome composition (Carroll et al. 2011; Lambeth et al. 2015), yet it is unknown whether specific microbial factors contribute to healthy or diseased outcomes. We asked whether individuals with IBD or prediabetes vary in the counts of BIS genes within their microbiome compared to healthy individuals. To this end, we analyzed 4,918 fecal metagenomes from 345 individuals comprising the HMP and the Integrative Human Microbiome Project (iHMP) dataset (Lloyd-Price et al. 2019); 214 healthy, 103 with IBD (Crohn's Disease or ulcerative colitis), and 28 with prediabetes. For those individuals with more than one metagenome sequence, BIS gene hits were averaged by donor to account for the existence of more than one sequenced metagenome per individual. While prediabetes and healthy individuals possessed comparable counts of BIS genes, we found that a higher percentage of healthy individuals harbored significantly more BIS genes than individuals with IBD **(Figure 5.8A, Table 5.5, Figure 5.9)**.

We next reasoned that differences in total Bacteroidetes abundances between healthy and IBD individuals could account for the differences in BIS counts we observed. We therefore quantified the abundances of Bacteroidetes between healthy, prediabetes, and IBD groups. Our analyses showed that the relative abundances of Bacteroidetes per individual were similar between the three groups **(Figure 5.8B)**, yet there were statistically different frequency medians per individual between the healthy, prediabetes, and IBD groups **(Table 5.5)**. Our results show that BIS gene counts are more abundant in healthy individuals than in those with IBD and these BIS gene counts cannot be explained by the difference in Bacteroidetes frequencies between healthy and IBD groups.

Discussion

Here, we show that a gene cluster encoding a putative Contractile Injection System, called Bacteroidales Injection System (BIS), is present in the gut microbiomes of nearly all healthy adult individuals from Western countries. We find that BIS genes are present in human microbiomes throughout mucosal tissues (oral, nasal, vaginal, ocular) and enriched in metagenomes from gut samples. Type 6 Secretion Systems have gained recent recognition as secretion structures that promote disease in several prominent human pathogens like *Pseudomonas aeruginosa* and *Vibrio cholerae*. However, our discovery that a distinct Bacteroidales encoded CIS gene cluster is present in a majority of human gut microbiomes, stems from studies of symbiotic interactions between environmental bacteria and diverse eukaryotic hosts like tubeworms, insects, and amoeba (Freckelton et al. 2019; Vlisidou et al. 2019; Böck et al. 2017; Nicholas J Shikuma et al. 2016; N. J. Shikuma et al. 2014). The close relatedness of BIS with other structures promoting microbe-eukaryotic interactions (MACs, Afp, PVCs and Subtype-4 T6SS) suggests that BIS could mediate interactions between Bacteroidales and their human host or bacterial species within the human microbiome. Importantly, our results warrant future investigations into the potential functions of BIS genes in the gut microbiome, which will require significant experimental validation.

Although Contractile Injection Systems (eCIS and T6SS Subtypes 1-4) share certain components, CIS subtypes are distinguished based on sequence similarity of gene or protein homologs and the presence or absence of specific CIS components. Specifically, the protein sequences of BIS tube, baseplate, and sheath genes possess significant similarity to the homologous proteins of other eCIS, based on e-value (**Table 5.1**). We used metagenomic and metatranscriptomic analyses with thresholds (e-value <0.001) that excluded homologous

genes from other secretion systems (e-value >0.01). Further, a distinguishing feature of gene clusters related to MACs, Afps, PVCs and BIS is the presence of a baseplate (gp27) gene. This gp27 gene is not present in canonical T6SS Subtypes 1-3 and is likely to correspond to BIS gene clusters when identified within our metagenomic analyses. Independent methods have been used previously to find, describe, and characterize 631 eCIS-like loci from the 11,699 publicly available complete bacterial genomes, including BIS (L. Chen et al. 2019). We acknowledge that these analyses come with potential limitations and that experimental validations are required to support the findings described here.

BIS may not have been extensively described before this work because they likely evolved independently from previously described CIS, such as Subtype-3 T6SS (Böck et al. 2017; Coyne, Roelofs, and Comstock 2016), and possess significantly divergent sequence homologies (**Figure 5.1B**). Like other described CIS, BIS gene clusters harbor genes encoding the syringe-like structural components and could encode for effectors that elicit specific cellular responses from target cells. For example, the closely related injection system called MACs possesses two different effectors; one effector protein promotes the metamorphic development of a tubeworm (Ericson et al. 2019), and a second toxic effector kills insect and mammalian cell lines (Rocchi et al. 2019).

We currently do not yet know the conditions that promote BIS production within healthy or diseased human individuals. However, we show here that BIS genes are expressed *in vivo* during colonization of humanized mice (Ridaura et al. 2013) and under laboratory conditions with various carbon sources (McNulty et al. 2013). We observed a heterogenous expression pattern of BIS genes in humanized mouse transcriptomes and *in vitro*, which may

be due to limitations in sequencing depth, and/or differential expression of BIS structural and effector proteins that compose a multi-subunit complex.

Our analyses show that BIS genes are more prevalent in individuals with healthy gastrointestinal tracts than in those suffering from IBD (Crohn's Disease and ulcerative colitis). Several studies have demonstrated that dysbiosis in the human gut is correlated with microbiome immaturity, type 2 diabetes, and diseases like obesity and Inflammatory Bowel Disease (IBD) (Ley et al. 2006; Hooper and Gordon 2001; Kau et al. 2011; Subramanian et al. 2014; J. Wang et al. 2012; Carroll et al. 2011). IBD is a chronic inflammation of the gastrointestinal tract that encompasses two diseases, Crohn's Disease and ulcerative colitis, both characterized by decreased microbial diversity, lower microbiome composition stability, and an increase in Enterobacteria (Morgan et al. 2012; Knights et al. 2014). Studies have shown that although dysbiosis and the metabolic profiles of the gut microbiome influence the disease, microbial functions have a greater contribution to the disease (Morgan et al. 2012). Our work warrants future investigations into whether BIS play a role in the promotion or maintenance of a healthy gastrointestinal tract.

If BIS do interact with human cells, they may promote or enhance symbiotic interactions with human gut commensals such as *Bacteroides cellulosilyticus* (Robert et al. 2007). Injection systems closely related to BIS are described to mediate both beneficial and infectious microbe-host relationships. For example, MACs mediate metamorphosis of a marine tube worms (N. J. Shikuma et al. 2014; Ericson et al. 2019), and a Subtype-4 T6SS (Böck et al. 2017) mediates membrane interaction between *A. asiaticus* and its amoeboid host. In contrast, Afp and PVCs inject toxic effectors into insects (Hurst, Glare, and Jackson 2004; Yang et al. 2006). Our findings evidence the presence of the BIS gene cluster in a majority of

human gut microbiomes; however, the potential function of the BIS genes needs to be investigated. The hypothesis that the BIS cluster could mediate microbe-human interactions arises from previous studies that describe, and characterize similar gene clusters (N. J. Shikuma et al. 2014; Böck et al. 2017; Yang et al. 2006).

Many correlations between Bacteroidales abundances in the human gut and host health are currently unexplained. Little is known about the crosstalk mechanisms between the microbiome and the human host, and the functional role of Bacteroidales in microbial dynamics in the human gut. Future research into the conditions that promote the production of BIS and its potential protein effectors may yield new insight into how Bacteroidales prevalence correlate with host health. In addition to their effect on host health, a functional BIS could provide the tantalizing potential as biotechnology platforms that may be manipulated to inject engineered proteins of interest into other microbiome bacteria or directly into human cells.

