Intraspecies Antagonistic Interactions Driven by the Type VI Secretion System in *Vibrio cholerae*

by

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Abstract

The type VI secretion system (T6SS) is a contact-dependent molecular weapon used by bacteria to transport a variety of effectors into neighbours. Attacked cells must have immunity proteins specific to each incoming effector in order to neutralize their cytotoxic effects. *Vibrio cholerae,* a ubiquitous species in temperate and tropical coastal waters, possesses a T6SS that includes at least three unique pairs of Effector-Immunity (EI) proteins. Maintaining a diverse population of EI in one location suggests that T6SS intraspecies competition can influence population structuring through spatial segregation. We investigated T6SS-mediated competition interactions in a pairwise manner for 14 isolates from a single population in Oyster Pond, MA, located off the eastern US coast. Of the 91 possible pairwise competitions, the majority of strains are unable to coexist, with some instances of isolates able to outcompete others, and minimal cases of coexistence based on predicted competitive outcomes of T6SS EI compatibility. This is due to rapid horizontal gene transfer (HGT) events allowing diversification of EI modules in a lineage-specific manner.

Pairwise competition assays were performed on all 14 isolates, with the outcomes generally matching the prediction made based on their T6SS compatibility. One exception to this was a unique isolate with an extensive, but potentially not completely expressed, EI array. In all other cases, strains predicted to win outcompeted their opponents, isolates predicted to coexist experienced minimal reduction, and incompatible strains underwent a more

diverse gradient of loss. A competitive fitness-based hierarchy of Oyster Pond resident strains demonstrates structured T6SS efficacy within the population. Temperature-dependent competitive outcomes suggest abiotic factors influence T6SS interactions, which will be useful to consider in future work. Together, this data presents the effects of T6SS-mediated competition on intraspecific structuring of environmental populations.

Preface

This research was a collaborative effort. Individual contributions are listed below.

A version of chapter two will be submitted for publication as

“Hussain NAS, Kirchberger PC, Case RJ, and Boucher YF. Modular molecular weaponry leads to dynamic competitive outcomes in an environmental *Vibrio cholerae* population.”

All authors were involved in the creation and optimization of the experimental design, and had input on how best to analyze raw data. NASH performed all bench work experiments and generated competition figures. PCK analysed *Vibrio cholerae* genomes and constructed phylogenetic trees. NASH and PCK wrote the manuscript. All authors were involved with manuscript editing. The study was supervised by RJC and YFB.

Dedication

To M. Myers, who showed me that persistence is the key to success.

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Chapter 1: Literature Review

1.1 Introduction

Community ecology attempts to unravel the complex processes and interactions underpinning a multi-species system within its habitat, and has been continuously reinterpreted through phylogenetic, genomic and biological advancements (1). While the field of community ecology was established in macroorganisms, the concepts are still applicable to microbial communities, although perhaps more difficult to translate due to the variation of scales assessed, the opportunity for lateral gene transfer, and the asexual reproduction of prokaryotes. In addition to the above, microbial communities must also account for the dynamic physiochemical gradients within a microcosm (2). These implications pose a unique challenge in assessing prokaryotic communities: how do microbes maintain diverse and distinct populations within a community considering the overlapping metabolic and genomic profiles? Niches emerge and persist based on interactions at the individual and population levels in a spatiotemporal dependent manner, and influence the functional output and evolution of these microbial communities.

My thesis aims to interrogate the role of type VI secretion system (T6SS) contact-dependent killing in intraspecific competition. The T6SS can be used to spatially segregate populations and maintain diversity between related bacteria in a single environment (3). My research explores antagonistically driven population

structuring of *Vibrio cholerae* isolates collected from a shared habitat to observe environmentally informed population shifts through the T6SS.

1.2 Maintaining microbial species in a sea of horizontal gene transfer

There is a notable amount of prokaryotic contributions in shaping major global evolutionary events: the rise of atmospheric oxygen was facilitated through cyanobacteria, and diversification of the lineage resulted in the first steps towards multicellularity (4). The discovery of Asgard archaea has begun to bridge the gap between the domains of life, linking proteins generally associated with eukaryotes to a prokaryotic ancestor (5). There is also a substantial amount of effort needed to begin to untangle our origins from those of our unicellular progenitors; the first isolate from Asgard superphylum took over a decade of cultivation to obtain (6). In light of progress towards understanding of our complex history with the most basal branch of life, we must first consider what defines a species in order to pragmatically describe members within the domain.

In contrast with classical species concepts with roots in flora and fauna, prokaryotes are unhindered by barriers such as sexual reproduction to produce viable offspring, or qualitative assessment, to be defined (7, 8). As such, species delineation requires consideration of their evolutionary history, and deliberation into generating standardized criteria to define a species. Unlike macro-scale organisms, the ease in which bacteria exchange genetic content occurs rapidly and is appreciable among human timescales. Horizontal gene transfer (HGT) is

in direct opposition to periodic selection, which favours adaptive mutations and purging less fit variants, has led to debate on whether species can accurately and consistently be described in prokaryotic contexts (9-12). A reanalysis of microbial systematics must be performed in order to approach taxonomic classification with incorporation of new scientific knowledge and advances in the field.

1.2.1 A brief history on the classification of microbial species

Microbiologists face unique challenges in the field of taxonomy to unravel what members exist in complex communities and what functions they carry out. The former requires a working criteria of what dictates a species to inform the latter, which has led to the 70% DNA-DNA hybridization (DDH) cutoff, and a distinguishing phenotypic trait as the seminal features to demarcate bacterial species (13). The hybridization experiments required were technically challenging, generally required collaboration with specialized personnel and equipment, could not be implemented for environmental sampling, and the arbitrary 70% cutoff mainly served to justify extant bacterial species that were previously classified based on phenotype (9, 13, 14). As an answer to the difficulties of performing DDH, the 16S rRNA gene has been used as a gold standard due to conserved regions for primer design interspersed with hypervariable regions for taxonomy delineation, and the 1.5kb gene being large enough for analysis on (15, 16). A 97% sequence identity between two nucleotide segments of 16S rRNA has been appointed as the cutoff to assign

isolates to the same species, and 95% similarity is used to classify strains to the same genus (15). The 97% value has recently been modified to a 98.7-99% due to poor quality of publicly available sequences (14). Other genomic cutoffs such as 95% average nucleotide identity (ANI) were found to correspond to the 70% DDH value imposed, which correlated the similarities of two genomes on a nucleobase scale and was less experimentally demanding. With the availability of whole genomes, additional methods such as multi-locus sequence analysis (MLSA) to assess multiple housekeeping genes and identify allelic profiles, and phylogenetic analyses to address evolutionary relationships have improved species delineation through culture independent techniques (9, 17).

A systematics-based approach to microbial taxonomy is clearly required, however, ecological profiles should also be considered to establish meaningful species definitions. Diversification of metabolic processes and niches within a single species suggests the long-reaching impact of environmental adaptation on speciation. The ecotype theory in microbes posits that ecologically distinct populations can coexist due to distinctive niches forged in an environment, which emerge through accumulation of physiological and genetic differences (18). These cohesive forces are maintained through intraspecific purges of diversity on a genome level, a process called periodic selection. Upon speciation and diversification away from a shared genome, these now distinct lineages accrue and maintain neutral mutations that prevent reconciliation of new lineages back to the ancestral form. These ecotypes can arise in microcosms consisting of a

single species maintained over time without the requirement for geographic isolation (19-21), and speciation resulting in these conditions (cladogenesis) can occur just as frequently as adaptive mutations within a single lineage (anagenesis) (22). High-resolution intraspecific niche differentiation has also been observed in *Vibrio cyclitrophicus* populations recovered from small (S) or large (L) fractions of seawater (23). Ecological single nucleotide polymorphisms (SNPs) have recently allowed the L population to associate with hosts by presence of colonization, chitin adherence, and biofilm formation genes, while S strains share fairly conserved accessory genomes. There are higher observed recombination rates within, rather than between, microhabitats, suggesting a preference for environmentally selective HGT between diverging *V. cyclitrophicus* populations.

1.2.2 Muddying the microbial waters with horizontal gene transfer

While the reconciliation of multiple forms of data has allowed microbes to be identified in a pragmatic fashion, the phenomenon of HGT hinders ease of classification. Three different *Escherichia coli* sequences have been observed to share less than 40% of the same genes, and yet their remarkably conserved synteny of core genes places them all in the same species (24). These auxiliary genes that are not shared within all members within a species, define pathotypes and functionalities that contribute to a greater intraspecific diversity. The accumulation of mutations is certainly a factor towards diversification, but the extent of genes shared amongst unrelated species and even across kingdoms

indicates the involvement of HGT (25, 26). Genomic plasticity through DNA exchange is mediated through three processes, namely, natural transformation, conjugation, and transduction. This section will briefly outline the mechanisms of non-vertical inheritance of traits in the context of bacteria.

Some species are naturally competent and encode DNA-uptake apparatus that may become activated in times of starvation, high cell density, or environmental stresses such as UV radiation, in a process called natural transformation (27). Single stranded DNA (ssDNA) is taken up from the extracellular milieu across the inner membrane into the bacterial cytoplasm, either in a selective manner based on specific motifs distributed within the DNA segment, or independent of sequence (28). Two non-mutually exclusive fates await these DNA fragments once within the cell: DNA degradation to a nutritive resource, or incorporation into the genome through natural transformation (29, 30). In the former scenario, internalized DNA can be degraded and its constituents utilized by the recipient to reduce the energetic burden of *de novo* nucleotide synthesis (29, 31). The latter condition involves the heritable integration of exogeneous DNA that may manifest neutral, beneficial, or disadvantageous effects after expression (30). Biased uptake of DNA sequences can occur due to the recognition of specific DNA uptake sequence (DUS) segments encoding conspecific markers, demonstrated by members within *Neisseriaceae* and *Pasteurellaceae* (30). This sequence selection precedes the final fate of externally acquired DNA, and this bias is expected to be present in some degree in all bacteria species expressing

DNA uptake apparatus: *Neisseria* mutants that lack the only described DUS specificity factor, ComP, are greatly impaired in natural transformation (30, 32). In contrast, *Bacillus subtilis* has been recently demonstrated to prefer uptake of less related kin strains in an effort to practice “promiscuous but safe sex”: diversifying genomic content while reducing the risk of incorporating potentially harmful DNA of unrelated species (33). In silico simulations of bacterial populations at equilibrium demonstrate that cells with natural transformation capabilities are more fit compared to propagation with binary fission exclusively (34). Natural transformation provides the opportunity for incorporation of foreign DNA independent of any requirement for the donor and the recipient cell to be physically present in a shared space.

Conjugation refers to the exchange of macromolecules between partners in physical contact (35). There are multiple conjugative systems, which result in promiscuous DNA transfer within bacteria, as well as inter-kingdom exchanges (36-38). Conjugation is mediated through the type IV secretion system (T4SS) to transport both nucleoprotein complexes and proteins (36, 39). Prior to transfer, donor cell double stranded DNA (dsDNA) is nicked and unwound to form a DNA-protein complex that is then recruited to the T4SS for translocation (35). T4SS genes are often encoded on self-transmissible or mobilizable plasmids or integrated transposons to facilitate the spread of potentially advantageous traits, such as virulence factors or antibiotic resistance genes in a contact-dependent manner.

Transduction consists of the transfer of genetic material through bacteriophage introduction. DNA flanking the phage attachment site may be incorporated, or host DNA can be integrated randomly during phage packaging (40). Lysogenic conversion into the host genome provides potential temporal adaptive traits, at the expense of eventual cell death upon the reversion to the phage lytic cycle (41). Transduction is limited by the ongoing bacterial defenses against incoming foreign DNA, and phage host range. However, as phages are the most abundant biological entities on earth, their effect on bacterial population dynamics is commanding: 10-20% of aquatic bacteria are estimated to succumb to phage predation daily (42, 43). Consideration into phage-mediated population turnover in addition to effects outside of host mortality should be accounted for in terms of novel gene acquisition in nature.

1.2.3 Intra-species bacterial identification

Researchers of macro-scale systems have emphasized the importance of maintaining intraspecific variation for ecological biodiversity (44) – a concept shared concept in microbial ecology. However, the challenge of defining bacterial taxa expands to multiple classifications due to the fluid nature of genomic plasticity. Estimates on global prokaryotic species diversity (45-49) do not address intraspecific variability. Endogenous mutations, in addition to larger scale genomic variation such as the presence or absence of plasmids, genes, prophages, genomic islands, induce further divergence within a single species

(50). Strain identification is crucial to understanding the habitat, virulence, evolution, and metabolic potential beyond a species concept.

Phenotypic methods, such as assays to test for the presence of biochemicals, antibiotic susceptibility, or ability to grow on selective media, have been substituted in favour of molecular methods (51). Genotyping offers the ability to discriminate strains based on DNA at a high resolution. Ongoing efforts to sequence bacterial isolates has allowed the establishment of the core genome, consisting of genes found within all strains of a species, accessory genes that are present in at least two members, and unique genes only found in a single strain (52). Altogether, the core, accessory, and unique genes comprise the pangenome, which represents the complete genetic material within a species. Understanding the genetic content of strains is crucial for scenarios requiring precise identification, such as epidemiological surveillance (53-56). Globalization provides pathogen access to non-endemic areas, and thus tracking clonal expansions of outbreaks is essential in generating a rigorous and rapid response to communicable diseases (53, 55). Multiple in silico methods to differentiate strains have been established (51, 57-63), and may be genera dependent, or utilized on a broader scale with parameter adjustments.

While the need for a sensitive and precise method to differentiate bacteria strains is evident in clinical microbiology, these ideas should also be translated to ecological applications. *Pseudomonas putida* is an opportunistic pathogen in

nosocomial infections, in addition to existing as an environmentally ubiquitous bacterium due to the wide range of metabolic potential found in its pangenome (64). Existing as a phylloplane resident – the microbial community inhabiting the surface of leaves – the strain *P. putida* KT2440 has been shown to outcompete coincubated phytopathogens on *Nicotiana benthamiana*, reducing leaf necrosis of its plant host, and function as a possible biocontrol agent on crops (65). *Aliivibrio fischeri* is responsible for the nightly colonization and *illumination* of the light organ in the Hawaiian bobtail squid *Euprymna scolopes*, however; despite numerous bioluminescent *A. fischeri* isolates being recovered from the surrounding seawater, only specific strains are ever isolated from the host (66). *Myxcoccus xanthus*, a social, predatory bacterium, displays distinct complex hunting strategies dependent on prey: *Sinorhizobium melitoti* colonies that lack the exopolysaccharide galactoglucan, are consumed by waves of aggregated *M. xanthus* invading undirectionally to lyse cells (67). In contrast, *S. melitoti* strains expressing galactoglucan are entirely surrounded by aggregates of *M. xanthus*, with the latter initiating a rippling behavior associated with scavenging cell debris before predation and subsequent lysis of prey (68). These selected scenarios serve as appreciable examples to support the need for interspecific resolution of bacteria to understand population dynamics, host-microbe associations, and expression regulation within a species.

With such a rich evolutionary background, tracing microbial lineages on global timescales requires consensus on how to define a species using polyphasic

approaches (69). With the advent of next generation sequencing (NGS) technology, the ease of sequencing whole bacterial genomes is now financially and methodologically more feasible (70). The refinement and ongoing development of NGS methods has allowed an intensive curation of genomes to investigate what members of a microbial community exists, and what ongoing metabolic functions are carried out. Reconciliation of phenotypic data in the genomic age of microbiology offers a holistic approach at the classification at the species and sub-species level, with consideration into ecological niches, evolutionary relationships, metabolic potential, and HGT transfer.

1.3 Community theories in microbial contexts

While the scales upon which microbial communities are assessed are miniscule, the concepts bridging ecological processes between micro- and macro-frameworks remain fairly similar. Both systems are subject to selection, mutation, and genetic drift, and define communities as multispecies assemblies interacting in a shared environment (1). However, interpretations of what a contiguous habitat is, and what is defined as an interaction, are less standardized in microbial ecology, leading microbiologists to not only attempt to unravel the significance behind microbiology communities, but also to defend the existence and pragmatic classification of them. The field of community ecology has been labelled “a mess” due to its innumerable influencing factors, with microbial diversity especially scrutinized and lamented as ‘being beyond practical calculation’ (71, 72). An additional challenge in this vein lies in its fundamental

tenet of *'everything is everywhere, but the environment selects'* – stated by Lourens Baas-Becking in 1934 (73). The notion that bacteria are all cosmopolitan, equally distributed inhabitants, and abiotic habitat properties alone predict their environmental presence, does not account for dispersal capabilities, nor specific host associations. Contemporary research has indeed found biogeographical distributions of bacteria both on global scales in aquatic and terrestrial environments (74, 75), and at more intimate scales (76-78). However, the flow of microbial genes between distinct, geographically separated environments is unimpeded by regional constraints and gene pools instead correlate to niches, supporting the idea of ubiquitous distribution of genes, with ecology serving as a major selection force (79). This incongruence between the distribution of genes and the members encoding them serve as further challenges in the framework of microbial ecology, and leads us to consider how variation is maintained at different scales.