Methods

Phylogenetic analyses of CIS sheath and tube proteins

Whole genomes and assembled contigs illustrating a diversity of representative phage-like clusters (**Table 5.2**) were downloaded from NCBI to construct a database using BLAST+(2.6.0). The *B. cellulosilyticus* WH2 Sheath1 (WP 029427210.1) and Tube1 (WP 118435218) protein sequences were downloaded from NCBI and a tBLASTn was performed against the genome database. The recovered nucleotide sequences were then translated using EMBOSS Transeq (EMBL-EBI, https://www.ebi.ac.uk/Tools/st/emboss_transeq/) to generate a list of protein sequences. While the sheath and tube proteins are functionally homologous, their genomic diversity required other protein queries against the custom genome database to capture protein homologs from more divergent secretion systems (sheaths: WP_012025251.1, YP_009591452.1, WP_001882966.1; tubes: WP_012473180.1, YP_009591453.1, WP_015969329.1, WP_003022149.1, WP_001142947.1). To capture these highly divergent protein homologs, we used T6SS-Hcp, VipA/B; and Phage- gp18, gp19 as reference proteins. The amino acid sequences were aligned using the online version of MAFFT (v7) with the iterative refinement alignment method e-ins-i for the Sheath1, and fft-ins-I for the Tube1 phylogeny. The aligned fasta file was converted into a phylip file using Seaview (Gouy, Guindon, and Gascuel 2010). PhyML was performed through the ATCG Bioinformatics web server and utilized the Smart Model Selection (SMS) feature and the Maximum-Likelihood method (Guindon et al. 2010; Lefort, Longueville, and Gascuel 2017). The model WAG+G+I+F was used for the Sheath1, and rtREV+G+I+F for the Tube1 phylogeny. Different alignment algorithms were used based on the conservation of the protein sequences. The smart model selection feature from the ATGC web server calculated the best

phylogenetic substitution model based on the alignment. Bootstrap values of 1000 resamples –instead of only 100- were calculated to ensure tree robustness. The Maximum likelihood tree topology was confirmed using other methods, including Neighbor joining, Maximum-Parsimony, Minimum-Evolution, and UPGMA. Trees were manipulated and viewed in iTOL (Letunic and Bork 2016).

BIS gene cluster synteny analyses

To identify CIS gene clusters in Bacteroidetes we used a modified protocol used to identify T6SS (Coyne, Roelofs, and Comstock 2016). Briefly, the assemblies for 759 *Bacteroides* and *Parabacteroides* genomes included in the Refseq database (release 92, 26553804) were downloaded. Proteins from each assembly were searched with HHMER v3.2.1 (http://hmmer.org/) for a match above the sequence gathering threshold (bit score > 31.4, e-value < 1×10^{-9})of the Pfam HMM profile 'phage_sheath_1' (PF04984) (El-Gebali et al. 2019). For each match, up to 20 proteins were extracted from either side. All proteins from the resulting set (phage sheath $\pm$ 20 proteins) were sorted by length and clustered at 50% amino acid identity using UClust v1.2.22q (Edgar 2010). Clusters containing $\geq$ 4 members were analyzed further. Cluster representatives were annotated using protein-profile searches against three databases: the Pfam-A database using HMMER3 (using family-specific gathering thresholds) (El-Gebali et al. 2019), the NCBI Conserved Domain Database using RPS-BLAST (e-value < 0.01) (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2011; 2017), and the Uniprot30 database (accessed February 2019, available from http://wwwuser.gwdg.de/~compbiol/data/hhsuite/databases/hhsuite_dbs/) using HHblits (Remmert et al. 2012; McGarvey et al. 2019). Multiple sequence alignments were

automatically generated from three iterations of the HHblits search and used for profile-profile comparisons against the PDB70 database (HHpred probability > 90, accessed February 2019, available from http://wwwuser.gwdg.de/~compbiol/data/hhsuite/databases/hhsuite_dbs/). Significant hits to cluster representatives were used to assign an annotation to all proteins contained within the parent cluster. Manual inspection of *Bacteroides* and *Parabacteroides* loci enabled consistent trimming of each genetic architecture; specifically, the genes intervening DUF4255 and FtsH/ATPase were retained.

Metagenomic mining analyses

To find the prevalence of the BIS genes within the Human Microbiome Project and the Integrative HMP, using NCBI's fastq-dump API we downloaded 11,219 and 3,059 metagenomes, respectively. The metagenomes were parsed where: left-right tags were clipped, technical reads (adapters, primers, barcodes, etc) were dropped, low quality reads were dropped, and paired reads were treated as two distinct reads. A subject database was created from the amino-acid sequences of the 18 BIS genes. Then the fastq files were piped through seqtk (Shen et al. 2016), to convert them to fasta format, which was then piped to DIAMOND via stdin. Then DIAMOND aligned the six-frame translation of the input reads against the subject database, with all default parameters, and an e-value cutoff of 0.001. For each metagenome the number of non-mutually exclusive hits to each CIS gene were then summed providing a hit 'count score'. From the hit counts, a heatmap was created by taking the number of hits of each gene per metagenome and dividing by the total number of reads and multiplying the result by one million, which was then log10(x+1) transformed. To

estimate the co-occurrence between pairs of genes, the hit count scores from the previous calculation were taken, and for each pair combination, the hit count of the lower of the two genes was added to a running total. The co-occurrence was then visualized on a network graph, where each edge corresponds to the number of times the pair of genes co-occurred in all the metagenomes (Hadley 2016; Csardi and Nepusz 2006) (R core Team 2017 - https://www.R-project.org/; ggraph - https://CRAN.R-project.org/package=ggraph). The prevalence of BIS genes was normalized by human donor to account for the presence of more than one sequenced metagenome per individual. For the MetaHIT and HMP datasets, the average number of BIS protein per person was calculated based on the metadata provided by the studies.

Metatranscriptomic mining analyses

Fastq files from transcriptomes were downloaded from the Sequence Read Archive using the SRA Toolkit (https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/). Low quality reads were removed using PRINSEQ++ (https://peerj.com/preprints/27553/). Reads were compared to the amino acid sequences of the *Bacteroides cellulosilyticus* WH2 BIS protein cluster using BLASTx with an e-value cutoff of 0.001. The best hit for each read was kept. Hits to each protein were normalized by the number of reads of each transcriptome and the length of each protein using the program Fragment Recruitment Assembly Purification (https://github.com/yinacobian/frap).

Bacteroidetes abundance in Healthy, IBD, and Prediabetes microbiomes

To estimate bacterial taxonomy abundance, MetaPhlAn version 2.6.0 was downloaded, along with the corresponding version 20 database, and run on each of the metagenomes from the Human Microbiome Project and the Integrative Human Microbiome Project (Segata et al. 2012)

Statistical analysis for comparison of BIS in Healthy, IBD, and Prediabetes

To test the difference between the medians of two groups (healthy-IBD, prediabetes-IBD, and healthy-prediabetes), a Confidence Interval (CI) for the difference in medians was constructed by the percentile nonparametric bootstrap method for the difference in medians using 10,000 bootstrap replicates for each group. Statistical analysis showed no difference between Crohn's Disease and ulcerative colitis for neither BIS protein count (*Crohn's - colitis*: -0.02519917 (-0.1524929, 0.1018519)) nor Bacteroidetes abundance (*Crohn's - colitis*: 0.01385387 (-0.1265633, 0.1489824)). These results were further validated with asymptotic Wilcoxon rank sum tests (**Table 5.6**).

Availability of supporting data

The datasets supporting the conclusions of this article are available in the Human Microbiome Project Data Portal (https://portal.hmpdacc.org/); the additional metagenomes and metatranscriptomes analyzed in the study are publicly available on the NCBI SRA database corresponding to previous studies (McNulty et al. 2013; Ridaura et al. 2013; Qin et al. 2010), and all accession numbers and protein IDs are listed in Additional files (Tables S1-S4). Phylogenetic and synteny analyses were performed with webserver programs cited in the

methods. Scripts used for metagenomic and metatranscriptomic data analyses are available in GitHub (https://github.com/yinacobian/MR-blastx).