1.3.1 Persistence of diversity in communities

There are several theories regarding how diversity persists in a habitat. In niche theory, adaptations facilitate bacterial heterogeneity by allowing different organisms to coexist spatially by occupying different roles in a microecosystem (80). Individuals with higher fitness outcompete others in a habitat with shared resources, and thus members performing the best in a specified ecological niche establish speciation (81). This is similar to the previously discussed ecotype model, in which ecological diversification within a population initiates the

irreversible process of divergence (18). Recent advancements in long-term environmental sampling have yielded an extensive community dataset of coastal plankton over 93 days (82). Across broad taxonomic rankings, composition was relatively stable, while finer scale analysis indicated rapid turnover at the operational taxonomic unit (OTU) associated with the species level classification that correlated with abiotic and biotic fluctuations on the microcosm scale. Specific algal peaks corresponded to rapid increases in bacterial OTUs able to metabolize exudates in the surrounding phycosphere (82), which is the area surrounding the primary producer enriched in nutrients (83). Several OTUs persisted at minimal abundance with short-lived expansions in appropriate conditions (82). Competition from higher trophic level organisms in a top down approach, in tandem with environmental parameters, prevents the expansion of rare microbes until conditions allow transient blooms (84). Structured niches allow the maintenance of biodiversity for both populations, allowing less abundant species to exist within narrow, defined niches.

In contrast, neutral theory suggests that stochastic processes such as death, speciation, or immigration into a population leads to diversity of species in an unpredictable manner, assuming species have similar functions, equivalent competitive ability, and that niches are negated (81). Introduction of external genes into a community is further complicated by dispersal limitations of bacteria, as although the genetic material itself may be promiscuously found across different environments (79), the ability for natural transformation is species

dependent. Additionally, the long-term evolution experiment (LTEE) established in *E. coli* in 1988 (85), illustrates that over tens of thousands of generations in stable environments, adaptive mutations among the 12 founding populations continue to develop and are not expected to reach saturation (86). Interestingly, nonsynonymous mutations in the core genome in the LTEE are acquired more frequently compared to accessory genes, while genes under positive selection are more stringently conserved compared to wild type populations in the environment (20). Consideration of stochastic forces as high dimensional data allows interrogation of underlying features such as growth rates, individual interactions and phenotypic profiles underpinning the large-scale processes of birth, death, and recombination (87). In a wider context, niche-neutral hypotheses have generated much debate over the process of speciation originating from a single population, or how the accumulation of adaptive traits leads to divergence (12, 88). However, reconciliation of niche and neutral theories allows researchers to understand microbial diversity at distinct scales: neutral theory has been suggested as a framework for the global forces influencing community ecology, while niche theory provides higher resolution analysis, at more localized scales (88).

In addition to niche-centric and neutral models, individualistic, temporally dependent considerations should be supplemented to further understand microbial diversity. In communities with non-transitive competition, different strategies may be given preference at different time-points, which allow

biodiversity to be maintained in a more rock-paper-scissors (RPS) community approach (89). This has been demonstrated in antibiotic mediated warfare in *E. coli* populations, where strains that produce the antibiotic colicin (C) outcompete sensitive (S) strains, sensitive strains overtake resistant (R) strains due to having faster growth rates, and R strains are unaffected by the antibiotics produced by C isolates (90, 91). All strains are able to be recovered if spatial heterogeneity is conserved and lethal interactions are localized to the interface of competing populations (91). *In vivo* experiments demonstrate that C, S, and R isolates of *E. coli* rapidly cycle within individual mice, but diversity persisted for longer between cohabiting mice (90). Of note is the inherent detriment colicin production entails for its synthesizers; secretion of the toxin into the extracellular milieu occurs only upon lysis, with activation of the SOS response due to DNA damage (92, 93). This mechanism is repressed under favorable conditions, but activated to preserve kin at local scale interactions (92).

Frequency dependent selection (FDS) highlights individual fitness as being dependent on the other existing phenotypes within a population, thus contributing to microbial diversification (94). Heterogeneity persists in stabilizing, or negative FDS systems, such as in habitats with bactericidal or bacteriostatic compounds (95). In harsh conditions, cooperation is favored amongst otherwise competing species to allow degradation of toxic compounds using a variety of metabolic pathways encompassed communally. A recent example of this is presented from Piccardi et al. (96), where four bacterial species selected for their bioremediation

potential, coexisted in industrial waste fluid as a community. In comparison, during monoculture experiments, only one isolate was able to reliably survive alone. When stress upon the community was alleviated through supplementation of alternative nutrients, competition – not collaboration – ensued. In disruptive, positive, FDS models, allelic diversity purges result in the preference for a common genotype (95). Rare alleles are unable to invade a population due to being at an inherent detriment upon introduction, as demonstrated with aposematic organisms (97). Species that present strong visual cues to warn predators of their toxicity (aposematism) are avoided, while new immigrants that do not present unpalatable signals are subject to increased predation. In microbial communities, this can be translated to antibiotic producers and a population sensitive to the secreted compound (95). Preference for the production of the antibiotic is favored over the sensitive genotype, resulting in the latter reduced or entirely displaced. Population shifts dependent on the extant community genotype pool can thus result in the maintenance of diversity, or the selection against it.

1.3.2 Communities based on behavior and function

If evolutionary processes still ensue in populations maintained under constant conditions with seemingly little pressure to adapt (85), how do bacteria survive in unpredictable, dynamic environments more reflective of natural habitats? Bet hedging involves stochastically driven phenotypic changes, to further chances of survival in rapidly changing or uncertain conditions (98). These switches are

independent of other members in a population, but undergo positive selection if the overall outcome is favorable. The assumption of environments in flux impedes consistent identification of which specific variables change, but loci under relaxed genetic constraint may be outcomes of adaptive evolution, allowing the exploration of phenotypic plasticity at relatively low cost (99). Hypermutator phenotypes have been explored in pathogens, such as the extensive variation of lipopolysaccharide constituents of *Haemophilus influenzae* to evade host immune responses (99, 100), as well as *Pseudomonas aeruginosa* recovered from a patient at early diagnosis of cystic fibrosis (CF) at six months, and after chronic infection 7.5 years after (101). Comparative genomics of both *P. aeruginosa* isolates determined a total of 68 mutations were accumulated during host incubation, with 33 instances being nonsense mutations. Outside of medical infections, *P. aeruginosa* monocultures still undergo rapid genomic diversification to produce different phenotypes within a single population to increase resilience to environmental perturbations (102). While rapid mutation may allow survival in unstable conditions, there are clear drawbacks to imposing random mutations: genome decay resulting in loss of multiple gene functions, loss of fitness, and displacement of these phenotypes in a population altogether. Interestingly, these deficits may not always occur, and mutator phenotypes in the LTEE have been shown to operate with increased fitness compared to nonmutators in their specified conditions, despite having evidence of genome decay, and are less fit than their counterparts when grown in different conditions

(103). Bet hedging may offer individualistic fitness advantages and increase diversity independent of population and community composition.

Metabolic potential of microbial consortia expands on community structuring above population level classification and temporal restraints. A wide variety of functional processes are polyphyletic and can be observed across multiple taxa through HGT, convergent evolution, genome streamlining, or loss of function (104). This phenomenon has been demonstrated through observing dissimilar bacterial colonizers of the green algae *Ulva australis*, presenting high conservation of metabolic capabilities (70%) and a subset functional genes maintained in all communities (105). Functional redundancy may also shed light into how successional shifts exist within finer time scales: discrete communities sampled from similar environments are the result of gradual displacements in the absence of excessive disturbance. Feng et al. (106) describe distinct microbiome shifts in early human infant development attributed to a breast milk and formula diet (S1) and the subsequent weaning and the provision of complementary food (S2). Coincubation of key species from the S1 and S2 microbiome resulted in the S2 community consistently outcompeting S1 taxa, with the exemption of four persisting S1 strains. Functional expression profiles were retained in monoculture and mixed culture treatments for these S1 isolates, although shifts towards specific metabolites were observed due to additional substrates available through S2 metabolism. Functional redundancies may mitigate dramatic community

alterations in response to ecosystem perturbations, with specific taxa being less significant to overall output (107).

1.4 Should I stay or should I go: colonization and dissemination strategies

Diversity is maintained in a spatiotemporal manner, which then begs the question of how space and time factor into community establishment and persistence. The competition-dispersal tradeoff concept in ecology dictates that organisms less effective at using resources can capitalize by migrating to new nutrient patches first, and are subsequently displaced by stronger competitors in a patch overtime, restarting the search for substrates (108). High-resolution microbial community screenings have provided observations supporting microbial biogeography across various ranges in contrast to homogeneous distribution. Physically limiting bacterial dispersal was found to reduce microbial abundance and diversity by contrasting microbial communities found in open and closed nylon bags filled with irradiated soil litter (109). Free-living or loosely attached cells isolated from soil with heavy metal contamination were more tolerant towards exposure to these same toxins compared to cells attached to soil particulates in biofilms in the same samples, indicating a bias of geographic distribution of cells at the microniche level (110). On grander scales, closely related *Vibrionaceae* in sympatry have been shown to have distinct and temporally dependent behaviours associated with colonization or free-living states for maximal resource acquisition (111). Within these aquatic systems, bacteria compete for resources

on a micro-landscape scale in which particulate matter can serve as nutrient rich, spatially isolated microcosms in an otherwise oligotrophic environment (112). Microbial dispersion and colonization are discrete, though not mutually exclusive, factors that shape community composition.

Resources in natural habitats are patchily distributed amongst fairly large scales for microorganisms, and motility is an advantageous foraging mechanism (113). The ability to disperse enhances the chance of encountering particles diffused into the medium 100-8000x more frequently than bacteria unable to swim, increasing the rate of substrate discovery and colonization (114). Vergin and colleagues (115) have demonstrated that 70% of all particle encounters are made by motile microbes, where these organisms make up only 0.1% of the relative abundance in a community. Dispersion is energetically costly, but can lead to new nutrient patches and movement may incorporate fine-tuning of motion to respond to micrometer adjustments in chemical gradients, termed chemotaxis (116). This mechanism is widely distributed amongst motile bacteria, and has roles that range from guiding pathogens to heat-stressed corals (117), to attracting symbiotic members of the plant rhizosphere microbiome (118). Chemotactic bacteria accounting for a nearly negligible 0.001% of the total bacterial community, have increased 18% higher encounter rates than only motile members (114). During environmental fluctuations, bacteria with faster dispersal rates may employ bet hedging strategies to find more favourable habitats: in nutrient limited conditions, *E. coli* upregulates motility in anticipation

of identifying resources through chemotaxis (119, 120). Speed is also an important consideration, as prokaryotic swimming rates have been observed up to 1000 μm/s, equivalent to 200 body lengths per second (121), with 3.3 - 27.3 hours calculated as the average time for fast swimming bacteria to encounter particles in the open ocean, while slower bacteria are estimated to discover particles within a day (114). Although heterogeneity of both particle composition and abundance in the environment must be assessed, motility allows primary sensing of resources in oligotrophic conditions, and the ability to colonize new microcosms.

While stochasticity influences the initial distribution of colonizing bacteria, duration and order of species arrival affects downstream composition. Bacterial immigrants with similar fitness entering recently created habitats can expand exponentially, to gain density advantages and prevent subsequent invasions (122). This phenomenon, known as the priority effect, extends to both genotypes and species and is favoured in stochastic environments. The monopolization effect further elaborates on the importance of rapid access to new resources, and denotes that early arriving individuals that are able to adapt to local conditions and in turn prevent displacement of later species that are intrinsically better competitors (119). Due to community heterogeneity, interspecific HGT and de novo mutations can streamline increased fitness and reflect contemporaneous evolution, or evolution occurring in parallel with *ecological* shifts (123).

The initial discovery of transient nutrient patches leads to attachment to physical substrates and competition for resources over extended periods, with the opportunity for subsequent detachment (124). Approaching members can transiently associate with encountered particles, circumnavigate around the object, and select their site of attachment. Initial members on newly formed marine particles are dependent on the relative abundance of species in the surrounding local environment, not particle composition (125). Chitin, a derivative of glucose, is found in both terrestrial and aquatic systems and is the second most abundant biopolymer on earth (126). The substrate forms insect exoskeletons and crustaceous shells, and serves as a nutrient source for bacteria with appropriate metabolic enzymes (127). In microcosm experiments with sterile chitin beads incubated with unfiltered seawater, Datta et al. (112) demonstrate highly diverse communities during the initial attachment phase onto the beads, weakly correlated to attachment capability. A shift towards motile microbes able to degrade chitin was found in the second phase of progression, which correlated with the metabolic selection and communities on beads diverged from the surrounding water bacteria. The third and final phase presented an increase and eventual saturation of diversity with isolates using byproducts of chitin catabolism as nutrient sources. Although the results were reproducible across replicates of individual beads, this three stage successional progression is conceptualized as derivatives of stochastic and deterministic factors (128). Microbial assembly during colonization is associated with the capacity of attachment to the substrate, but can be made of members with

incongruent metabolic profiles. Deterministic factors initiate as microbes begin to change the physiochemical conditions within a microcosm, selecting for specific abiotic factors while competing with other residents for resources. As environments become more stable, ongoing selection tempers communities towards equilibrium if ecosystem perturbation is not experienced. Mature, high density microbial communities attached to substratum can function as “baby machines” (129), with motile progeny dispersing away from attached progenitors and colonize new particles (130).

1.5 Biofilms as natural microbial communities

Historical methods of studying microbes have been in isolation, which has influenced contemporary research on how microbiology is interpreted (131). It is now evident that most environmental bacteria do not exist as solitary, free living organisms (132). A microbial consortium attached to a surface is referred to as a biofilm, which is regarded as the preferential lifestyle of the majority of microbes outside of laboratory conditions. While these intricate microcolony assemblies take days to develop, and cells in biofilm generally grow at slower rates than planktonic cells (133), lower-end estimates suggest 70% of total extant prokaryotes thrive in a “city of microbes” (124, 132), and this microbial communal lifestyle has been immortalized in the oldest fossils in the world (134, 135), with filamentous microbial biomass from hydrothermal vent precipitates discovered in Quebec, Canada posited to be 3 770 – 4 280 million years old (134). Interestingly, interspecies bacteria coexisting in planktonic phase may have a

greater propensity to form biofilms together, indicating a socially driven behaviour (136). What *is* the enduring advantage then, of growing together? Biofilm members develop emergent properties that are not evident in free-living counterparts, and are reflective of supraorganism behaviours (137). Quorum sensing (QS), for example, occurs when bacteria are able to secrete and sense incoming signalling molecules, allowing synchronized behaviours from a population (138). These actions are cell density dependent and only performed if the signalling molecules in the environment are of high enough concentration. Biofilms can be initiated nonspecifically through abiotic stresses, or through biotic interactions in a species-specific manner (136). The scales and functions of these communities vary greatly, and can arise in antibiotic resistant polymicrobial diseases such as CF (139), play roles in bioremediation by degrading unwanted constituents in contaminated substrates (140), solubilize atmospheric carbon into the ocean as part of the biological pump (114), and fix nitrogen in plants (141).

Cells in biofilms are embedded in extracellular polymeric substances (EPS) secreted by enveloped members and represent a complex admixture of proteins, polysaccharides, lipids, and extracellular DNA (142). This matrix accounts for over 90% of the dry mass of biofilms, with living organisms accounting for less than 10% (143). EPS is considered to be “the dark matter of biofilms”, as individual components vary between communities, and are difficult to isolate and quantify (144). Altogether, it serves as a heterogeneous and dynamic mesh to manipulate abiotic factors such as diffusion, sorption, electrical charge, and water

retention, while limiting spatial distribution of cells. EPS also provides a “house” structure to members within a biofilm, offering mechanical stability to serve as the foundation to these metaphorical cities. The matrix can also activate functions independent of biotic constituents: extracellular enzymes in the matrix can be bound to individuals, and perform as an external digestive system to process nutrients in close proximity to cells for subsequent uptake (144). Diffusion is constrained in these microcosms, with internal gradients can forming where the topmost layers receive the majority of environmental exposure and incoming molecules, and concentrations diminish with inward progression (145). This allows division of labour and specialization in a three-dimensional habitat, as bacteria can also utilize the attached surface for resources. These multifarious gradients allow waste and nutrient exchange throughout the EPS, offering energy rich storage in close proximity (146).

1.5.1 Interactions of microbes in an aggregated community

Within these polymicrobial aggregates, biotic and abiotic associations significantly influence internal dynamics. Biofilms assemble into highly structured microcosms facilitating synergistic and antagonistic interactions. This environment serves as a hotspot for HGT due to enclosed, enriched conditions with diverse populations in high density (27). This enables the consortia to establish a relatively efficient cycling of nutrients with the opportunity for gene exchange, while maintaining spatial and ecological niches. Consideration of the

interactions within these communities allows investigation on how diversity affects biofilm productivity (147).