Figures and Tables

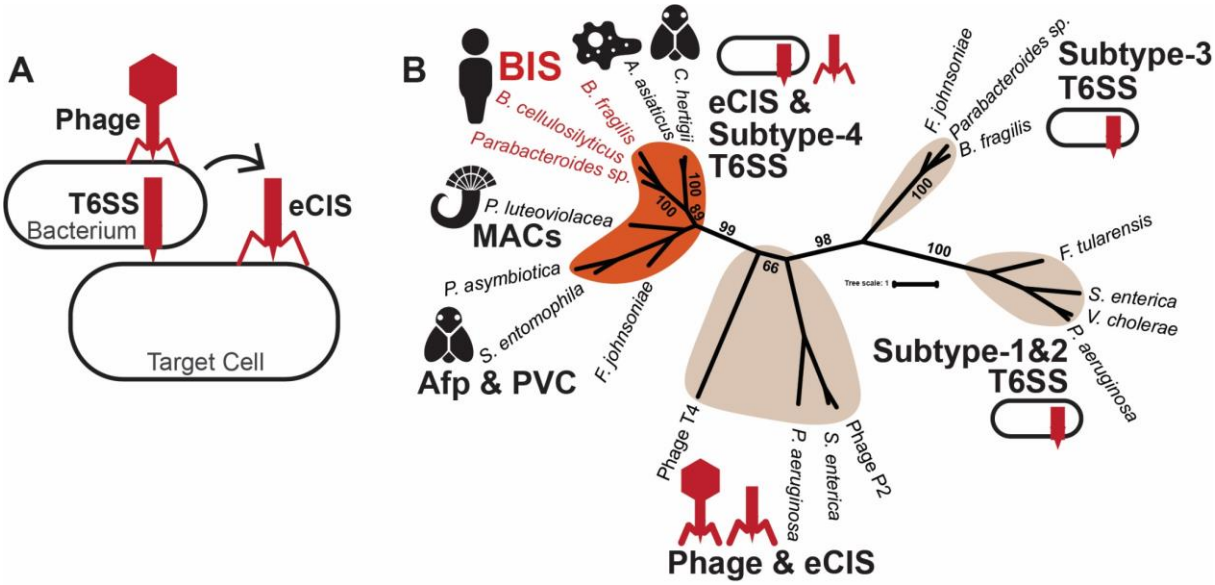

Figure 5.1 Bacteroidales possess a distinct Contractile Injection System gene cluster. **(A)** Contractile Injection Systems are related to the contractile tails of bacteriophage. There are two main types of CIS: Type 6 Secretion Systems (T6SS) are bound to the bacterial cell membrane and act from within the producing cell while extracellular CIS (eCIS) are released by bacterial cell lysis and bind to target cells. **(B)** Unrooted phylogeny of CIS sheath protein sequences. BIS group with known Subtype-4 T6SS and eCIS (orange) are distinct from known Subtype-1, Subtype-2 and Subtype-3 T6SS (**Table 5.1**). Bacteria with BIS identified in this study are highlighted in red. Bootstrap values are expressed in number of occurrences- that support the phylogenetic structure- out of 100, from 1000 resampling events.

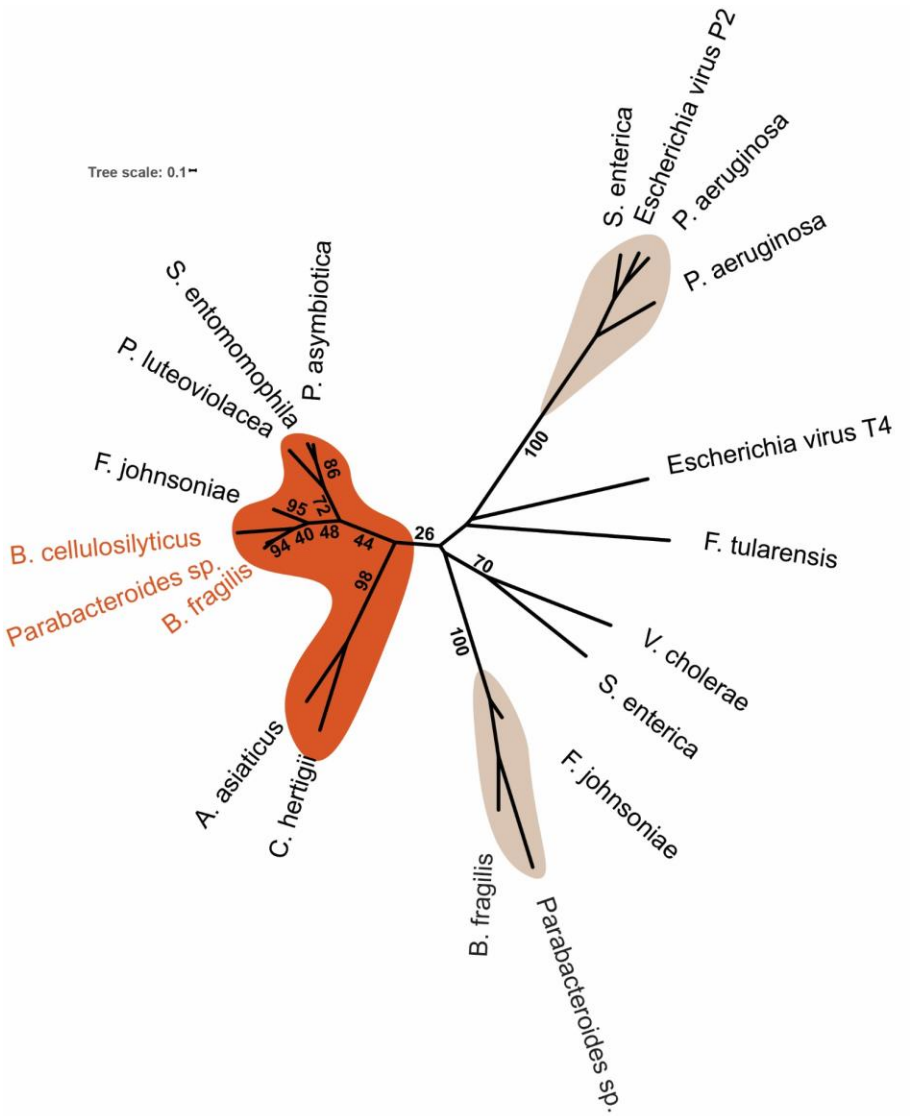

Figure 5.2 Unrooted phylogeny of CIS tube protein sequences. BIS group with known T6SS Subtype-4 and CIS (orange) are distinct from known T6SS Subtype-1 and Subtype-2 present in human pathogens, and known Subtype-3, characterized to mediate bacteria-bacteria interactions.

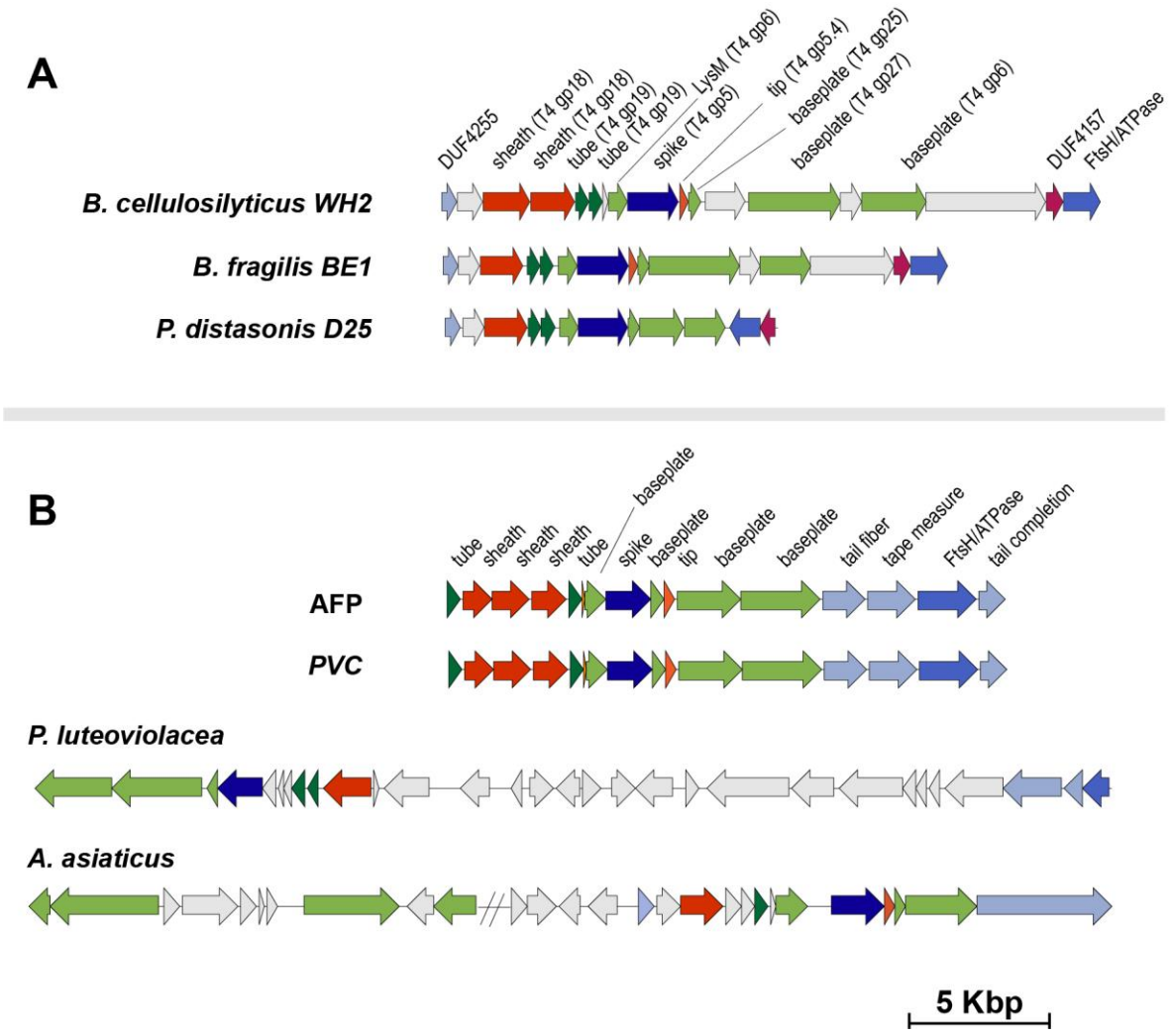

Figure 5.3 BIS gene clusters are found in three genetic architectures. **(A)** Synteny plot of BIS gene clusters in *Bacteroides* and *Parabacteroides* species compared to **(B)** those of *P. luteoviolacea* MACs, *S. entomophilia* Afp, *Photorhabdus* PVCs, and *A. asiaticus* Subtype-4 T6SS. Representative CIS gene cluster architectures are shown, with genes color coded according to function. Genes with no significant sequence similarity at the amino acid level to any characterized proteins are colored light grey. Sequence coordinates of all gene clusters are provided in **Table 5.3**.