1.5.2 The importance of spatial structuring in biofilms

Spatial segregation is generally established between non-isogenic strains in a biofilm to ease the burden of competing for the same nutrients. Clonal expansion happens within a biofilm and creates heterogeneous patches of cells. This structured arrangement allows incompatible groups to coexist, and cooperative actions to be maximized in a habitat. Spatial arrangement can also be temporally dependent as seen with successional communities, but will greatly influence the output of interactions between members (148, 149). As incongruent cells colonize surfaces, the heterogeneous distribution may persist, or clonal segregation may occur upon biofilm progression (148). Both scenarios have been observed in *B. subtilis* colonization with a biofilm forming strain and a mutant unable to secrete EPS (150). EPS can be seen as a public good product, with the synthesis of it being energetically expensive to make, but beneficial for the group. However, cheaters that do not produce the substrate can still exploit it, at no metabolic deficit. The *B. subtilis* biofilm is homogenized at high starting inoculations of both strains, with the mutant benefitting from the biofilm protection. At low concentrations of both founder strains however, spatial segregation occurs and EPS producers aggregate and interact with each other preferentially over mutants. Genotype separation has been implicated with overall biofilm productivity by allowing cooperative behaviors to ensue with

relations instead of competition (148). In contradiction, the overarching theme of segregated cooperation is orchestrated by competition between unrelated bacteria to preserve spatial kin integrity (151).

1.5.3 Alone we can do so little, together we can do so much.

Coexistence can be a result of indirect actions benefitting surrounding members. Incompatible groups of bacteria interacting on microscale levels have been demonstrated by co-occuring *Streptomyces* strains producing different antibiotics to compete with one another (152). Kelsic and colleagues (153) present an interesting community dynamic of antibiotic degrading and producing organisms allowing antibiotic sensitive strains to survive in well mixed environments. Unlike aforementioned RPS communities, antibiotic resistance is conferred not by intrinsic resistance, but through the breakdown of the antibiotic itself, serving as an indirect protector for sensitive strains. In contrast to cyclical wins based on resistance or growth rate advantages in RPS models, degraders, secretors, and sensitive strains coexisted in tightly intermixed models without spatial segregation. These communities are resilient to invasion from cheaters that do not produce or degrade antibiotics, as they are unable to displace their parental phenotype. Additionally, the secretion of metabolites in co-culture has been shown to increase both self and surrounding species growth (154, 155). In unidirectional by-product cross-feeding, a secreted by-product is used by a different cell in a commensalism relationship, with one cell benefiting and the other incurring no effect (155). These interactions are prevalent within the human

gut microbiome that is exposed to a variety of complex substrates – such as plant-based starches – requiring multiple species and metabolic pathways to extract nutrients (156, 157). Mathematical models by Goyal and Maslow (158) suggest that diversity can be stabilized in cross-feeding situations, as waste products of one bacterium can serve as nutrients for other taxa, to fulfill specific niches. In a simulation examining the effects of pH on community composition, Dohi and Mougi (159) present a scenario with an acidophile and alkaphile able to coexist at intermediate pH levels in approximately equivalent compositions. This state of equilibrium provided the highest community resilience with both populations maintained despite preference for either extreme ends of pH. This can be postulated to occur in the gut microbiome, where most perturbations result in a shift back to homeostasis (160).

Clinical microbiology lends itself to many examples of how biofilms promote virulence in hosts. Siderophores secreted into the EPS allow organisms to chelate iron, a limiting factor in both aquatic and terrestrial environments (161). Iron is also an important molecule for pathogenic organisms to initiate virulence: *P. aeruginosa,* the predominant agent of CF morbidity (162), utilizes siderophores to initiate biofilm formation, and sequestration of iron by host chelators prevents the formation of biofilm microcolonies (163). Once a biofilm formation is established, *P. aeruginosa* can maintain chronic infections in CF patients with minimal host immune response. Additional contributors to the disease such as *Streptococcus milleri* can then invade and exacerbate conditions

and increase lung damage (164). When a biofilm establishes maturity and competition amongst members is low, conditions are permissive for less fit genotypes to exist: *P. aeruginosa* mutants defective in iron scavenging have been recovered in late stage CF mucous, where they would be outcompeted in at earlier stages (165). As biofilms function as independent ecosystems, HGT facilitates community resistance to multiple antibiotics in a short space of time (166). Resistance to antibiotics can be conferred by plasmid or gene exchange, or through de novo mutations considering the strong selective pressure to survive (167). Pathogen resistance to antibiotics that are prescribed as last resort measures is a developing cause for concern in contemporary times. Vancomycin, a last resort antibiotic due to the potential for severe side effects, has been shown to be ineffective in some species of bacteria, such as vancomycin resistant *Enterococcus faecalis* (VRE). Vancomycin resistance is due to the *vanA, vanB, vanD, vanF*, and *van F* genes in enterococci (168). In 2002, researchers discovered *vanA* in methicillin-resistant strain of *Staphylococcus aureus* (MRSA) coisolated with VRE from a single patient. The presence of *vanA* alone allowed MRSA to withstand high concentrations on vancomycin, in addition to eight other antibiotics. As nosocomial infections account for the fourth leading cause of death in the US, mitigating antibiotic resistance in settings with immunocompromised people is of utmost importance (166).

Increases in antimicrobial resistance in biofilms are evident in polymicrobial infections, but also in bacteria sensitive to antibiotics in planktonic forms

becoming tolerant in biofilm (169). *Klebsiella pneumoniae* sensitive to ampicillin is able to withstand 2500 times the minimum inhibitory concentration of the antibiotic in biofilm, compared to its planktonic counterpart during sustained 4 hr exposure (170). Since the strain used in this study does not encode mechanisms to inactivate ampicillin (i.e. a β-lactamase enzyme), and resistance is rapidly conferred in a monoclonal biofilm, this suggests that bacteria can acquire this adaptive trait not by mutations or natural transformation, but through the act of establishing a biofilm (169, 170). It was previously discussed that high stress situations (e.g. heavy metal toxicity) drives facilitation if all species possess equal fitness, with relaxed conditions allowing competition to ensue (96). This phenomenon has also been reflected in the dynamic in vitro response to antibiotic challenges (171). Spatial segregation of an auxotrophic strain resistant to the antibiotic ceftriaxone sodium (CRO) and a sensitive isolate able to provide nutrients for the former was determined to be dependent on CRO presence. In microfluidic chambers generating a passive gradient of both CRO and lactose, resistant bacteria mobilized closer towards the antibiotic source, protecting the sensitive nutrient strains. In the absence of CRO, a parasitic relationship formed with the auxotroph reliant on the nutrient producer. Computational simulations modelling an antibiotic sensitive and antibiotic resistant strain also support the cooperation over competition: in mutualistic relationships, antibiotic treatment decreases the concentration of both strains, while in neutral conditions the resistant strain is favoured (172). Li and colleagues also suggest that the incorporation of CRO diffusion to skew biofilm formation elicits cooperation in a

survival dependent manner, and the chemical gradients generated are reflective of natural conditions (171). The effects of antibiotic resistance can then also be extended towards environmental consequences. Anthropogenic input into reservoirs from wastewater treatment plant effluent expose bacteria to minimal, but consistent concentrations of antibiotics and other chemicals (173). Continual contact with foreign chemicals allows environmental biofilms to serve as a source of antibiotic resistance without direct exposure to clinical settings.

Environmental factors have selected for biofilm formation as the primary mode of bacterial existence in nature (132). Biofilms have been demonstrated to form due to synergistic effects: Ren et. al (174) describe a subset of four soil isolates (*Stenotrophomonas rhizophila, Xanthomonas retroflexus, Microbacterium oxydans* and *Paenibacillus amylolyticus*) preferentially aggregating together, with the total cells per strain was higher in the admixture than in monoculture biofilms. This additive effect for interspecific biofilm formation is reflected in most environmental consortia across freshwater, marine, and soil communities (136). Spatial organization in itself can also present opportunities for altruistic interactions: *B. subtilis* exhibit diverse colony morphologies due to genetic factors and abiotic stresses (175). The wrinkled phenotype allows the bacterium to survive gas and liquid penetration into the biofilm (176), as well as providing structural support (177). Wrinkles are a result of genetically controlled cell death, alleviating compressive forces from dividing cells encased in a restrictive matrix (178). Sacrificing cells in a localized manner allows the persistence of the biofilm,

and this phenotype is preferentially formed from nonflagellated *B. subtilis* strains (179). This may provide adaptive advantages for sessile colonizers to outcompete motile genotypes by limiting mechanical compression (178, 179). As environmental bacteria generally subside on ephemeral nutrient resources, biofilms provide an attractive option for more long-term benefits.

1.5.4 I believe the common denominator of the universe is not harmony, but chaos, hostility, and murder.

It has been stated that in microbial systems, “it is easier to evolve to kill your enemies than to favour your friends” (180). This sentiment is perhaps more of what is expected in bacterial communities vying for limited space and assets, with antagonism serving as an underscoring theme in many interactions. Competition for the same resource without explicit association between competitors is termed as exploitation or scramble competition (181). Interference, or contest, competition refers to direct antagonistic actions between individuals allowing the winner sole access to the reward. In microbial contexts, rewards would denote substrates for growth. Environmental bacteria exist in heterogeneous and oligotrophic conditions, and the nutrient in limited concentration is linearly correlated with bacterial yield as determined by kinetic growth equations proposed by Monod (182). Therefore, effective competition strategies are required to capitalize on transient resources.

Cooperative interactions among microbes still entail restrictions to ensure exploitation is regulated, and can be policed through indirect competition. Public goods that benefit the community as a whole are still regulated to avoid excessive cost to producers: *Vibrio cholerae* producing chitinase secrete the enzyme extracellularly, allowing sugars to be consumed by non-producers (183). Spontaneous mutations of *V. cholerae* can result in excessive EPS secretion and form thick biofilms to sequester the enzyme and its digested monomers, reducing the chance of resources reaching exploiters. An alternative strategy used by isolates not producing excess EPS relies on flow of nutrients by external forces. Transporting both the enzyme and substrates away impacts all cells negatively, but non-producers are more disadvantaged as chitinase – and therefore digestible sugars – are not available in the immediate vicinity. Survival of less fit genotypes generally depend on the fitness of the entire population (138), which has been shown for examples such as *E. coli* mutants unable to produce siderophores relying on co-operators (184), antibiotic sensitive isolates requiring antibiotic degrader association for resistance (153), and invading yeast exploiters that metabolize sugars generated from public goods unable to outcompete enzyme producers (185). Despite cooperative policing, cheating can still ensue between similarly fit competitors: for example, the ability of *P. aeruginosa* to secrete and absorb strain specific siderophores, varies among isolates (186). Some genotypes of *P. aeruginosa* have been shown to be able to uptake more forms of siderophores than they secrete, offering an exploitative advantage in competition between relatives (187).

Antimicrobial peptide (AMP) production has been a common mechanism among all domains of life to influence immune regulation in addition to inter- and intra-specific competition (181, 188). AMPs are naturally synthesized peptides and proteins with both bactericidal and bacteriostatic effects (189). In addition to inhibitory effects, AMPs have been posited to serve as signalling molecules in low concentrations (190): sub-inhibitory concentrations of antibiotic exposure. These inhibitory compounds have been isolated from *Staphylococcus* in nasal passages (191), to soil (192, 193) and marine (194, 195) constituents, and offer a potential reservoir of novel antibiotics for discovery (196). Bacteriocins are a class of bacterially secreted AMPs that can offer broad or narrow spectrum activity, and producers encode specific immunity mechanisms in order to survive these toxins (197): the previously discussed colicin is a common bacteriocin produced by enteric bacteria (198). Release of these bacteriocins can be accomplished passively upon lysis like for colicin, or via active secretion like for the wide spectrum lantibiotics produced by Gram-positive bacteria (199). Entry into target cells is mediated through specific receptors to cause cytotoxic effects, through DNA degradation, pore formation, 16s rRNA cleavage, or inhibiting peptidoglycan formation (200). Bacteriocin production may offer an advantage during colonization to outcompete sensitive strains, which can produce population shifts that can be maintained for extensive periods of time (197). An example is highlighted below through the process of replacement therapy to prevent tooth decay (201). A *Streptococcus mutans* isolate with increased bacteriocin production was provided orally to induce competitive exclusion of

pathogens. The introduced strain aggressively displaced indigenous *S. mutans* in patients, and was able to be recovered 14 years later. Bacteriocins and other AMPs mediate population and community level dynamics through interference competition.

Tailored responses can be observed in competition at species and sub-species levels of antagonism. *Pseudomonas fluorescens* transcriptional expression is altered in a species dependent way in secretion of secondary metabolites amongst other soil inhabitants in the same space (193). Pairwise competitions against *Pedobacter*, *Brevundimonas*, or *Bacillus* resulted in the differential expression of 325-571 genes by *P. fluorescens* depending on which competitor it was faced with, including an unknown broad spectrum antibiotic for both Gram negative bacteria. RPS dynamics in the context of colicin production was previously discussed as a framework to maintain diversity in competition (90, 91). Interestingly, *E. coli* from mammalian gut microbiomes skew towards colicin producing (10-50% of the population), and resistant (50-90%) strains, with sensitive isolates limited to less than 5% of the population (202). Stringent ‘rock beats scissors’ outcomes in RPS systems do not take into account the possibility of reciprocal partner interactions, which must be considered in prolonged exposure to nonlethal competition. Majeed and colleagues (202) reevaluate the RPS model in the context of colicin production, and demonstrate that isolates secreting more of the potent toxin in homogenized mixtures were outcompeted by weaker strains. This observation was posited to be the result of weaker strains

increasing colicin production when in contact with a stronger competitor, with no reciprocal counterattacks. The authors suggest that more fit individuals may still be outcompeted due to differences in responses upon interaction. Refining antagonistic attacks can also be demonstrated in *Streptomyces spp.* cohabiting together (203). Antibiotic production between 13 strains was measured through zones of clearance produced in pairwise competition. In pairwise competition, approximately 30% of strains inhibited the competitor. However, with the addition of a third strain, a two to nearly three-fold increase in inhibition occurred. Previously passive strains in co-culture became antagonistic, and roughly 10% of interactions with initially aggressive strains could be suppressed when a third species was included. Attacks between isolates were frequently observed in low nutrient conditions, indicative of more natural environments. Tailoring antimicrobial attacks in response to incoming species may allow niches to be maintained and increase overall biodiversity over spatiotemporal scales (204).

In addition to secondary metabolite secretion, contact dependent competition offers a close range mechanism for bacterial warfare. *Caulobacter crescentus* presents a pore forming bacteriocin localized to the outer cell surface that requires expression of an immunity protein to survive upon contact with the toxin (205). Social bacteria such as *M. xanthus* display complex social behaviours in in multicellular biofilms (67). Under nutrient deprivation, *M. xanthus* undergoes cellular differentiation, allowing the formation of fruiting bodies and spores encased within to survive until more acceptable conditions. Over half the cells in

a population sacrifice themselves to form the stalk structures holding the fruiting bodies. This act of mass altruism requires cells to recognize other members as kin, which is mediated by the TraA polymorphic receptor on the cell surface (206). Closely related cells are able to recognize presented receptors, and exchange substrates through outer membrane exchange (OME). The outcomes of this interaction can vary between repairing damaged siblings, exchanging resources, and competition. Competition involves kin discrimination through the delivery of SitA, a polymorphic toxin that inhibits swarming of the recipient, and death through DNA degradation or arrest in cell division (207). Survival requires the specific immunity gene to prevent intoxication upon delivery. This dual kin identification mechanism allows initial discrimination through TraA receptors using a greenbeard recognition system, which refers to selective altruism to members with the same genotype, in addition to a second verification through the SitA toxin (208). An additional form of self-nonself discrimination lies in contact dependent inhibition (CDI) systems, first discovered in a rat fecal *E. coli* strain (209). CDI is employed by Gram negative species with two-partner secretion systems (type V secretion systems) delivering surface exposed polymorphic toxins to adjacent cells, with cognate immunity proteins providing protection from intoxication (210). The toxins form long filamentous protrusions from the producers akin to a stick tipped with poison, and result in impeded cell growth or death (211). Although a competitive tool, it can also be used in self-nonself discrimination, as the *E. coli* CDI is effective intraspecifically, but not against other enteric bacteria (212). CDI has been observed in some organisms such as

Burkholderia thailandesis to be associated with biofilm formation and extracellular DNA modification independent of its competitive role, suggesting immune cells can both defend against invaders and cooperate amongst each other (213). Close quarter combat is effective in high-density situations to spatially structure environments with prolonged cell contact, and allow niche expansion due to competition (214).