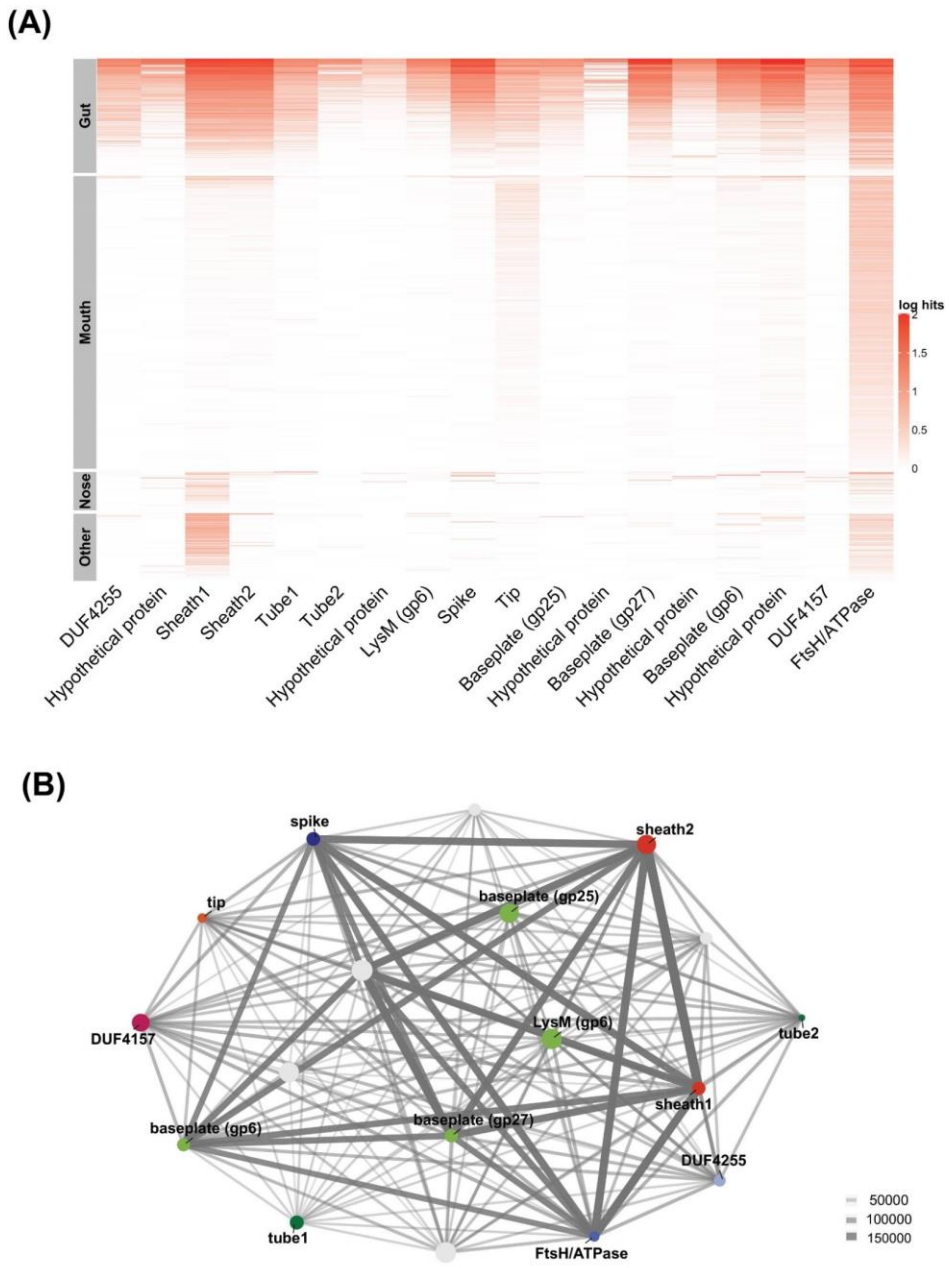

Figure 5.4 BIS genes are abundant in human gut and mouth microbiomes, and present in other human microbiomes. **(A)** Coverage plot of BIS genes (Log10 of 1,000,000*hits/reads) in 8,320 microbiomes associated with mucosal tissue: gut, mouth, nose, and other (includes vaginal and skin tissues) from 232 healthy humans. **(B)** Ten BIS genes are found more often together in human metagenomes (co-occurrence network). Node size represents the number of hits for each protein across all runs. Line weight represents the number of times any two proteins occurred together within a dataset.

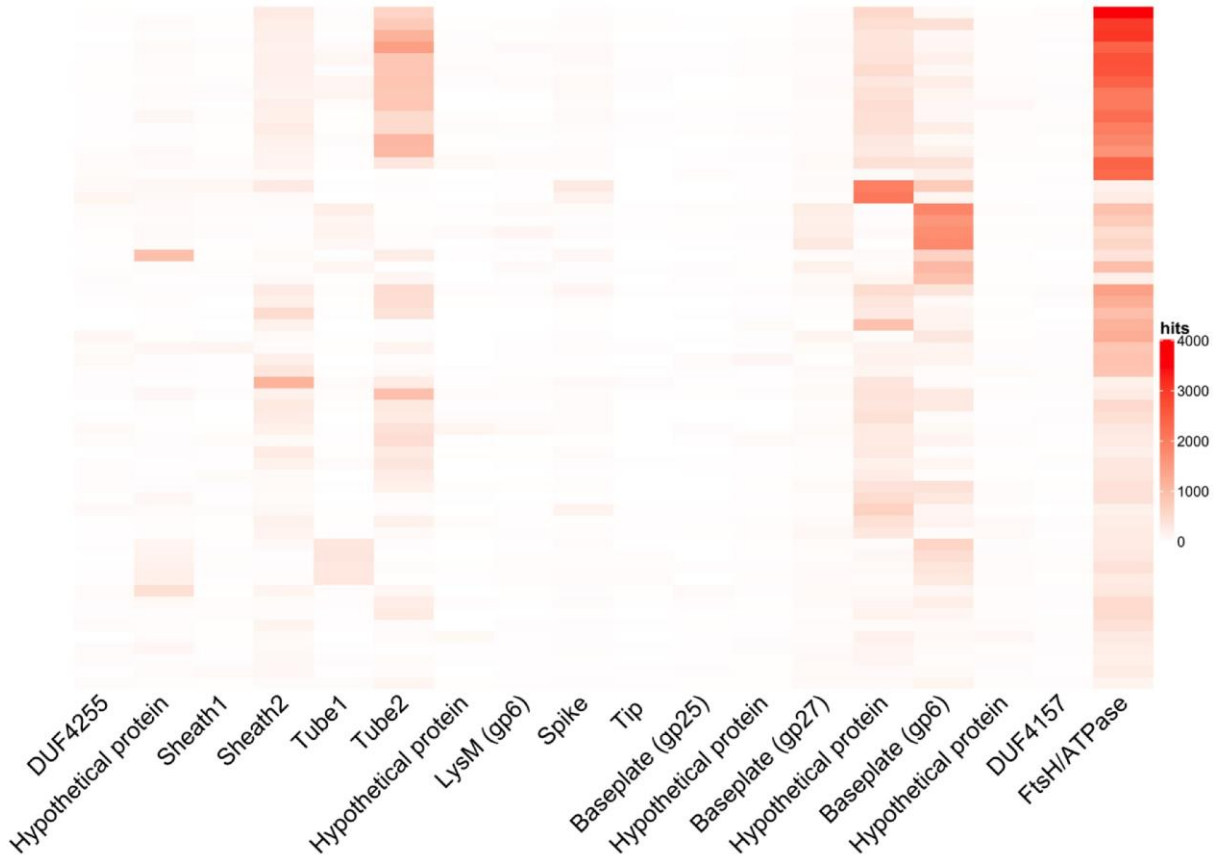

Figure 5.5 BIS genes are expressed *in vivo* in humanized mice. Coverage plot of BIS genes (normalized by number of reads and protein nt size) from 59 stool metatranscriptomes of humanized mouse microbiomes.

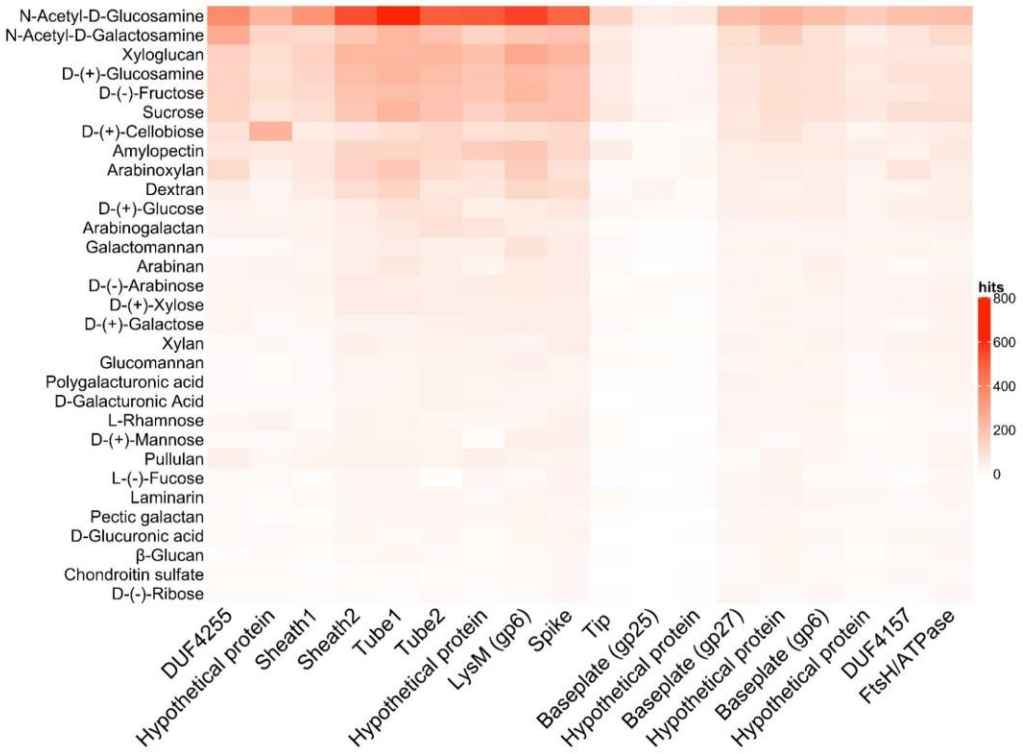

Figure 5.6 BIS genes are expressed during *in vitro* culture of *B. cellulosilyticus* WH2. Relative abundance of RNA hits to 18 major genes of the BIS in *B. cellulosilyticus* WH2 culture in MM supplemented with 31 different simple and complex carbohydrates.

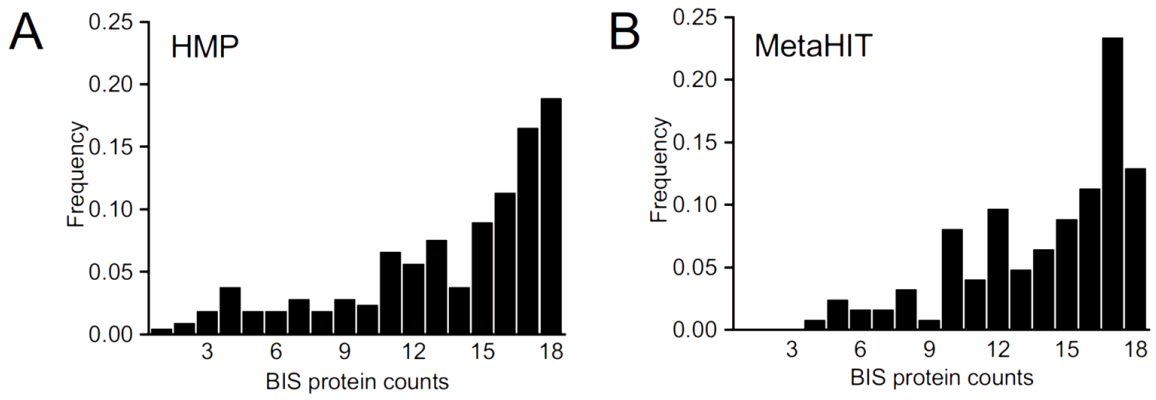

Figure 5.7 BIS genes are present in the microbiomes of a majority (99%) of adult individuals from the United States and Europe. Frequencies of 18 BIS proteins from fecal samples of 338 individuals. **(A)** HMP n=214, from healthy North American individuals (Huttenhower et al. 2012); **(B)** MetaHIT n=124, from a study of European individuals (Qin et al. 2010). Protein hits are normalized by individual donor.

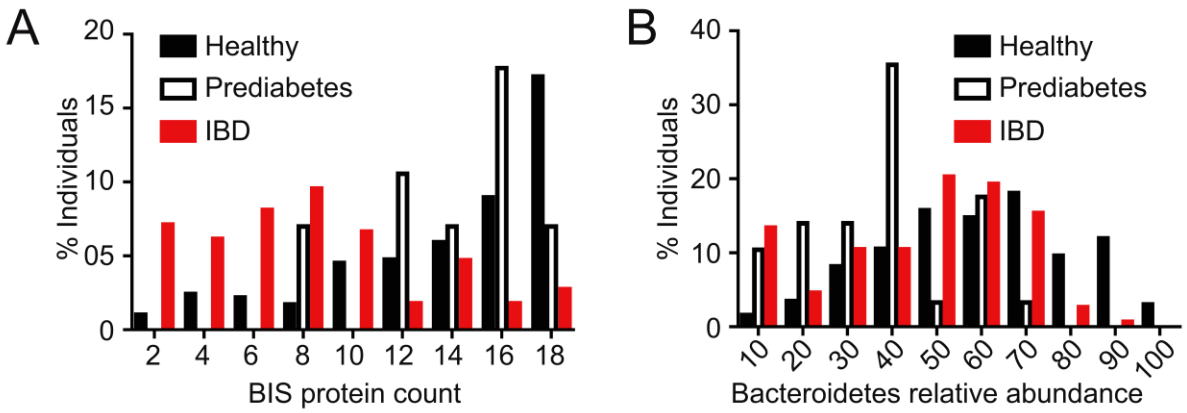

Figure 5.8 BIS genes are present in higher abundance in healthy individuals compared to individuals with IBD. **(A)** Percentage of individuals possessing a given number of BIS proteins from 214 healthy, 103 IBD, and 28 Prediabetes fecal microbiome samples. **(B)** Percentage of individuals possessing a given relative abundance (%) of Bacteroidetes within their gut microbiomes.