1.6 The spear and the shield: the type VI secretion system

The T6SS is a widespread mechanism used in contact-dependent competition by ~25% of Gram-negative bacteria (215). A cluster of T6SS genes was first discovered in *Rhizobium leguminosarum* in 2003 and posited to be associated with a temperature dependent secretion system (216), and T6SS secreted proteins and gene clusters were later identified in *Edwardsiella tarda* the following year (217). In 2006, Pukatzki and colleagues (218) proposed the cluster of genes to be a novel “type VI” secretion system in *V. cholerae* after observing the bacterium killing *Dictyostelium discoideum* and successfully evading predation from the amoebae. Although initially discovered to be a virulence factor against eukaryotes (218), the role of T6SS has been expanded on to reflect bacterial competition within and between species (219) (65, 220-223). Strains can also encode multiple T6SS clusters (T6SS2-6) with different sets of toxins (224-228).

The apparatus consists of 13 conserved proteins (229) displaying homology to a bacteriophage tail spike (230), which has evolved to deliver a cocktail of toxigenic proteins, otherwise known as effectors, to neighbouring cells (231). The inner tube is comprised of hemolysin coregulated protein (Hcp) subunits surrounded by a heterodimer outer sheath, tipped with a valine-glycine-repeat-G (VgrG) trimer (229). Effectors can be directly associated with the spear tip proteins, or delivered with a chaperone (230, 232, 233). A proline-alanine-alanine-arginine (PAAR) protein can be added on top of the VgrG tip to both sharpen the spear and serve as an attachment point for additional effectors (233, 234). Contraction of the outer T6SS sheath propels the tip of the spear and interior tube into adjacent cells (229, 235). Survival is dependent on possessing the correct immunity genes to neutralize incoming toxins, thereby allowing the killing of non-related bacteria and the survival of kin (220), outlining the dual role of the T6SS in attack and defense. Effector-immunity (EI) gene sets vary even between closely related strains, and modules are subject to replacement through HGT events (236).

T6SS duelling spans cooperative, competitive, and pathogenic interactions across various scales, with some examples highlighted below. *Shigella,* a bacterial resident of the human gut microbiome, is responsible for dysentery, which is associated with bloody diarrhoea (237). Dysentery is mostly endemic in places with poor water sanitation, and is transmitted through the fecal-oral route. While all four species of *Shigella* can cause disease, *Shigella flexneri* and

Shigella sonnei infections encompass the majority of clinical cases (222). There has been a recent increase towards *S. sonnei* infections in developed parts of the world, possibly due in part to interbacterial interactions: Anderson et al. (222) determined *S. sonnei* is able to outcompete the T6SS deficient *S. flexneri* and *E. coli* in mice models based on the T6SS. This may allow *S. sonnei* to cause disease in humans with improved nutrition as an outcome of industrialization by actively competing with the resident gut microbiome, while *S. flexneri* infection is more present in developing parts of the world. Pathogens such as *Yersinia* and *Burkholderia* have differentially expressed T6SSs depending on the environment. *Yersinia pseudotuberculosis* harbours four T6SS gene clusters, each likely conferring different functions for the bacterium (238). Loss of T6SS-4 in *Yersinia pseudotuberculosis* results in near complete attenuation of virulence in murine models. However, the T6SS-4 is also expressed at room temperature, indicating a potential role in the environment outside hosts (228). *Burkholderia* species can encode up to 5 T6SS gene clusters, although the amount within species in the genus can vary. For example, *Burkholderia thailandensis* encodes T6SS-1, T6SS-2, and T6SS-4 to T6SS-6. The T6SS-1 in *B. thailandensis* plays a role in interbacterial warfare, and disrupting the mechanism results in a 100-1000 fold reduction in fitness against competitors (239). In contrast, out of the four encoded T6SS clusters of *B. thailandensis*, only T6SS-5 is required for virulence in mice. *A. fischeri* is a bioluminescent bacterium that forms an association with the Hawaiian bobtail squid *E. scolopes* at night (240). Colonization by the bacterium into the light organ of the squid illuminates the squid when high enough bacterial

cell density has been reached in the host (66). This light projection mimics the moon and allows the squid to evade nocturnal predators. Within the light organ, the spatially segregated crypts are rarely dominated by more than one strain of *A. fischeri* as previously discussed in section 1.2.3. (241). In vitro competitions with *A. fischeri* strains isolated from squids identified that a second T6SS – termed T6SS-2 – determined killing of strains that only possessed the primary T6SS (242). *A. fischeri* isolates lacking the T6SS-2 gene cluster could coexist if the T6SS-2 encoding strains killed off each other with incompatible toxins, and may occur *in vivo* as the squid flushes out the bacterial population in the light organ each morning. While the host-bacteria interaction is mutual, the niche establishment is antagonistically driven. The refinement of contact-dependent competition allows the T6SS to mediate multiple different interactions in environmental and host conditions.

1.6.1 The T6SS as a spatial structuring tool

T6SS competition is mainly restricted to the edge of microcolonies of the incompatible groups encountering each other, forming a no man's land where advancement of both parties is hindered (3, 243). This spatial limitation restricts clonal expansion, allowing heterogeneous communities to develop and support microbial cooperation in an established microcosm. Genetic phase separation allows coexisting cells to function as ecologically contained units and even recycle expended T6SS proteins among sister cells to allow coordinated attacks against competitors (244). In this manner, the T6SS can be used for self/non-self

discrimination in heterogeneously distributed communities For example, *Proteus mirabilis*, a multicellular swarming bacterium, uses the T6SS as an exploratory tool during communal engagement (221). Aggregates of *P. mirabilis* engage in T6SS combat with encountered strains, and segregate into compatible populations denoted by a macroscopic demarcation on agar termed a Dienes line. *In vivo* and *in vitro* competitions using mammalian gut microbiomes demonstrate that *Bacteroides fragilis* strains engage in T6SS competition that results in a community population shift (245). The human gut microbiome is fairly resilient, and the same Bacteroidetes strains have been able to be recovered after decades in patients (246), potentially due to the T6SS mediated spatial segregation among members in the microbiome. Physically structuring environments through antagonism allows clonal segregation to occur and the maintenance of biodiversity on microcosm scales.

1.6.2 *Vibrio cholerae* and its T6SS

Vibrio cholerae, the focus of this thesis, can exist within the human gut as a pathogen in addition to within marine, brackish, and freshwater environmental reservoirs (247). This bacterium is the causative agent of the human diarrheal disease cholera, which has spanned over 200 years, with the first recorded pandemic beginning in 1817, and has been estimated to cause 21 000 to 143 000 deaths per year (248). Like the previously discussed dysentery, cholera is transmitted through the fecal-oral route, and cases are usually associated with poor water quality or insufficient sanitary practices. Patients affected with cholera

generally undergo oral rehydration therapy (ORT) and drink a salt and sugar solution during the course of the disease (249). This ensures that the excessive and rapid loss of bodily fluids due to diarrheal purges does not develop into severe dehydration, which is the main cause of death of cholera. While there are over 200 serogroups of *V. cholerae* categorized based on variations of the O-antigen on the outer membrane lipopolysaccharides, only the O1 and O139 lineages are responsible for cholera epidemics (250). Together, strains within these two serogroups account for all six historical pandemics of cholera, in addition to the ongoing seventh pandemic (248) However, not all O1 or O139 *V. cholerae* are pathogenic, and only strains within the cholera pandemic generating (PG) lineage cause worldwide outbreaks of the disease (250). *V. cholerae* within the PG lineage encode the cholera toxin (CT), conferred through the lysogenic cholera toxin phage (CTX). The CT is classified as a two-component toxin with A and B subunits (251). After ingestion of *V. cholerae*, it passages into the small intestine, where it can initiate colonization if there is sufficient concentration of the bacterium. Upon successful colonization, the B subunit of the CT binds to the epithelial cells in the gut, where the A subunit enters the host cell cytosol and increases chloride ion secretion, leading to fluid accumulation in the lumen of the gut and the watery diarrheal symptom associated with cholera (251, 252).

While it is more known for its role as a pathogen, less is understood about the presence of *V. cholerae* in environmental reservoirs (253). The bacterium is readily isolated from both patient and environmental samples during epidemics,

but between outbreaks, it evades isolation using conventional culturing techniques. There have been multiple hypotheses to explain this phenomenon, where animals, humans, and the environment have all been implicated in the maintenance and transmission of cholera (253, 254). However, the most substantiated hypothesis has been attributed to the environment as reservoirs, where *V. cholerae* ubiquitously exists. The bacterium has been recovered from multiple environmental sources (63, 255-258), with strains within and outside of the O1/O139 serogroups able to be isolated. Within the environment, *V. cholerae* can attach to small crustaceans known as copepods (255). The shells of these copepods are composed of chitin, a substrate that can be digested for nutrients by *V. cholerae,* and serve as hosts for the bacterium in aquatic environments (259). In addition to copepods, other flora and fauna such as birds, algae, insects, and fish, have been identified as organisms that *V. cholerae* associate with (251, 253, 260-262). There has also been support for the notion that *V. cholerae* is more viable in environments with more particulate matter (63, 253), suggesting that attachment to surfaces is an important mechanism for *V. cholerae* in natural reservoirs.

The *V. cholerae* dual lifestyle of pathogen and environmental habitant is in part mediated by the T6SS (247, 263). The bacterium can utilize the single T6SS encoded to establish a niche in the human intestine in addition to defending against environmental bacteria (231). Strains of this species can also encode the actin crosslinking domain (ACD) located on the C-terminal end of some VgrG

proteins, which is directly involved in causing eukaryotic cell death utilized in the gut, as well as predation defense in the environment (264). The T6SS effectors encoded by *V. cholerae* can exhibit a variety of cytotoxic effects, such as peptidoglycan degradation, pore formation, or lipase activity (220). These effector and immunity genes are encoded on three separate loci (in addition to the T6SS structural components) in the *V. cholerae* genome, termed auxiliary-1, auxiliary-2, and the large cluster (63, 220, 229, 265). Additional EI genes can be encoded outside of these aforementioned loci (266-268), which results in a wide diversity of T6SS module compositions.

1.8 Thesis Objectives

Inter- and intra- species contact-dependent antagonism has been studied in pairwise competitions previously (220, 222, 242, 269, 270). However, the impact of T6SS effects on intraspecific populations, and therefore the downstream effects on population structuring within environmental reservoirs, have not been well elucidated. My research aims to interrogate the T6SS as a determining factor in preserving genotypic heterogeneity at ecologically relevant scales. Previous sampling endeavours have allowed us to accrue an environmental *V. cholerae* strain library of nearly 500 strains, all sampled from a single location on the eastern US coast, Oyster Pond, MA (271). These isolates within Oyster Pond encode different effector and immunity genes, suggesting these highly diverse strains are generally incompatible at the intraspecies level based on T6SS EI modules. Less than 5% of the identified isolates from our collection are able to

coexist based on their T6SS repertories. Furthermore, 90% of *V. cholerae* in the population was found to be particle-associated through culture independent quantification (63), providing potential microcosms of high competition. Cultured isolates from Oyster Pond were then further categorized on a sub species scale through sequencing of the vibriobactin utilization gene B, *viuB* (63), in addition to clonal complexes (CCs) assigned through multilocus sequence typing (MLST) analysis of housekeeping genes (271). Both levels of classification offer a high-resolution approach to distinguish ecologically relevant lineages of *V. cholerae*. Through this rapid screening, we selected 14 lineages representing the diversity found within this population, each displaying discrete T6SS repertoires. Of note, the *viuB*-73 allele, which corresponds to *V. cholerae* lineage that gave rise to pandemic strains (pandemic generating or PG lineage) (250) was identified within the Oyster Pond intraspecies community. Only culture-independent amplification distinguished the presence of *viuB*-73; isolates of this genotype could not be cultivated from Oyster Pond.

To understand the influence of the T6SS in shaping natural bacterial populations, I performed the following:

(I) Competition experiments between pairs of environmental *V. cholerae* strains considering ecologically relevant parameters.

(II) Competition experiments between *Vibrio cholerae* V52, a PG associated strain, and Oyster Pond isolates.

(III) Analysis of the outcomes of competition on individual and population scales.

This project investigates how *V. cholerae* strains from a single coastal location harboring diverse incompatible EI sets coexist. Our results provide fundamental insight on how conflict influences population structures. Using *V. cholerae* as a model offers knowledge into how pathogens survive outside their host species, and compete within their environmental reservoirs. This may help elucidate how pathogenic *V. cholerae* survive and outcompete other environmentally sourced strains in aquatic systems to reach infectious doses. The incorporation of environmental *V. cholerae* strains supplements a vast repertoire of knowledge known of the medically relevant lineages in a holistic approach to understanding all facets of the species.

Chapter 2: Modular molecular weaponry leads to dynamic competitive outcomes in an environmental *V. cholerae* population

2.1 Abstract

Sympatric populations of the bacterium *V. cholerae* consist of a large number of phylogenetically different but ecologically similar lineages struggling for a limited pool of common resources. Type VI Secretion system (T6SS) mediated antagonism between such competing bacteria is thought to play an important role in this lifestyle; however, these dynamics have never been studied in a population level framework. Here we show that numerous *V. cholerae* lineages isolated from a single source possess unique repertoires of cytotoxic T6SS effector and immunity gene arrays, undergoing rapid turnover as a result of horizontal gene transfer (HGT). Using pairwise in-vitro competition assays, we demonstrate that the vast majority of T6SS mediated duels end in stalemates, with lineage-specific effector-immunity gene arrays serving as an effective means of kin-discrimination and defense of resources. However, past HGT events of effector-immunity gene modules can significantly alter the outcome of these competitions in favour of the recipient strain. Additionally, HGT can lead to the formation of temporary “alliances” between distantly related strains through acquisition of effector-immunity gene modules from each other, reducing T6SS mediated competition until the next HGT event. As a result of this, a hierarchical competitive network is established, in which specific strains are at a temporary

advantage over some but not all other members of the population. These interactions give room for density-dependent fitness effects and a constant T6SS HGT mediated arms race, both of which could ultimately serve to preserve diversity within bacterial populations.

Natural bacterial populations are generally divided into microdiverse clusters of closely related organisms, below the threshold of what is usually considered a bacterial species. A central question in microbial ecology and evolution is how these clusters are retained despite the homogenizing force of frequent genetic exchange and the purging effect of selection. It has been suggested that both niche differentiation and mosaic sympatry, where co-occuring organisms are irregularly distributed into small patches, limiting direct interactions, are prerequisites for this diversity. We show that in natural *Vibrio cholerae* populations, the T6SS is an effective means towards this end. T6SS are present in ~25% of Gram-negative bacteria, and we expect within-population diversity in T6SS repertoires and the resulting competitive dynamics to be a common theme in free-living bacteria.

2.2 Introduction

Environmental reservoir populations of *V. cholerae,* the etiological agent of cholera, are composed of different toxigenic and non-toxigenic lineages striving for resources in brackish water habitats. Among their competitive arsenal, shared with ~25% of all Gram-negative bacteria (215), is the type VI secretion system

(T6SS), the structural genes of which are spread over three loci termed aux-1, aux-2 and large cluster in the *V. cholerae* genome (220). The T6SS apparatus, which has evolved from a bacteriophage tail spike (230), consists of a hollow tube tipped with a membrane puncturing protein (akin to a spear), surrounded by an outer sheath (229, 272). Contraction of the outer sheath propels the tip of the spear and interior tube into adjacent cells (including cells of the same species or even kin), injecting a combination of lethal effector proteins in a contact dependent manner (220). Effectors confer a variety of cytotoxic abilities, and are each generally encoded upstream of a specific immunity gene, forming effector-immunity (EI) modules. Survival of T6SS mediated attacks depends on cells possessing the correct combination of immunity proteins to neutralize incoming effectors (232). As such, even closely related cells with different immunity proteins are killed through T6SS mediated antagonism (220). In terms of EI module content, the aux-1, aux-2, and large clusters of *V. cholerae* are polymorphic, each capable of encoding a large variety of different EI modules. Additionally, three monomorphic T6SS loci - encoding only a single EI module - have also been identified in *V. cholerae* and designated as aux-3, aux-4 and aux-5 (266-268). Given the large number of EI modules, strains of *V. cholerae* could theoretically display millions of different combinations, and indeed the observed strain level diversity in T6SS module combinations is vast (236).

This degree of variation in EI profiles is mainly attributable to horizontal gene transfer (HGT) (273), which in *V. cholerae* is tightly linked with T6SS activity

(274, 275). The acquisition and replacement of effector and/or immunity genes is orchestrated through various HGT mechanisms, namely, homologous recombination and homology-facilitated illegitimate recombination for polymorphic loci, and site-specific recombination in the case of monomorphic loci (276, 277). Ancestral immunity genes can be entirely replaced or retained with the addition of new EI modules within a locus (276). Retention of immunity modules during recombination events, replacing their cognate effector, can lead to the accumulation of multiple orphan immunity genes at a single locus (236). These orphan immunity genes are hypothesized to provide a fitness advantage, protecting strains from additional T6SS attacks.