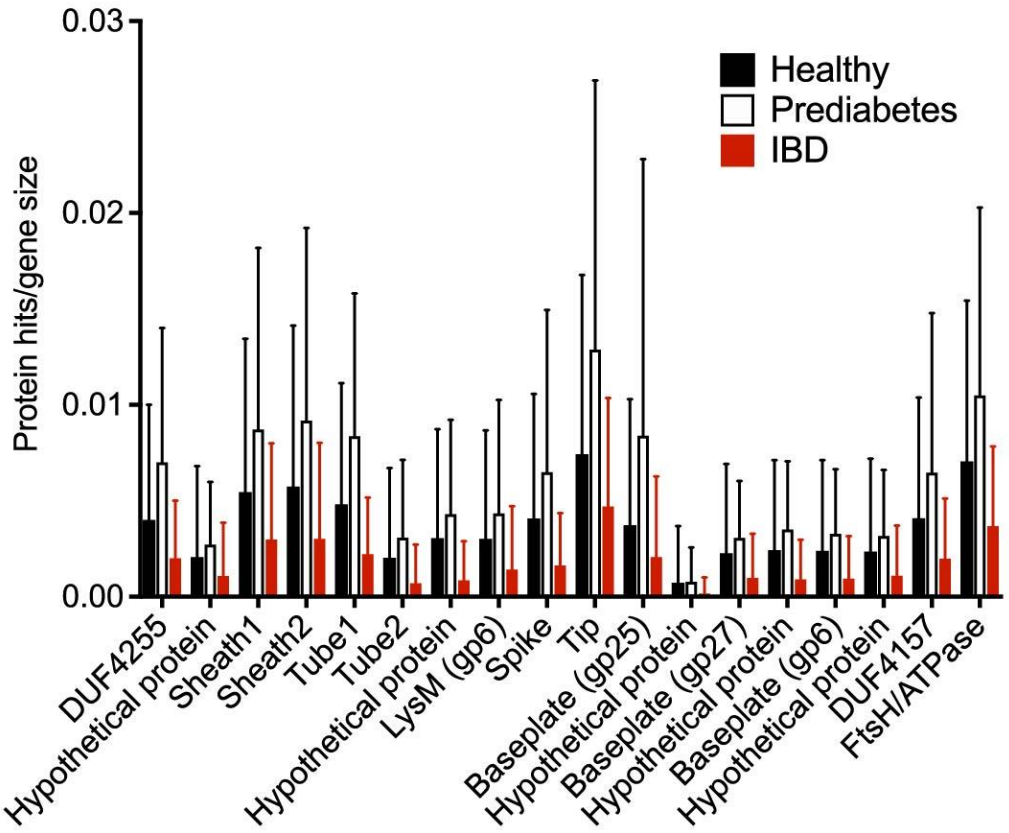

Figure 5.9 BIS protein abundance per individual in microbiomes of healthy, prediabetes, and IBD groups. Mean number of hits for each protein are normalized by gene size (nt). Error bars indicate standard deviations from 214 healthy, 103 IBD, and 28 Prediabetes individuals.

Table 5.1 Sequence similarity of sheath and tube proteins from representative secretion systems used to construct phylogenetic trees against BIS proteins.

Organism	Protein	Locus tag (query)	e-value
***B. cellulosilyticus* WH2 BASEPLATE locus tag (subj.): WP_029427202.1**			
B. fragilis BIS	hyp. prot.	CUA19277.1	3.0E-90
A. asiaticus T6SS^{iv}	baseplate	WP_012472726.1	2.0E-67
C. hertigii T6SS	baseplate	WP_014934193.1	2.0E-57
Parabacteroides sp. BIS	baseplate	WP_009276509.1	1.0E-42
Flavobacterium johnsoniae eCIS	baseplate	ABQ06175	1.0E-42
P. luteo MACs	MacB	WP_029427202.1	5.0E-32
S. entomophila Afps	baseplate	WP_010895813.1	7.0E-18
P. asymbiotica PVCs	baseplate	WP_015834374.1	3.0E-16
V. cholerae T6SS^{i}	baseplate	NP_232421.1	6.1E-01
***B. cellulosilyticus* WH2 SHEATH-1 locus tag (subj.): WP_029427210.1**			
Parabacteroides sp. BIS	sheath2	WP_009276514.1	0.0E+00
B. fragilis BIS	sheath2	WP_005803145.1	2.0E-109
P. luteo MACs	MacS	WP_039609824.1	6.0E-94
A. asiaticus T6SS^{iv}	sheath	WP_012473177.1	5.0E-66
C. hertigii T6SS^{iv}	sheath	WP_014934609.1	8.0E-65
Flavobacterium johnsoniae eCIS	sheath2	WP_012025137.1	2.0E-64
P. asymbiotica PVCs	sheath1	WP_015834924.1	2.0E-51
S. entomophila Afps	sheath1	WP_010895805.1	9.0E-49
Pseudomonas aeruginosa eCIS	sheath1	WP_003113197.1	8.0E-10
Salmonella enterica subsp. enterica serovar Typhi eCIS	sheath1	WP_000046142.1	8.0E-06
Francisella tularensis subsp. tularensis T6SS^{ii}	sheath	WP_003023948.1	2.1E-01
Parabacteroides sp. T6SS^{iii}	sheath1	WP_008669225.1	2.3E-01
B. fragilis T6SS^{iii}	sheath1	WP_053873779.1	4.9E-01
Flavobacterium johnsoniae T6SS^{iii}	sheath1	WP_012025251.1	4.9E-01
Pseudomonas aeruginosa T6SS^{i}	sheath2	WP_003087596.1	8.3E-01
Salmonella enterica subsp. enterica serovar Typhi T6SS	sheath2	WP_000013884.1	1.3E+00
V. cholerae T6SS^{i}	sheath	WP_001882966.1	3.3E+00
***B. cellulosilyticus* WH2 SHEATH-2 locus tag (subj.): WP_029427209.1**			
B. fragilis BIS	sheath2	WP_005803145.1	0.0E+00
Parabacteroides sp. BIS	sheath2	WP_009276514.1	0.0E+00
C. hertigii T6SS^{iv}	sheath	WP_014934609.1	7.0E-122
A. asiaticus T6SS^{iv}	sheath	WP_012473177.1	3.0E-114
Flavobacterium johnsoniae eCIS	sheath2	WP_012025137.1	6.0E-70
P. asymbiotica PVCs	sheath2	WP_015834924.1	3.0E-64
P. luteo MACs	MacS	WP_039609824.1	1.0E-63
S. entomophila Afps	sheath2	WP_010895805.1	1.0E-57
Pseudomonas aeruginosa eCIS	sheath1	WP_003113197.1	2.0E-08
Salmonella enterica subsp. enterica serovar Typhi eCIS	sheath1	WP_000046142.1	4.4E-02
V. cholerae T6SS^{i}	sheath	WP_001882966.1	2.5E-01
B. fragilis T6SS^{iii}	sheath1	WP_053873779.1	2.9E-01
Parabacteroides sp. T6SS^{iii}	sheath1	WP_008669225.1	3.1E-01

Table 5.1 Sequence similarity of sheath and tube proteins from representative secretion systems used to construct phylogenetic trees against BIS proteins. (Continued)

Organism	Protein	Locus tag (query)	e-value
Flavobacterium johnsoniae T6SS[iii]	sheath1	WP_012025251.1	5.2E-01
Pseudomonas aeruginosa T6SS[i]	sheath2	WP_003087596.1	2.2E+00
Francisella tularensis subsp. tularensis T6SS[ii]	sheath	WP_003023948.1	2.7E+00
Salmonella enterica subsp. enterica serovar Typhi T6SS	sheath2	WP_000013884.1	6.4E+00
***B. cellulosilyticus* WH2 TUBE-1 locus tag (subj.): WP_007212392.1**			
Parabacteroides sp. BIS	tube2	WP_005861441.1	3.0E-62
B. fragilis BIS	tube1	WP_005803146.1	4.0E-59
Flavobacterium johnsoniae eCIS	tube2	WP_007806487.1	6.0E-51
S. entomophila Afps	tube	WP_010895803.1	3.0E-32
P. luteo MACs	MacT1	WP_039609825.1	2.0E-31
P. asymbiotica PVCs	tube	WP_015835472.1	4.0E-31
P. luteo MACs	MacT2	WP_039609826.1	1.0E-09
A. asiaticus T6SS[iv]	tube	WP_012473180.1	3.0E-08
C. hertigii T6SS[iv]	tube	WP_014934612.1	6.0E-05
V. cholerae T6SS[i]	tube	WP_001142947.1	2.2E-01
B. fragilis T6SS[iii]	tube2	WP_005787106.1	4.2E-01
Salmonella enterica subsp. enterica serovar Typhi eCIS	tube2	WP_001207653.1	5.7E-01
Flavobacterium johnsoniae T6SS[iii]	tube1	WP_012025247.1	1.0E+00
Salmonella enterica subsp. enterica serovar Typhi T6SS	tube1	WP_000338756.1	1.3E+00
Parabacteroides sp. T6SS[iii]	tube1	WP_005836793.1	3.8E+00
Pseudomonas aeruginosa eCIS	tube2	WP_003083317.1	7.0E+00
Francisella tularensis subsp. tularensis	tube	WP_003022149.1	NA
Pseudomonas aeruginosa T6SS[i]	tube1	WP_003085175.1	NA
***B. cellulosilyticus* WH2 TUBE-2 locus tag (subj.): WP_007212393.1**			
A. asiaticus T6SS[iv]	tube	WP_012473180.1	3.0E-09
S. entomophila Afps	tube	WP_010895803.1	9.0E-09
P. asymbiotica PVCs	tube	WP_015835472.1	1.0E-08
Flavobacterium johnsoniae eCIS	tube2	WP_007806487.1	1.0E-08
P. luteo MACs	MacT2	WP_039609825.1	6.0E-08
Parabacteroides sp. BIS	tube2	WP_005861441.1	2.0E-07
B. fragilis BIS	tube1	WP_005803146.1	2.0E-06
P. luteo MACs	MacT1	WP_039609826.1	6.0E-06
C. hertigii T6SS[iv]	tube	WP_014934612.1	1.0E-04
Pseudomonas aeruginosa eCIS	tube2	WP_003083317.1	3.6E-01
B. fragilis T6SS[iii]	tube2	WP_005787106.1	5.7E-01
Salmonella enterica subsp. enterica serovar Typhi Ecis	tube1	WP_000338756.1	6.9E-01
V. cholerae T6SS[i]	tube	WP_001142947.1	7.6E-01
Flavobacterium johnsoniae T6SS[iii]	tube1	WP_012025247.1	3.9E+00
Pseudomonas aeruginosa T6SS[i]	tube1	WP_003085175.1	6.0E+00
Parabacteroides sp. T6SS[iii]	tube1	WP_005836793.1	6.1E+00
Francisella tularensis subsp. tularensis	tube	WP_003022149.1	NA
Salmonella enterica subsp. enterica serovar Typhi T6SS	tube2	WP_001207653.1	NA