Despite the large EI assortment within *V. cholerae*, almost all members of the pandemic-generating (PG) lineage (250) have retained one specific EI module combination, and due to their nature as the causative agent of cholera, their T6SS has been the most closely studied (264, 278-280). The T6SS is under strict regulation in PG strains, with activation modulated through several variables, including temperature, osmolarity, cell density as well as mucin, indole, and bile salts present in the human digestive tract (263, 281, 282). This contrasts with the perpetually active T6SS in most non-toxigenic environmental strains thriving in oligotrophic aquatic environments (282). In competition with environmental strains, the specific EI module combination of PG *V. cholerae* is thought to be superior to that of other strains, making PG *V. cholerae* expelled from patients successful competitors against environmental bacteria (220, 231, 263).

This apparent dominance of a specific EI module combination stands in clear contrast with the vast, HGT-mediated diversity in EI modules of *V. cholerae* overall, which hints at the ecological and evolutionary significance of this abundance of T6SS associated genes. If specific EI modules such as that of the PG lineage were universally beneficial, the combination of frequent HGT and strong selection would be expected to homogenize EI module content within individual populations. Under such circumstances, the observed EI diversity in strains from across the world would be a reflection of the locally optimal EI modules for individual populations. If, on the other hand, other factors such as density dependent fitness effects or rock-paper-scissor-like dynamics were at play (89), global diversity would be mirrored in local populations, with a multitude of combinations circulating among co-occurring strains. In this study, we investigate competitive dynamics created by the diversity of T6SS EI modules in a population-level context and as such elucidate the biological significance of this modular molecular weaponry.

2.3 Methods and materials

2.3.1 Strain selection and growth

All environmental strains of *V. cholerae* used originate from Oyster Pond, Massachusetts, USA, with isolation protocols are previously described (271). The clinical isolate *V. cholerae* V52 was chosen to represent pandemic generating cholera strains expressing a constitutively active T6SS. *Escherichia coli* K12 substrain MG1655 was used in control experiments to ensure T6SS were actively expressed. Spontaneous rifampicin-resistant mutants were generated by

recovering mutants spread plated on rifampicin supplemented Luria-Bertani (LB) (Difco) plates as performed in MacIntyre and colleagues (278).

2.3.2 Effector immunity (EI) typing

To identify conserved chromosomal T6SS loci, genes downstream of aux-1, aux-2, and the large cluster EI modules (VC1421, VCA0022 and VCA0125 of *V. cholerae* strain N16961 respectively), were mapped to each environmental *V. cholerae* genome in Geneious 6.1.8 (https://www.geneious.com). Putative immunity genes were extracted and classified based on previous reference sequences for each effector-immunity family as described by Unterweger et al. (220), followed by effectors for each locus. The presence of aux-5 was queried through the T6SS predictor pipeline from Crisan *et al*. (268) in addition to Geneious mapping.

2.3.3. Phylogenetic tree construction

Genomes for all isolates have been sequenced as previously described (271). Whole genome alignment of strains was performed using mugsy version (283) and gaps were removed, resulting in an alignment of 2,948,969 bp. A whole genome phylogeny was subsequently built using the GTR+GAMMA substitution model implemented in RAxML (284), with branch support assessed with 100 fast-bootstrap pseudoreplicates. Individual alignments of effector and immunity genes from Oyster Pond strains and reference strains from Kirchberger et al. (236) were

aligned using ClustalOmega (285), standard settings, and phylogenetic trees were constructed as above.

2.3.4 Competition assay

Competition assay protocols were adapted from previously described methods (278). Overnight cultures of *Vibrio cholerae* or *Escherichia coli* were grown at 37°C on LB agar supplemented with rifampicin when appropriate. Cells were harvested and the concentration of strains were normalized by OD_{600} to 10^7 cfu/ml. Rifampicin-resistant and rifampicin-sensitive wild type (WT) strains were resuspended in LB broth in 1:1 ratios of 10^7 cfu/ml, and 25 µl of the mixture were spotted on prewarmed LB agar plates and incubated at either 25°C or 37°C for four hours. For each replicate, 25 µl of the starting mixture was suspended in 975 µl of LB, serially diluted and spot plated onto LB or LB with rifampicin (LB+R) to determine starting concentrations of each strain. After incubation, each spot was harvested completely and resuspended in 1 ml of LB, which was then serially diluted tenfold to 10^{-7} concentration. A 10 µl aliquot from each dilution was spot plated on both LB and LB+R plates in duplicate. Each pairwise experiment was performed with six replicates. Plates were incubated at 37°C, and colony forming units (CFUs) were counted at the highest dilution recovered. Single CFUs were counted if the previous dilution was accurately observed to be tenfold greater, i.e., approximately 10 CFU were found before the single CFU. Concentration of WT strain was determined by subtracting CFUs on LB+R from CFUs enumerated on LB. Control competition assays were performed to ensure all T6SS were

functioning as expected by competing strains with a T6SS- *E. coli* MG1655 (Figure 2.3).

2.3.5 Competitive index and survivor percentage calculation

Competitive indexes (CI) were determined by comparing concentrations of survivors to starting cultures as performed previously (220). CFUs/ml of rifampicin resistant strains were divided by CFUs/ml of WT, for both starting and recovered time points. The ratio after 4 hours was divided by the starting concentration to calculate CI. The average of surviving strains was calculated by dividing the CFUs at 4 hours over the starting concentration per strain, and converted into a percentage.

2.3.6 Supernatant assay

Cell free supernatant was harvested from overnight cultures grown as described above through serial centrifugation at 13,000 rpm for two 10-minute intervals. Target strains were spread on LB+R plates, and 2ul supernatant aliquots were spotted on the dried spread plates. Plates were incubated overnight at 37°C, and plaque formation was visually inspected the following day.

2.3.7 Statistics and visualization

A one-sample student T-test determined statistical significance of CI values against an assumed comparative mean of 0. For figure 2.5B, a one factor

ANOVA was performed to assess statistical significance of mean reduction of strains based on predicted interaction, in addition to a two sample unpaired T-test. Dot plots were generated in R using ggplot2 (286, 287). The competitive hierarchy graph was generated in Cytoscape (288). All figures were processed afterwards in Adobe Illustrator CS6.

2.3.8. Data availability

Accession numbers for all strains used in this study along with relevant metadata are listed in Table S2.1 in Supplemental Material.

2.4 Results and Discussion

2.4.1 Almost all lineages in a *V. cholerae* population possess mutually incompatible T6SS effector-immunity module compositions

To understand type VI secretion system mediated competitive dynamics in *V. cholerae* populations, we analyzed the structure of T6SS loci in 14 strains from an extensively sampled coastal population in the eastern United States (63, 271). For this, we followed previously developed typing schemes (220, 236), denoting effector and cognate immunity gene families in the T6SS-associated large cluster and aux-1, aux-2 with capital letters. In the case of aux-1 and aux-2, this scheme describes auxiliary toxins loaded onto the T6SS spear by adapter proteins, and in the case of the main locus, variable C-terminal ends of the spear-forming VgrG

protein itself (236, 289). Additional loci aux-3, aux-4 and aux-5 were also characterized based on preceding studies (266, 267).

In accordance with what we previously observed in *V. cholerae* on a global scale, every member of this individual population encodes a unique combination of T6SS EI modules (Figure 2.1), with only three possessing a counterpart in a previously described global *V. cholerae* dataset (236). EI structure ranges from simple one effector- one immunity gene pairings (for example CC3 or CC5) to a complex array containing not only a complete EI-pair but also three truncated effectors and their cognate immunity genes, and seven additional orphan immunity genes in the main locus of CC4. Overall, out of the 19 effector and cognate immunity gene families previously observed in the *V. cholerae* pangenome, only three effectors (C in aux-2, and F and H in the main cluster), and only one cognate immunity gene (F in the main cluster) are absent in the population. We also observed a previously undescribed putative EI-module type (consisting of a unique C-terminal region and a cognate immunity gene, here termed M-type in accordance with the alphabetical naming scheme) in the main array of CC4.

As previous experimental results show that strains possessing different EI module combinations are capable of mutual killing, and assuming that orphan immunity genes are capable of detoxifying cognate effectors, the vast majority of strain combinations within this population should not be able to coexist in close

contact without mutual T6SS mediated killing (i.e. they should be incompatible) (Figure 2.2).

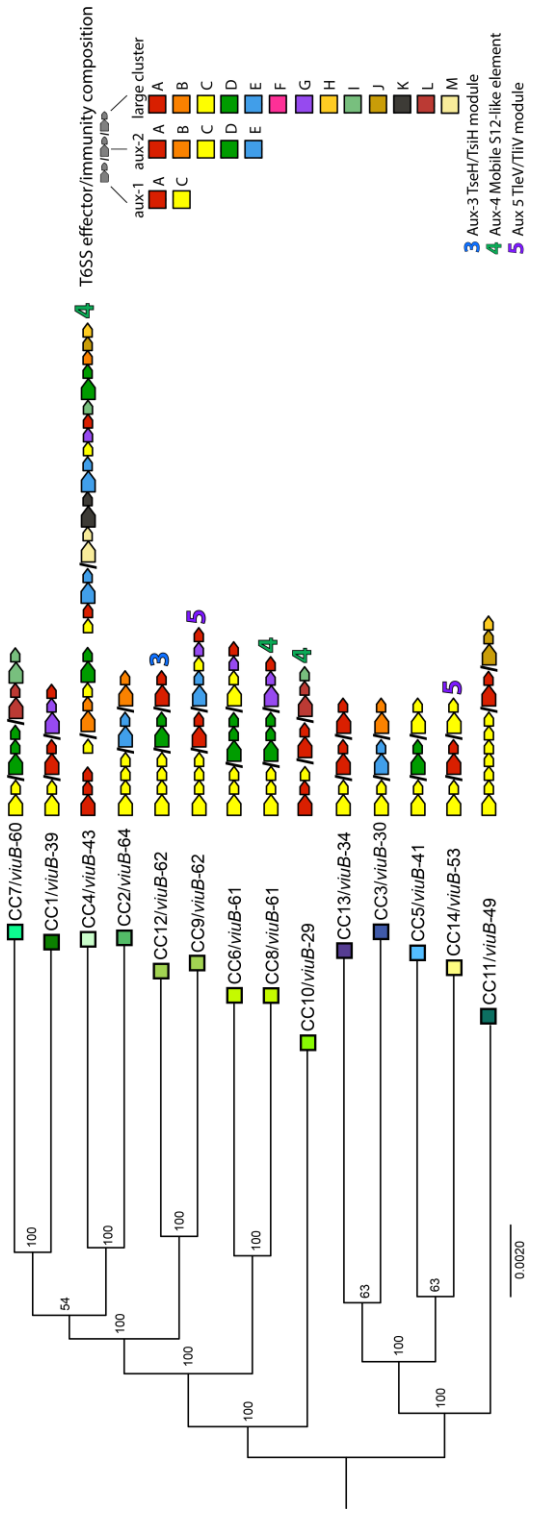
T6SS effector/immunity composition
aux-1
aux-2
large cluster
A
C
A
B
C
D
E
A
B
C
D
E
F
G
H
I
J
K
L
M
3 Aux-3 TseH/TsiH module
4 Aux-4 Mobile S12-like element
5 Aux 5 TleV/TliV module
CC7/viuB-60
CC1/viuB-39
CC4/viuB-43
CC2/viuB-64
CC12/viuB-62
CC9/viuB-62
CC6/viuB-61
CC8/viuB-61
CC10/viuB-29
CC13/viuB-34
CC3/viuB-30
CC5/viuB-41
CC14/viuB-53
CC11/viuB-49
100
54
100
100
100
100
100
63
63
100
100
0.0020

Figure 2.1: Type VI secretion system effector and immunity genes in a *Vibrio cholerae* population. Large arrows next to strain names indicate T6SS effectors, small arrows immunity genes, and colour of arrows represent different effector and immunity protein coding gene families as defined by >30% shared amino acid identity. Auxiliary cluster 1, 2 and the large cluster genes are separated by slashes, spaces within clusters denote interruptions in canonical locus structure. Note that similarly named/coloured families denote different families in different clusters. Numbers indicate auxiliary T6SS loci. Phylogeny of strains was constructed from a 2,948,969 bp whole genome alignment using the GTR+GAMMA model implemented in RAxML (284). Statistical branch support was obtained from 100 bootstrap repeats. Scale bar indicates nucleotide substitutions/site.

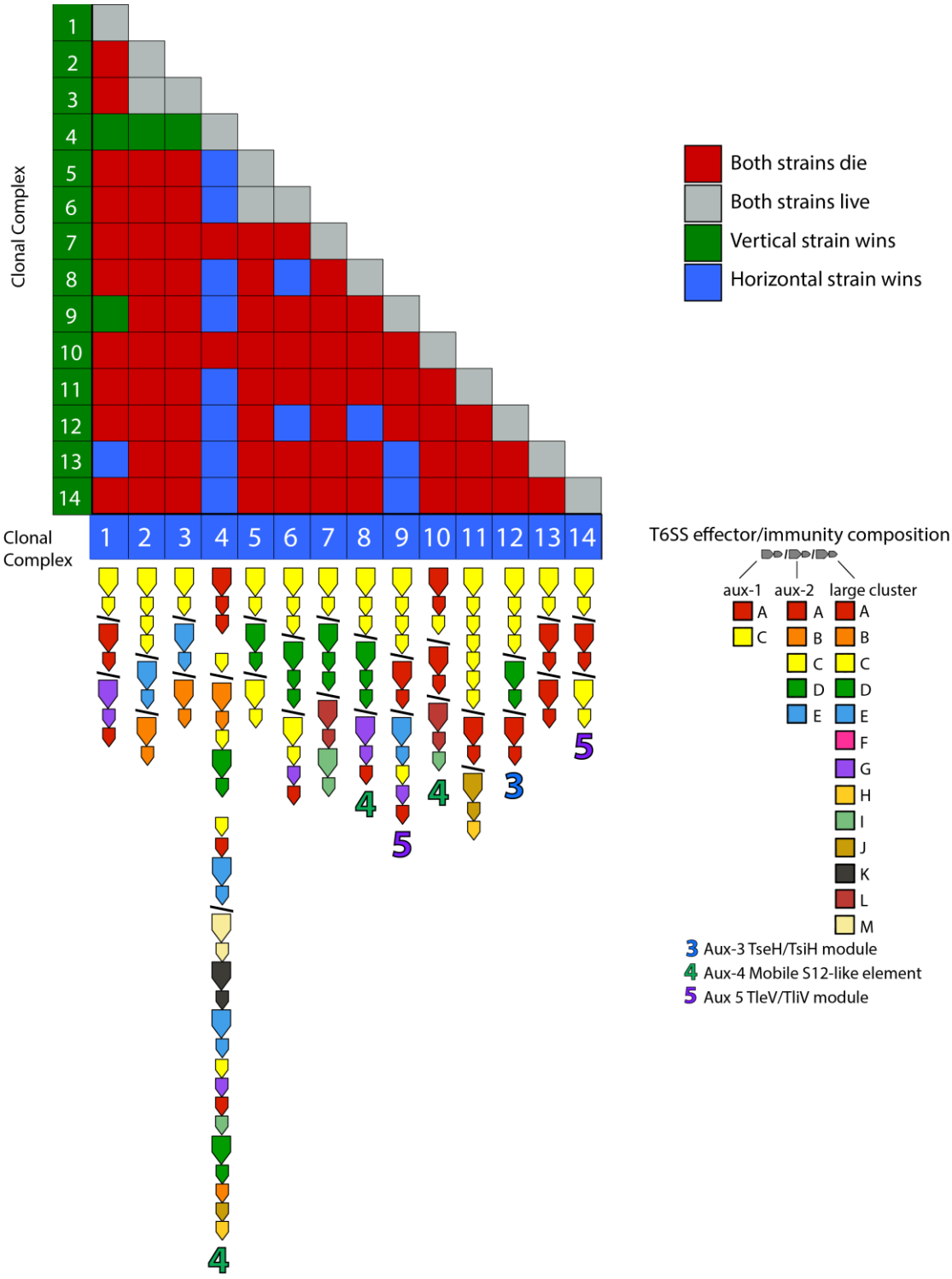
Clonal Complex
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Clonal Complex
Both strains die
Both strains live
Vertical strain wins
Horizontal strain wins
T6SS effector/immunity composition
aux-1
aux-2
large cluster
A
C
A
B
C
D
E
A
B
C
D
E
F
G
H
I
J
K
L
M
3 Aux-3 TseH/TsiH module
4 Aux-4 Mobile S12-like element
5 Aux 5 TleV/TliV module

Figure 2.2: Predicted outcomes of pairwise competitions based on effector-immunity gene combinations. EI module composition is indicated below strain names in accordance with Figure 2.1. Grey squares indicate strains coexisting based on compatible EI modules, red squares indicate incompatible EI modules, green squares indicates a favourable competitive outcome for strain on the left, blue squares for strains on the bottom.

2.4.2 Temperature affects the outcome of competition

When pitted against *Escherichia coli* K12 through co-incubation on agar plates, all *V. cholerae* strains showed the ability to outcompete this bacterium, which does not encode a T6SS itself (Figure 2.3). This reduction was dependent on physical contact, killing 80-90% of all cells (Figure 2.3B), and we observed no reduction in *E. coli* cell numbers from *V. cholerae* supernatants alone or in *E. coli* only competitions, indicating that the T6SS of these isolates is active and responsible for the outcome of these competition assays (the typical state for environmental *V. cholerae*) (282). As such, the T6SS should provide *V. cholerae* with the ability to defend against invaders and prevent them from gaining a foothold in their population. To test this assumption, we competed all environmental strains against *V. cholerae* strain V52, a toxigenic PG lineage isolate not native to the Oyster Pond population. Previous studies have shown that V52 displays constitutively active T6SS expression and superior competitive abilities compared to a number of environmental strains (220, 263, 278, 279). The V52 EI module combination is not found in any Oyster Pond strains, and therefore should compete with them in a T6SS dependent manner. Indeed, we confirmed that when co-incubated at 37°C (the temperature more closely resembling that of a human host for pathogenic vibrios), V52 clearly outcompeted nine out of 14 strains (Figure 2.4). Only CC4, a strain displaying unusually gene-rich T6SS clusters (see Figure 2.1) appeared to show a degree of (non-significant) competitive ability against V52 at 37°C. However, at 25°C (reflective of the average temperature of Oyster Pond (63)) we observed a general trend

towards increased competitive ability for environmental strains. Seven out of 14 strains fared significantly better against V52 at this temperature, with only CC11 showing a degree of reduced competitive ability (Figure 2.4). Overall, only two out of 14 strains are outcompeted by V52 at 25°C. This altered outcome of competitions at lower temperature was not the result of a decrease in cytotoxicity (as one would expect from a downregulation of T6SS at lower temperatures). At both 37°C and 25°C, about half the cells of environmental competitors were killed by V52 (Figure 2.4B), indicating unaltered T6SS activity. In contrast, the number of surviving V52 cells changed from 200% of the original input (indicating growth) at 37°C to lower than 100% at 25°C, indicating they were being killed more efficiently by their environmental opponents. Therefore, either a decrease in growth rate for V52, increased T6SS mediated competitive ability of environmental strains or a combination of both resulted in much more even matchup between the competitors. It has previously been hypothesized that the superior competitive ability of V52 is due to the highly conserved and lethal EI module combination within the PG lineage. However, from our results it appears that at least in the aquatic reservoir of cholera, this combination is no more effective than that of environmental strains overall.

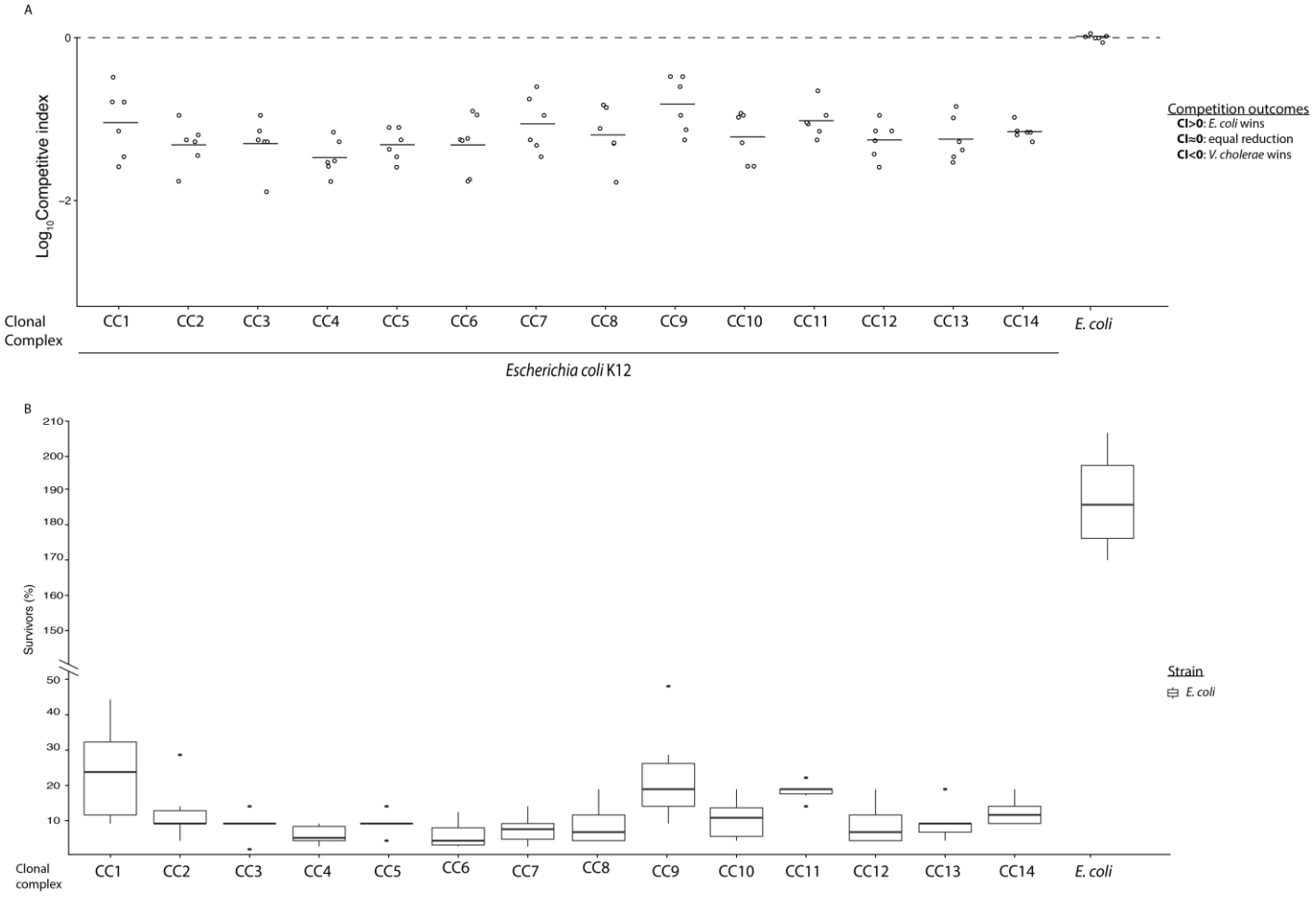
A
0
−2
Log10 Competitve index
Clonal Complex
CC1
CC2
CC3
CC4
CC5
CC6
CC7
CC8
CC9
CC10
CC11
CC12
CC13
CC14
E. coli
Escherichia coli K12
Competition outcomes
CI>0: E. coli wins
CI≈0: equal reduction
CI<0: V. cholerae wins
B
Survivors (%)
210
200
190
180
170
160
150
50
40
30
20
10
Clonal complex
Strain
E. coli

Figure 2.3: Competition of *V. cholerae* strains against T6SS-negative *E. coli* K12. *E. coli* lacking a type VI secretion system was competed against all *V. cholerae* isolates used in this study in equal concentration at 25°C over 4 hours. (A) Dots indicate the log competitive index for each replicate (N=6), with the average shown as a bar on the y-axis, with a negative CI indicating a favourable competitive outcome for the *V. cholerae* strain. (B) Percentage of surviving *E. coli* cells compared to the original input of cells in competition against various *V. cholerae* strains. Self-crosses of *E. coli* are included in both figures as a control. Box-and-whiskers plots show median, 25^{th} and 75^{th} percentiles (upper and lower hinges) 1.5 inter-quartile range (whiskers). Outliers are shown as individual dots.

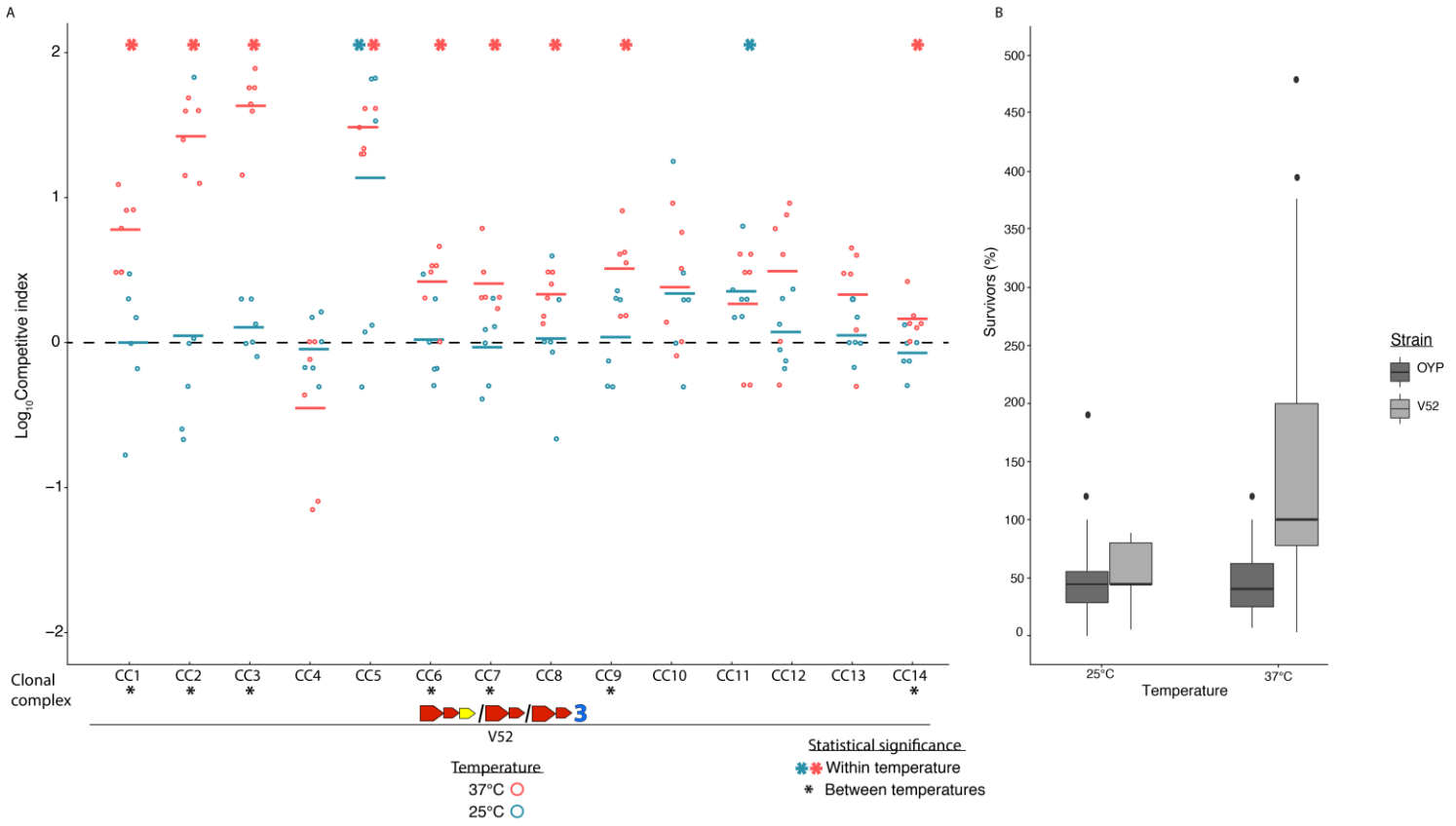
A
Log10 Competitive index
2
1
0
−1
−2
Clonal complex
CC1
CC2
CC3
CC4
CC5
CC6
CC7
CC8
CC9
CC10
CC11
CC12
CC13
CC14
V52
Temperature
37°C
25°C
Statistical significance
Within temperature
Between temperatures
B
Survivors (%)
500
450
400
350
300
250
200
150
100
50
0
25°C
37°C
Temperature
Strain
OYP
V52

Figure 2.4: Competition of toxigenic Pandemic Group *V. cholerae* against non-toxigenic natural isolates at environmental and human host temperatures. *Vibrio cholerae* V52 was competed on agar plates against *V. cholerae* isolates from Oyster Pond in equal concentration at either 25°C or 37°C over 4 hours. (A) Dots indicate the log competitive index for each replicate (N=6) at 37°C (red) and 25°C (blue), with the average shown as a bar on the y-axis. A positive log CI indicates favourable outcome towards V52, a negative CI denoting environmental strains as the winner. Coloured asterisks indicate statistically significant outcomes in favour of V52 at each temperature based on a one-sample T test. Black asterisks below strain names indicate statistically significant differences in mean CI values between 25°C and 37°C competitions from two tailed, unpaired T test ($p<0.05$). (B) Percentage surviving cells compared to the original input for V52 and all environmental strains after 4h of competition at 25°C and 37°C. Box-and-whiskers plots show median, 25^{th} and 75^{th} percentiles (upper and lower hinges) 1.5 inter-quartile range (whiskers). Outliers are shown as individual dots.

2.4.3 Horizontally transferred EI modules reduce competition between distantly related strains

Previous work has shown that while closely related strains of *V. cholerae* (members of the PG lineage) engage each other using their T6SS, this interaction does not result in damage, presumably because their identical EI modules neutralize one another (220). Assuming that identity in EI module composition solely determines whether strains coexist (rather than other genetic factors), it can be predicted that almost all combinations of *V. cholerae* isolated from the Oyster Pond population should be incompatible (Figure 2.2). In two instances (CC5 and CC6 as well as CC2 and CC3), strains possess similar EI module composition, differing only in orphan immunity genes, and could therefore potentially coexist (Figure 2.1 and Figure 2.2). To test this prediction, pairwise competition assays were performed using all possible combinations of strains. In accordance with the findings that temperature significantly influences the outcome of competition, all assays were performed at 25°C to emulate environmental conditions. Indeed, both specific predictions of coexistence as a result of identical EI modules resulted in competitive indices close to 0, an identical outcome to competitions of strains with themselves (Figure 2.5A). However, while competition of a strain with itself resulted in near doubling of cell numbers, competition of different strains with identical EI module yield reduced numbers of both cell types (Figure 2.5B). Nonetheless, the number of surviving cells from competition of strains with compatible EI module composition was still significantly higher ($p<0.05$) than that of incompatible strains, with (on average)

75% of cells of each lineage surviving instead of only 50% (Figure 2.5B). Sub-optimal binding between effectors in one and immunity proteins in another strain due to divergence in amino acid sequences and resulting incomplete detoxification would be an obvious reason for the remaining degree of competition. However, almost all relevant proteins share 91-100% identity between compatible pairs at all loci, with only the aux1 immunity proteins of CC2 and CC3 (68.5-72.8% identity) and the aux2 immunity proteins of CC5 and CC6 (82.4% identity) being more divergent. Since binding interactions between effector and immunity proteins have not been studied so far, the reason behind the incomplete reduction of competition is unclear. An alternative consideration could also be differential generation times of strains. As growth rates differ between Oyster Pond isolates (Figure 2.6), this variation may also influence co-culture dynamics.

A
Single culture
Coexist
Incompatible
Log10Competitive Index
2
0
−2
Clonal Complex
CC1
CC2
CC3
CC6
CC8
CC12
CC6 v CC5
CC3 v CC2
CC14 v CC10
CC14 v CC2
CC8 v CC2
CC8 v CC3
B
Survivors (%)
400
375
350
325
300
275
250
225
200
175
150
125
100
75
50
25
Self
Coexist
Incompatible
Interaction

Figure 2.5: Reduction of competition between strains with identical effector-immunity module composition. Identical (white), compatible (green) and select incompatible (red) strains were competed on agar plates at equal concentrations at 25°C for 4 hours. (A) Dots indicate the log competitive index for each replicate (N=6), with the average shown as a bar on the y-axis. EI module composition is indicated below strain names in accordance with Figure 2.1. (B) Percentage of surviving cells compared to the original input of identical (white), compatible (green) and incompatible (red) strains with non-significant competitive outcomes (excluding instances of one strain outcompeting the other). Box-and-whiskers plots show median, 25^{th} and 75^{th} percentiles (upper and lower hinges) 1.5 inter-quartile range (whiskers). Outliers are shown as individual dots. Asterisks indicate statistically significantly different means between each group (unpaired T test $p<0.05$).