Table 5.2 Distinctive structural proteins—tube and sheath—representing diverse Contractile Injection Systems.

Species	Strain	Genome Accession	Sheath Protein Accession	Tube Protein Accession	Reference
Candidatus *Amoebophilus asiaticus*	5a2	CP001102.1	WP_012473177.1	WP_012473180.1	(Böck et al., 2017)
Bacteroides cellulosilyticus	WH2	CP012801.1	WP_029427210.1	WP_007212392.1	(McNulty et al., 2013)
Bacteroides fragilis	BFBE1.1 (BE1)	LN877293.1	WP_005803145.1, WP_053873779.1	WP_005803146.1	This study (synteny analysis)
Cardinium hertigii	cEper1	NC_018605.1	WP_014934609.1	WP_014934612.1	(Böck et al., 2017)
Enterobacteria phage P2		NC_041848.1	YP_009591452.1	YP_009591453.1	Reference phage
Enterobacteria phage T4		NC_000866.4	NP_049780.1	WP_015969329.1	Reference phage
Flavobacterium johnsoniae	UW101	NC_009441.1	WP_012025137.1, WP_012025251.1	WP_012025138.1	(Böck et al., 2017)
Francisella tularensis subsp. tularensis	SCHU S4	AJ749949.2	WP_003023948.1	WP_003022149.1	(Böck et al., 2017)
Parabacteroides sp.	D25	NZ_JH976500.1	WP_009276514.1, WP_008669225.1	WP_005861441.1	This study (synteny analysis)
Pseudoalteromonas luteoviolacea	HI1	KF724687.1	WP_039609824.1	WP_039609825.1, WP_039609826.1	(Böck et al., 2017)
Pseudomonas aeruginosa	PAO1	NC_002516.2	WP_003113197.1, WP_003087596.1	WP_003083317.1, WP_003085175.1	(Böck et al., 2017)
Salmonella enterica subsp. enterica serovar Typhi	CT18	NC_003198.1	WP_000046142.1, WP_000013884.1	WP_000338756.1, WP_001207653.1	(Sana et al., 2016)
Serratia entomophila (pADAP)	A1MO2	NC_002523.4	WP_010895805.1	WP_010895803.1	(Hurst et al., 2004)
Vibrio cholerae O1 biovar El Tor	N16961	NC_002506.1	WP_001882966.1	WP_001142947.1	(Ishikawa et al., 2012)
Photorhabdus asymbiotica	ATCC43949	NC_012962.1	WP_015835470.1	WP_015835472.1	(Vlisidou et al., 2019)

Table 5.3 Sequence coordinates of genes within the BIS clusters that form three different genetic arrangements.

Accession	Species	Strain	start	stop	strand	arch.
NZ_ATFI01000004.1	***Bacteroides cellulosilyticus WH2***	**WH2**	**271076**	**294193**	**-**	**1**
NZ_QRXS01000004.1	*Bacteroides cellulosilyticus*	AF17-25	276218	298574	-	1
NZ_QRVJ01000001.1	*Bacteroides cellulosilyticus*	AF22-3AC	604020	580904	+	1
NZ_QRSV01000007.1	*Bacteroides cellulosilyticus*	AF29-17	269200	292317	-	1
NZ_LRGD01000010.1	*Bacteroides cellulosilyticus*	CL09T06C25	44848	67991	-	1
NZ_JH724085.1	*Bacteroides cellulosilyticus CL02T12C19*	CL02T12C19	94259	117409	+	1
NZ_EQ973491.1	*Bacteroides cellulosilyticus DSM 14838*	DSM 14838	290576	313959	+	1
NZ_KQ968695.1	*Bacteroides intestinalis*	KLE1704	249385	272510	-	1
NZ_QSUL01000020.1	*Bacteroides oleiciplenus*	OM05-15BH	29099	52460	+	1
NZ_JH992940.1	*Bacteroides oleiciplenus YIT 12058*	YIT 12058	1533689	1555212	-	1
NZ_JAGH01000002.1	*Bacteroides sp. 14(A)*	14(A)	2478682	2501030	+	1
NZ_RAZN01000003.1	*Parabacteroides goldsteinii*	0.1X-D42-15	28300	49757	+	1
NZ_JH976474.1	*Parabacteroides goldsteinii CL02T12C30*	CL02T12C30	486316	507720	+	1
NZ_KE159513.1	*Parabacteroides goldsteinii dnLKV18*	dnLKV18	1159956	1181413	-	1
NZ_QSWF01000006.1	*Parabacteroides gordonii*	OM02-37	268051	289934	-	1
NZ_KE386763.1	*Parabacteroides gordonii DSM 23371*	DSM 23371	428020	449720	+	1
NZ_KQ033920.1	*Parabacteroides gordonii MS-1*	MS-1	770218	791918	-	1
NZ_QUHH01000016.1	*Parabacteroides sp. AF14-59*	AF14-59	19676	38492	+	1
NZ_QTMM01000005.1	*Parabacteroides sp. AF17-3*	AF17-3	296338	317796	-	1
NZ_QUGR01000001.1	*Parabacteroides sp. AF18-52*	AF18-52	476916	495726	-	1
NZ_QUDI01000023.1	*Parabacteroides sp. AF48-14*	AF48-14	70961	91946	+	1
NZ_KB822571.1	*Parabacteroides sp. ASF519*	ASF519	5930811	5952268	-	1
NZ_KQ033902.1	*Parabacteroides sp. HGS0025*	HGS0025	2808277	2829908	+	1
NZ_LT669941.1	*Parabacteroides timonensis*	Marseille-P3236	862055	883911	-	1
NZ_LN877293.1	***Bacteroides fragilis***	**BE1**	**3202545**	**3220402**	**-**	**2**
NZ_QRZO01000002.1	*Bacteroides fragilis*	AF14-14AC	123788	141668	-	2
NZ_QRZH01000002.1	*Bacteroides fragilis*	AF14-26	126212	144092	-	2
NZ_AKBY01000003.1	*Bacteroides fragilis CL05T00C42*	CL05T00C42	80558	98415	-	2
NZ_JH724200.1	*Bacteroides fragilis CL05T12C13*	CL05T12C13	80522	98379	-	2
NZ_JGDN01000068.1	*Bacteroides fragilis str. 3397 N2*	3397 N2	69183	87040	-	2
NZ_JGEG01000075.1	*Bacteroides fragilis str. 3397 N3*	3397 N3	69898	87755	-	2
NZ_JGDO01000041.1	*Bacteroides fragilis str. 3397 T14*	3397 T14	2229	20086	-	2
NZ_JH976506.1	***Parabacteroides sp. D25***	**D25**	**344282**	**355992**	**+/-**	**3**

Table 5.3 Sequence coordinates of genes within the BIS clusters that form three different genetic arrangements. (Continued)