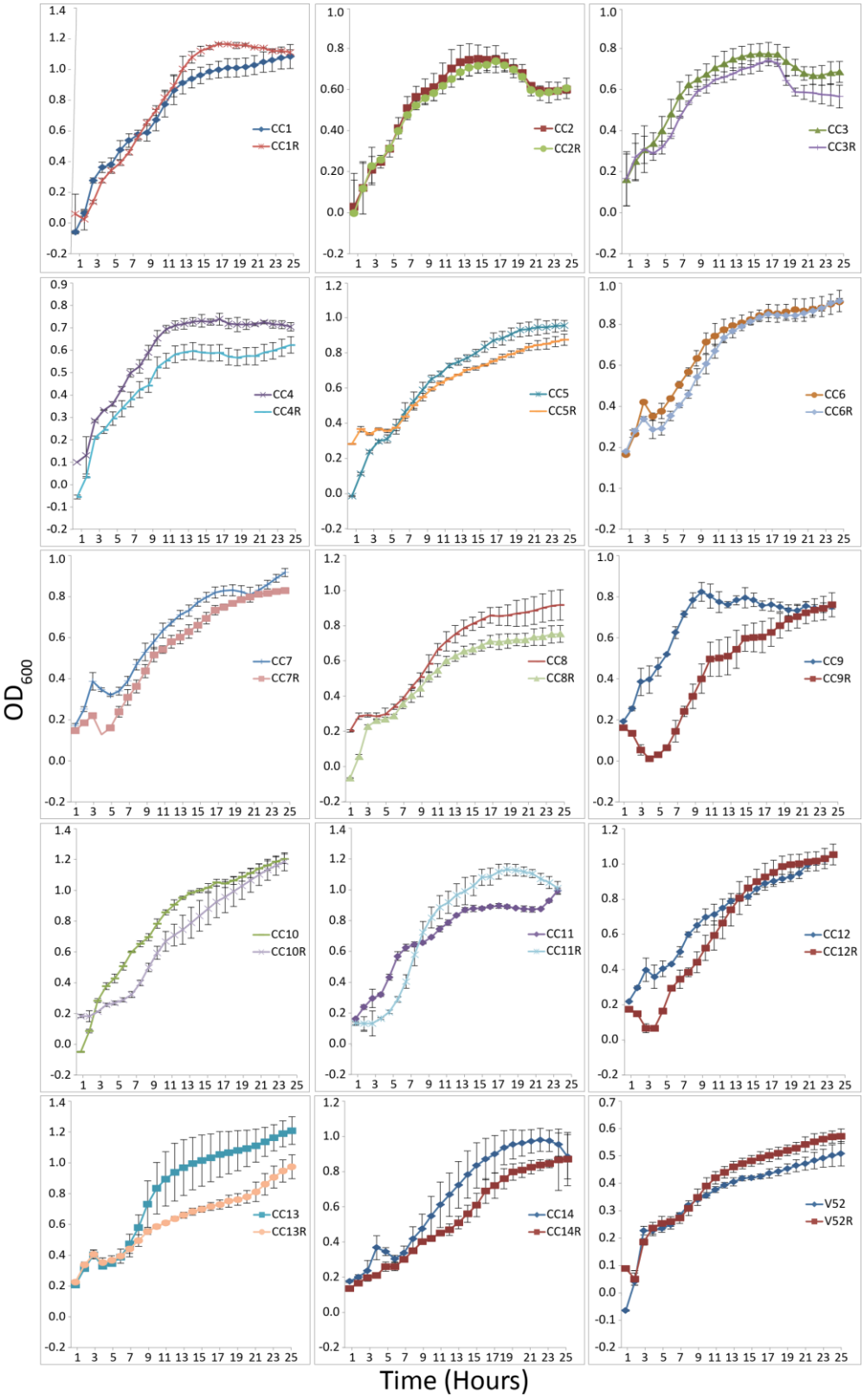
CC1
CC1R
CC2
CC2R
CC3
CC3R
CC4
CC4R
CC5
CC5R
CC6
CC6R
CC7
CC7R
CC8
CC8R
CC9
CC9R
CC10
CC10R
CC11
CC11R
CC12
CC12R
CC13
CC13R
CC14
CC14R
V52
V52R
OD600
Time (Hours)

Figure 2.6: *Vibrio cholerae* wildtype and rifampicin mutant growth curves. *V. cholerae* was cultured in LB broth for 25 hours at 25°C in a shaking incubator at 120 RPM. A starting inoculum of approximately $1x10^3$ cells was used for each strain in triplicate, and cell density was measured hourly through OD_{600}. Strains with an R at the end denote rifampicin resistant mutants. Standard deviation is indicated through error bars.

The potential role of HGT in reducing competition was also investigated. Both pairs of compatible strains are only distantly related in the context of the Oyster Pond population (approximately 50,000 base pair differences between each strain (271)), with their respective closest relatives displaying different EI module compositions (Figure 2.1). Since EI modules of the same type are patchily spread among *V. cholerae*, it stands to reason that their distribution is caused by HGT events. However, it is unclear whether these events happened in the distant past, with compatible strains acquiring modules elsewhere and migrating into the extant population independently, or whether strains acquired them from each other in the currently existing population, homogenizing EI module content. To investigate these possibilities, homologous effector and immunity genes were aligned from all Oyster Pond strains and reference strains from Kirchberger et al. (236) and their phylogeny was reconstructed. Recent, in-population HGT events should be apparent by identical or nearly identical EI module sequences in compatible strains, resulting in monophyletic clades consisting only of Oyster Pond isolates. In the case of aux-1, which displays the most sequence variation and least EI module type diversity, recent in-population HGT is apparent between CC3 and CC7, which sport almost identical C-type effector and immunity genes but incompatible EI modules in other loci (Figure 2.7A). In contrast, genes for compatible strain pairs CC2 and 3 as well as 5 and 6 appear to have diverged long ago or have been acquired independently, as evidenced by their phylogenetic distance. In aux-2, D-type effector and immunity genes for incompatible strain pairs CC4 and CC12 as well as CC6 and CC7 (but not for

CC2-3 and CC5-6) cluster together and apart from the alleles of all other strains, further evidence of recent in-population HGT (Figure 2.7B). In the large cluster, the B-type EI pair of compatible strains CC2 and CC3 forms a well-supported, monophyletic clade distinct from all other Oyster Pond or reference EI modules of the same type (Figure 2.7C). Both strain's immunity genes as well as the 3'-half of their effectors are identical in sequence, which is strongly suggestive of a recent recombination event. Interestingly, previous research has shown that CC2 and CC3 are among the most abundant strains in Oyster Pond (271). It is tempting to imagine that their shared environmental success could be the result of a past confrontation, leading to one strain replacing its EI module with that of another and thus creating a temporary truce between former competitors. Reduced competition between distantly related strains with compatible EI modules could provide a mutual advantage in situations where more than two bacterial strains are present whereby their compatibility allows them to outcompete others.

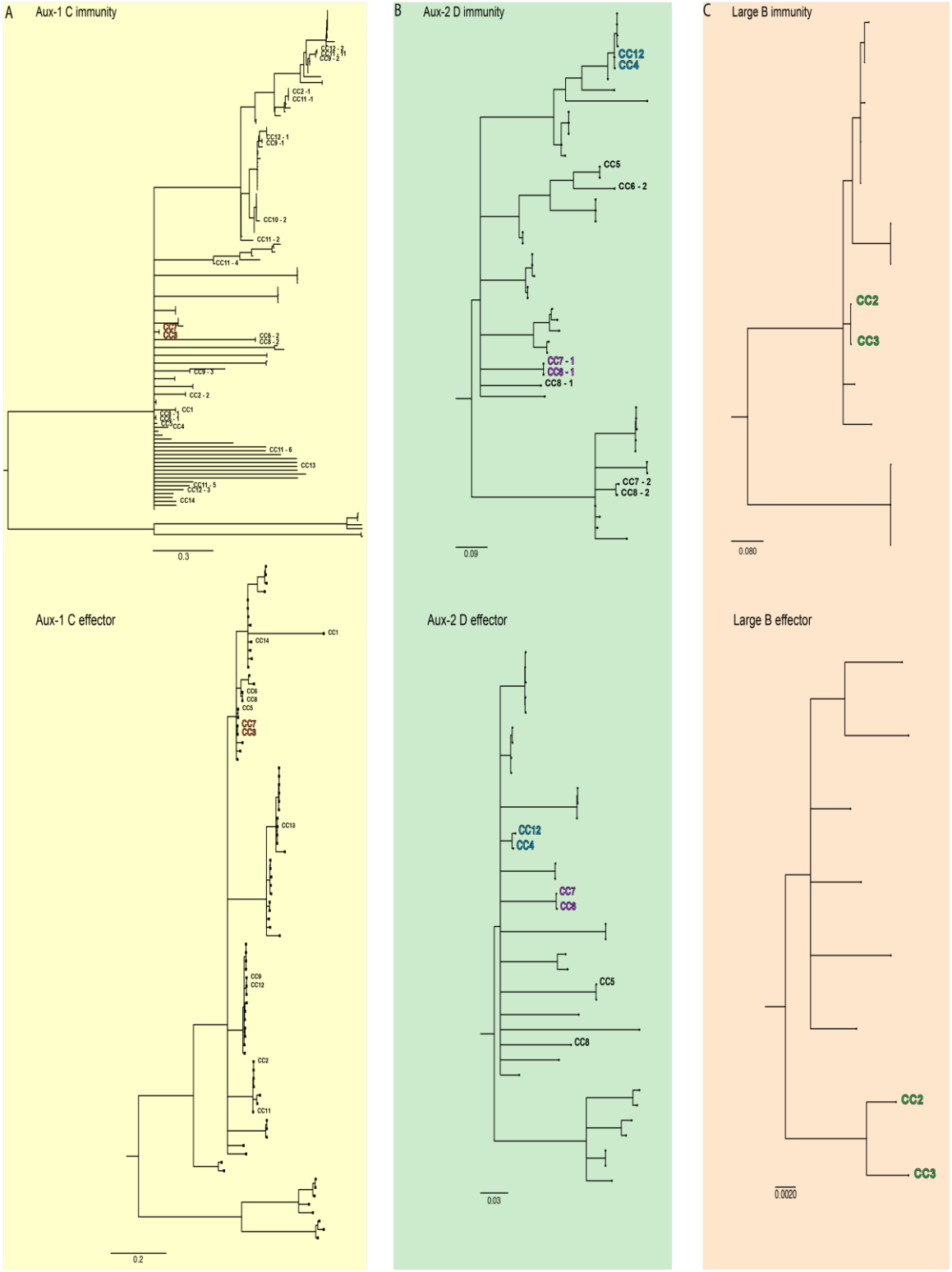
A
Aux-1 C immunity
CC2 -1
CC11 -1
CC12 - 1
CC9 -1
CC10 - 2
CC11 - 3
CC11 - 4
CC7
CC8
CC6 - 3
CC8 - 3
CC9 - 3
CC2 - 2
CC1
CC6 - 1
CC8 - 1
CC4
CC11 - 6
CC13
CC11 - 5
CC12 - 3
CC14
0.3
B
Aux-2 D immunity
CC12
CC4
CC5
CC6 - 2
CC7 - 1
CC8 - 1
CC8 - 1
CC7 - 2
CC8 - 2
0.09
C
Large B immunity
CC2
CC3
0.080
Aux-1 C effector
CC1
CC14
CC6
CC8
CC9
CC7
CC8
CC13
CC9
CC12
CC2
CC11
0.2
Aux-2 D effector
CC12
CC4
CC7
CC8
CC5
CC8
0.03
Large B effector
CC2
CC3
0.0020

Figure 2.7: Instances of recent horizontal gene transfer of *V. cholerae* effector and immunity genes. Trees were constructed based on single gene alignments of (A) aux-1 C-type, (B) aux-2 D-type and (C) large cluster B-type effector and immunity genes based on single gene alignments using the GTR+GAMMA substitution model implemented in RAxML (284). Leaves with strains from the oyster pond populations are indicated, with instances of horizontal gene transfer coloured. Unlabeled leaves represent reference strains from a global *V. cholerae* dataset (236). Tree support was assessed with 100 fast bootstrap replicates, and branches with support <70 were collapsed. Scale bars indicate nucleotide substitutions/site.

2.4.4 Orphan immunity genes in the large cluster sway outcomes of T6SS competition

We next investigated whether orphan immunity genes, present in a large number of *V. cholerae* strains in this population, are capable of conferring protection to the effectors of other bacteria. From the EI profiles present in this set of strains, it is predicted that in around a fifth of all possible unique combinations (18/91), one strain would be immune to all effectors of another strain but not vice versa (Figure 2.2). Indeed, with two notable exceptions, pairwise competition resulted in a positive outcome for the strain possessing orphan immunity genes compatible with their opponent's effector from the large cluster. For example, CC12 is outcompeted by CC8, which is protected from CC12's A-type effector in the large locus by an orphan A-type immunity gene. In turn, CC8 possesses a G-type effector that CC12 is not protected against. However, both CC8 and CC12 are outcompeted by CC6, which possesses orphan A and G-type immunity genes and a C-type effector. Notably, aux-3 of CC12 and aux-4 of CC8, which are mobile genetic elements encoding additional, unrelated EI modules (266, 267), did not sway the outcome of the competition (Figure 2.8). Similarly, CC13 is outcompeted by CC1, which is outcompeted by CC9, whose three orphan immunity genes protect against the effectors of three different strains (Figure 2.8). While these results appear rather clear cut and make it tempting to conclude that the outcome of T6SS competition between distantly related strains can be reliably predicted by their EI module composition alone, there are exceptions. In the one case where a strain possesses an orphan immunity gene

compatible to it's opponent's aux-1 effector (the non-Oyster Pond isolate V52 possessing a C-type orphan immunity gene compatible to CC13's C type effector), the strain predicted to win did not do so in a statistically significant way (Figure 2.4). However, V52's orphan C-type immunity protein displays only 80% amino acid identity from the C-type immunity protein that protects CC13 against its own effector, raising the possibility that the protection conferred to V52 is suboptimal. As genes in the main locus show the lowest level of divergence (i.e. proteins of the same family are almost identical) (236), it is perhaps not surprising that predictions based on the main locus alone are more accurate than those based on other loci with a higher level of genetic divergence.

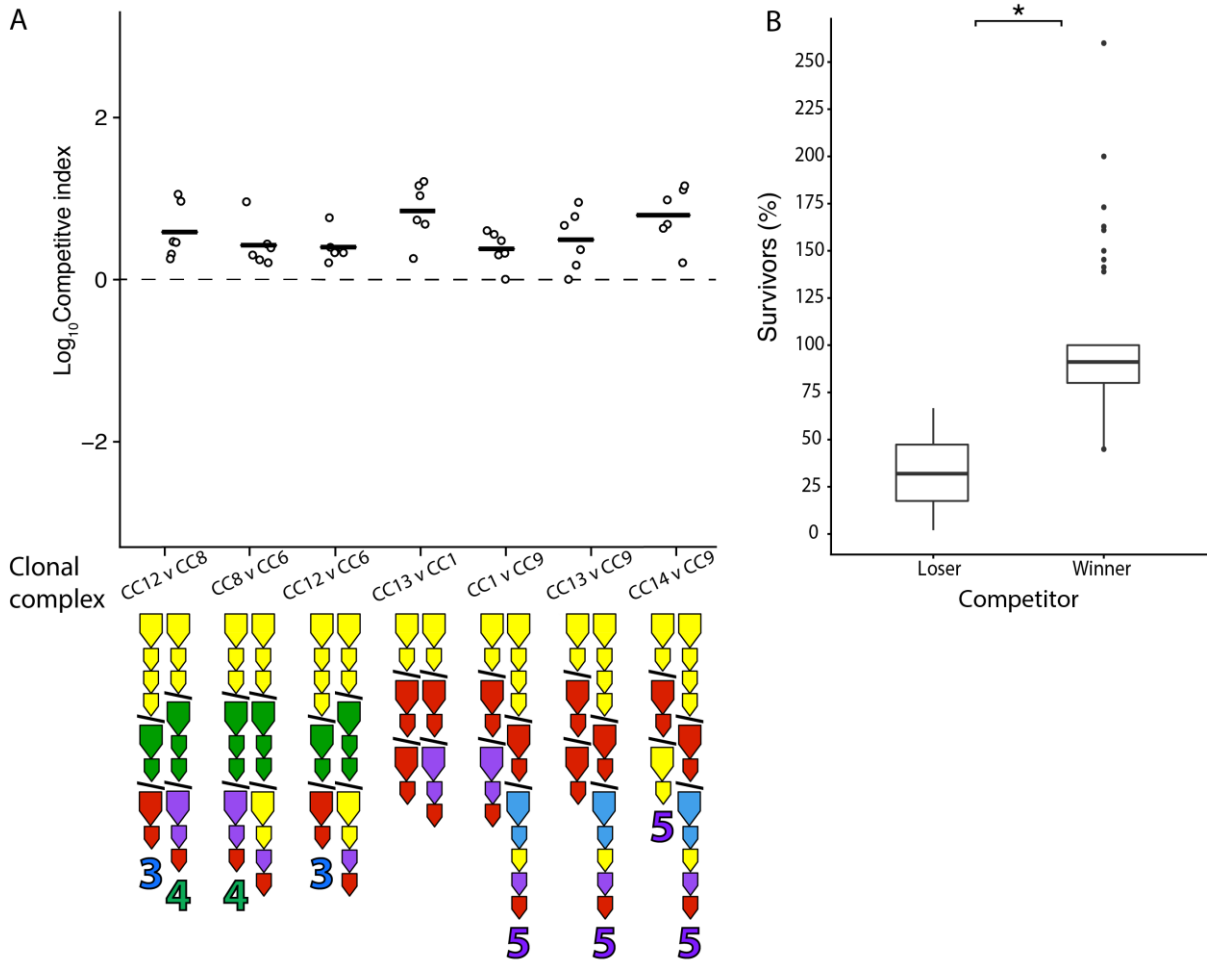
A
Log10Competitive index
2
0
−2
Clonal complex
CC12 v CC8
CC8 v CC6
CC12 v CC6
CC13 v CC1
CC1 v CC9
CC13 v CC9
CC14 v CC9
3
4
4
3
5
5
5
B
Survivors (%)
250
225
200
175
150
125
100
75
50
25
0
*
Loser
Winner
Competitor

Figure 2.8: Orphan immunity genes confer an advantage in pairwise competition. Strains differing only in orphan immunity gene composition were competed in equal concentration at 25°C over 4 hours. (A) Dots indicate the log competitive index for each replicate (N=6), with the average shown as a bar on the y-axis, with a positive CI indicating a favourable competitive outcome for the second strain in the competition. All CI values in 4A were statistically significant based on a two tailed, one sample student's T test ($p<0.05$). EI module composition is indicated below strain names in accordance with Figure 2.1. (B) Percentage of surviving cells compared to the original input of losing and winning strains in individual competitions. Box-and-whiskers plots show median, 25^{th} and 75^{th} percentiles (upper and lower hinges) 1.5 inter-quartile range (whiskers). Outliers are shown as individual dots.