Accession	Species	Strain	start	stop	strand	arch.
NZ_GG705151.1	*Bacteroides sp. 2_1_33B*	2_1_33B	811723	828493	+/-	3
NZ_CYXP01000003.1	*Parabacteroides distasonis*	2789STDY5608872	797	17567	+/-	3
NZ_CZAR01000002.1	*Parabacteroides distasonis*	2789STDY5834901	1098	17868	+/-	3
NZ_CZBM01000003.1	*Parabacteroides distasonis*	2789STDY5834948	348507	365278	+/-	3
NZ_QRXK01000024.1	*Parabacteroides distasonis*	AF18-10	690	12400	+/-	3
NZ_QRPA01000023.1	*Parabacteroides distasonis*	AF36-3	57479	74255	+/-	3
NZ_NFJX01000005.1	*Parabacteroides distasonis*	An199	298514	315279	+/-	3
NZ_NNCA01000001.1	*Parabacteroides distasonis*	CBA7138	2878268	2889978	+/-	3
NZ_JH976489.1	*Parabacteroides distasonis CL09T03C24*	CL09T03C24	423568	440338	+/-	3
NZ_JNHK01000089.1	*Parabacteroides distasonis str. 3776 D15 i*	3776 D15 i	3655	25273	+/-	3
NZ_JNHU01000057.1	*Parabacteroides distasonis str. 3776 D15 iv*	3776 D15 iv	236310	253075	+/-	3
NZ_JNHL01000054.1	*Parabacteroides distasonis str. 3776 Po2 i*	3776 Po2 i	82522	99287	+/-	3
NZ_KQ236096.1	*Parabacteroides sp. 2_1_7*	2_1_7	1621793	1633503	+/-	3
NZ_QSQY01000029.1	*Parabacteroides sp. 20_3*	TF09-4	28656	45426	+/-	3
NZ_QSQL01000017.1	*Parabacteroides sp. 20_3*	TF12-11	628	17398	+/-	3
NZ_QTMJ01000002.1	*Parabacteroides sp. AF19-14*	AF19-14	758	17529	+/-	3
NZ_QTMG01000015.1	*Parabacteroides sp. AF21-43*	AF21-43	616	17386	+/-	3
NZ_QTLZ01000003.1	*Parabacteroides sp. AF27-14*	AF27-14	356010	372781	+/-	3
NZ_QTLP01000005.1	*Parabacteroides sp. AF39-10AC*	AF39-10AC	654	17424	+/-	3
NZ_QTLI01000013.1	*Parabacteroides sp. AM17-47*	AM17-47	642	17412	+/-	3
NZ_QTLD01000037.1	*Parabacteroides sp. AM25-14*	AM25-14	1411	18181	+/-	3
NZ_RAYG01000046.1	*Parabacteroides sp. CH2-D42-20*	CH2-D42-20	801	17566	+/-	3
NZ_KQ236106.1	*Parabacteroides sp. D26*	D26	373154	389924	+/-	3
NZ_QTMX01000003.1	*Parabacteroides sp. OF01-14*	OF01-14	867	12577	+/-	3
NZ_LIDT01000035.1	***Bacteroides fragilis***	**20793-3**	**14633**	**36151**	**-**	**Tn**
NZ_PDCT01000010.1	*Bacteroides fragilis*	CM1`3	14711	36229	-	Tn
NZ_QTLE01000029.1	*Bacteroides sp. AM23-18*	AM23-18	24670	49228	+	Tn
NZ_QRZK01000006.1	*Bacteroides thetaiotaomicron*	AF14-20	31752	54562	+	Tn
NZ_QROV01000008.1	*Bacteroides thetaiotaomicron*	AF37-12	31743	54552	+	Tn
NZ_QRKS01000015.1	*Bacteroides thetaiotaomicron*	AM15-10	56434	79243	+	Tn
NZ_QSLC01000002.1	*Bacteroides thetaiotaomicron*	AM26-17LB	486821	509631	-	Tn
NZ_FOAL01000016.1	*Bacteroides thetaiotaomicron*	KPPR-3	50034	72843	-	Tn
NZ_JH976467.1	*Parabacteroides johnsonii CL02T12C29*	CL02T12C29	152432	172162	-	Tn

Table 5.4 Available annotations for the BIS gene cluster.

Locus Tag (IMG: GOLD Analysis Project ID)	KEGG ID	Protein ID	Gene annotation	T4 homolog	pADAP homolog
Ga0123724_112369	BcellWH2_03967	>WP_029427212.1	DUF4255	-	Afp16
Ga0123724_112370	BcellWH2_03968	>WP_029427211.1	Hypothetical protein	-	-
Ga0123724_112371	**BcellWH2_03969**	>WP_029427210.1	**Sheath1**	**gp18**	**Afp2/3/4**
Ga0123724_112372	**BcellWH2_03970**	**>WP_029427209.1**	**Sheath2**	**gp18**	**Afp2/3/4**
Ga0123724_112373	**BcellWH2_03971**	**>WP_007212392.1**	**Tube1**	**gp19**	**Afp1/5**
Ga0123724_112374	**BcellWH2_03972**	**>WP_007212393.1**	**Tube2**	**gp19**	**Afp1/5**
Ga0123724_112375	BcellWH2_03973	**>WP_007212394.1**	Hypothetical protein	-	Afp6
Ga0123724_112376	BcellWH2_03974	>WP_029427208.1	LysM	gp6	Afp7
Ga0123724_112377	BcellWH2_03975	>WP_029427207.1	Spike	gp5	Afp8
Ga0123724_112378	BcellWH2_03976	>WP_029427206.1	Tip	gp5.4	Afp10
Ga0123724_112379	BcellWH2_03977	>WP_029427205.1	Baseplate	gp25	Afp9
Ga0123724_112380	BcellWH2_03978	>WP_029427203.1	Hypothetical protein	-	-
Ga0123724_112381	**BcellWH2_03979**	**>WP_029427202.1**	**Baseplate**	**gp27**	**Afp11**
Ga0123724_112382	BcellWH2_03980	>WP_029427201.1	Hypothetical protein	-	Afp13
Ga0123724_112383	BcellWH2_03981	>WP_029427200.1	Baseplate	gp6	Afp12
Ga0123724_112384	BcellWH2_03982	>WP_029427199.1	Hypothetical protein	-	Afp14
Ga0123724_112385	BcellWH2_03983	>WP_029427198.1	DUF4157	-	-
Ga0123724_112386	BcellWH2_03984	>WP_007215181.1	FtsH/ATPase	-	Afp15

Table 5.5 Statistical Analyses of BIS protein counts and Bacteroidetes abundance. Confidence Interval (CI) for the difference in frequency medians between Healthy, Prediabetes, and IBD groups using a percentile nonparametric bootstrap method. The estimated difference in medians is reported and the corresponding 95th percentile Confidence Intervals. Confidence Intervals that do not cover zero have significantly different medians, denoted with an asterisk. See **Table 5.6** for Asymptotic Wilcoxon Rank Sum Test results.

	Healthy - IBD	**Healthy - Prediabetes**	**Prediabetes - IBD**
BIS protein count	0.444* (0.349, 0.505)	0.04 (-0.044, 0.163)	0.404* (0.259, 0.484)
Bacteroidetes abundance	0.126* (0.058, 0.184)	0.218* (0.153, 0.302)	-0.092 (-0.186, -0.033)

Table 5.6 Asymptotic Wilcoxon Rank Sum Test results of the analyses of BIS protein counts and Bacteroidetes abundance. *Alternative hypothesis: true must not equal to 0.

	Healthy - IBD		Healthy - Prediabetes		Prediabetes - IBD		Crohn's - colitis	
	W	**p-value**	**W**	**p-value**	**W**	**p-value**	**W**	**p-value**
BIS protein count	17758	< 2.2e-16	3307	0.3716	2419.5	4.05E-08	1144	0.5339
Bacteroidetes abundance	14712	1.37E-06	4652	1.99E-06	1092	0.04941	1175	0.6817

Acknowledgements

Chapter 5, in full, is published in mSystems, 2020. Rojas MI*, Cavalcanti GS*, McNair K, Benler S, Alker AT, Cobián-Güemes AG, Giluso M, Levi K, Rohwer F, Bailey BA, Beyhan S, Edwards RA, Shikuma NJ. The dissertation author was the co-primary investigator and co-first author of this paper.

I would like to thank Dr. Martin Pilhofer for providing constructive comments on the manuscript. This work was supported by the Office of Naval Research (N00014-17-1-2677, N.J.S. and S.B.), Office of Naval Research (N00014-16-1-2135, N.J.S), the National Science Foundation (1942251, N.J.S., 2017232404, A.T.A., and OISE1243541, F.L.R.), Alfred P. Sloan Foundation, Sloan Research Fellowship (N.J.S.).

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