Furthermore, CC4, a strain that possesses orphan immunity genes to 18 out of all 20 known putative T6SS effectors encoded in the aux-1, aux-2 and main locus, theoretically protecting it from all effectors in the population but the main locus L-effectors of CC7 and CC10, also defies predictions. While CC4 outcompetes four strains according to expectations, it is equally matched with five others, and is outcompeted by two other strains. However, the structure of T6SS loci in CC4 is more irregular than in other strains, making it unclear whether particular EI modules are part of an actively expressed operon (a reasonable assumption for strains with uninterrupted T6SS gene arrays). In aux-1, two A-type immunity genes are followed by a ~2300bp region encoding four hypothetical genes, followed by a C-type immunity gene. Failure to express this last gene due to potential exclusion from the normal aux-1 promoter activity would leave CC4 unprotected from the C-type immunity genes of all but one other strain in the population. Similarly, the aux-2 locus is split in two, with only the region containing B- and D-type EI module and one A-type orphan immunity gene appearing intact and part of the standard operon. The second part containing C and A-type orphan immunity genes and an E-type EI module is located downstream of an integrase and several genes encoding proteins of unknown function, in a reading frame opposite to that of the T6SS associated genes. As the region downstream of the T6SS aux-2 loci is conserved among other all strains, it appears that this is an insertion interrupting this region, potentially interfering with expression of these genes.

As such, these results indicate that to some degree, the simple presence or absence of orphan immunity genes in *V. cholerae* can sway the outcome of contact-dependent competition. However, complicating factors such as uncertainty regarding expression in atypical T6SS associated gene regions and divergence between effector and immunity proteins of the same type ultimately weaken the overall predictability of outcomes. Nonetheless, our results show that the presence of additional immunity genes, presumably the remnants of HGT-mediated replacement of ancestral EI modules, can provide a considerable advantage to *V. cholerae* strains.

2.4.5 *V. cholerae* populations as dynamic, competitive interaction networks

Most pairwise interactions between *V. cholerae* strains, both in this experiment and presumably in nature, occur between bacteria sharing few or no EI modules. In addition to the few instances where T6SS mediated competitive success can be predicted based on EI module composition, the majority of pairings resulted in even matchups between strains, indicating that the majority of the population is able to survive incoming attacks of co-occurring strains. Additionally, in around a quarter of competitions between strains with different EI modules and no protection by orphan immunity genes, one strain outcompetes the other (Figure 2.9). Based on the assumption that both the ability to outcompete a strain (potentially allowing invasion of novel environments) or, to a lesser extent, fighting it to a standstill (allowing defense of resources), are beneficial, we visualized the competitive network of Oyster Pond strains using a simple (and

arbitrary) scoring scheme (Figure 2.10). Using that scheme, CC9, which outcompetes seven of 13 strains and is not outcompeted by any of them, is placed squarely at the top of the hierarchy while CC12, which is beaten by six strains and only outcompetes a single strain, is at the bottom. While there appears to be a general upwards trend in the hierarchy, exceptions with circular networks of strains outcompeting each other exist - for example, CC1 < CC11 < CC12 < CC13 < CC1. As such, the possession of nominally weaker EI module combinations could under certain situations be beneficial. For example, in situations where CC1 becomes numerically dominant, the weak strain CC11 should be at an advantage due to its ability to specifically outcompete that strain. As strain composition in *V. cholerae* populations have been shown to be highly dynamic (271, 290), such scenarios do not appear to be particularly unlikely. Signs of density dependent fitness effects are not only visible on the level of strains, but also EI modules. Notably, the three most successful strains CC9, CC6 and CC4 all encode C, G and A immunity genes in their main locus, and 50% of all strains in the dataset contain an effector neutralized by these immunity proteins (see Figure 2.1). In summary, our results show that even strains with comparatively weak EI module combinations such as CC7 or CC3 are nonetheless capable of stalemating more than half of the strains in this population. Thus, normally non-competitive strains could thrive under conditions where they come up against competitors with favourable EI module combinations.

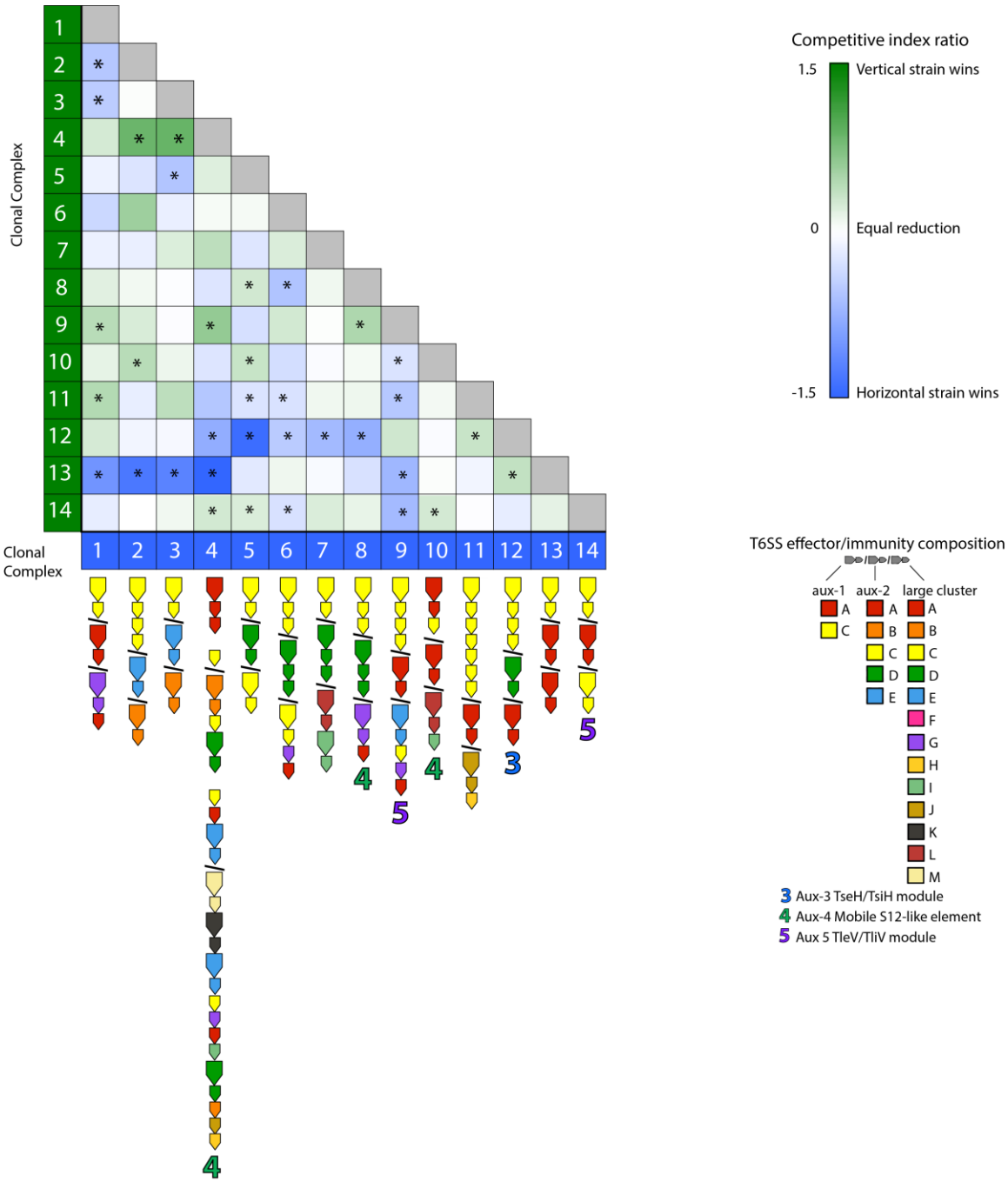
Clonal Complex
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Clonal Complex
Competitive index ratio
1.5
Vertical strain wins
0
Equal reduction
-1.5
Horizontal strain wins
T6SS effector/immunity composition
aux-1
aux-2
large cluster
3 Aux-3 TseH/TsiH module
4 Aux-4 Mobile S12-like element
5 Aux 5 TleV/TliV module

Figure 2.9: Pairwise competition of all members of an environmental *V. cholerae* population. Strains were competed in equal concentration at 25°C over 4 hours. The mean log competitive index was visualized for all competitions, and color and intensity within each cell indicates a favourable outcome for the strain listed on the left (green) or bottom (blue). EI module composition is indicated below strain names in accordance with Figure 2.1. Asterisks indicate statistically significant results from a two tailed, one sample student's T test ($p<0.05$).

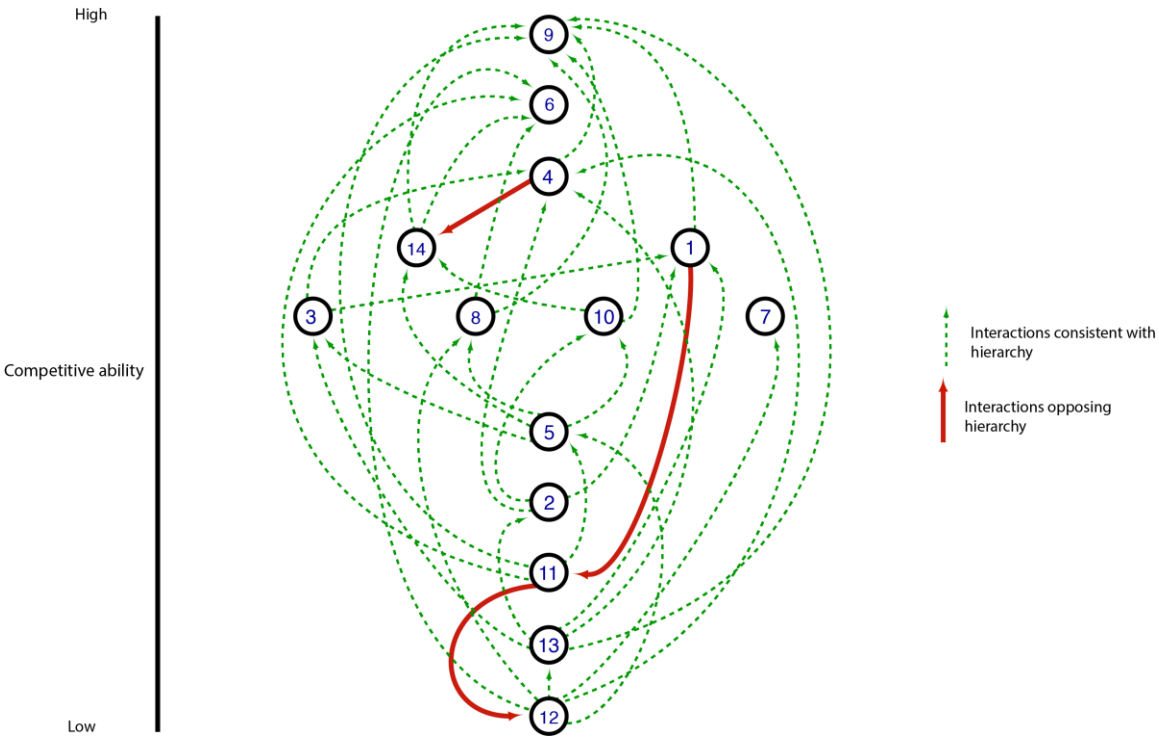

Figure 2.10: Competitive network of an environmental *V. cholerae* population. Numbered nodes indicate individual strains; edges indicate pairings with statistically significant competitive outcomes. Strains are ranked from most to least competitive on the y-axis based on a scoring system where a statistically significant competitive outcome is worth three and a draw one point. Arrows point towards winning strain in an individual competition, with green arrows indicating outcomes upwards or level in the hierarchy, red arrows highlighting a lower strain winning against a higher ranked strain.

2.4.6 Conclusions

Here, we demonstrate the effect that HGT-mediated diversity of EI modules can have on the competitive dynamics in a bacterial population. Given an otherwise identical EI module composition, orphan immunity genes, remnants of past HGT-events that replaced an ancestral effector gene with a new EI module, confer a predictable, positive effect to the recipient. Similarly, HGT can reduce competition between strains by homogenizing EI module content. Given the dynamic nature of EI module composition, it stands to reason that the rough hierarchy established in this work is very short lived - while the incorporation of new EI modules through eDNA uptake of lysed neighbours may refine and improve upon the repertoires of less fit strains, it may also cause higher performing strains to exchange stronger EI with weaker replacements at their detriment (274, 275). In particular, strains with highly competitive EI module combinations are more likely to take up DNA from defeated weaker ones. As the replacement of an EI module often leads to the retention of an immunity gene, and this immunity gene in combination with a new effector can provide a considerable competitive advantage against strains with otherwise similar EI modules, this downgrade in EI efficacy would be momentarily selected for. Thus a previously dominant strain could quickly find itself with a suboptimal EI module composition, leading to the rise of different strains.

Our results also demonstrate the clear limits of purely genomic-based prediction of competitive outcomes - a strain that is dominant at 37°C is equally matched at

25°C. Numerous other factors and environmental variables could have complex effects on T6SS mediated competition: For example, biofilms have been shown to act as a protective layer around *V. cholerae* to shield cells from exogenous T6SS attacks, with no reduction to endogenously mediated assaults (291). Furthermore, growth rate and motility of competing strains have been identified as factors important to displace groups (149). As such, EI module composition alone is not the sole deciding factor determining the outcome of T6SS mediated competition, or competition as a whole.

Perhaps the most interesting finding is that while some strains predictably outcompete others, most pairings are not only incompatible due to their EI module composition, they are also equally matched. These instances demonstrated bistable interactions where both genotypes persisted in a heterogeneous mixture. Bistability amongst cohabiting bacteria has been observed in *Streptomyces* antimicrobial production, and the survival of potentially conflicting groups allows diversification of metabolic pathways and substrates utilized in a complex microbial community (292). The T6SS has previously been demonstrated to maintain distinct bacterial groups through spatial segregation, with antagonism occurring at the edge of incompatible groups (3, 243). Our results thus support a model where the diversity of *V. cholerae* populations is maintained through the creation of a patchwork of microniches by T6SS mediated competitive exclusion. Biodiversity and community productivity is fostered within microbial systems lacking hierarchies (89-91, 202). In

heterogeneous, oligotrophic environments, bacteria have been shown to favor cooperation over competition, which may translate more accurately to natural conditions (96). Within these habitats, competitors vie for finite resources and space while avoiding extensive overlap between extant niches. Stronger competitors are established in a context dependent manner, as preserving extraneous elements such as constitutively expressed weaponry or antibiotic-resistance plasmids can be metabolically expensive to maintain (263, 280, 281). In multispecies communities for example, it may be more advantageous to cooperate than to antagonize to increase community productivity (148). Coexistence of incompatible strains can thus be maintained over long periods of time when ecological processes such as interactions and dispersal are spatially limited (91). As the initial homogenized inoculum of duelling strains was confined to a small physical space during competition, strains maintained heterogeneity through genotype segregation.

Environmental, ecologically differentiated non-pathogenic strains of *V. cholerae* thus could play an important role in preventing the spread of pandemic *V. cholerae* across the globe. The prolonged presence of pandemic *V. cholerae* in the previously cholera-free Haiti after the introduction by UN peacekeepers (293-295) indicates that environmental factors alone are likely not responsible for the patchy distribution of this pathogen. Global ocean currents and ship ballasts are likely to have spread occasional pandemic *V. cholerae* bacteria across the globe (248, 296, 297) but local *V. cholerae* (298, 299) might have prevented

them from becoming a permanent part of the immediate flora. Only when overwhelming concentrations of pandemic *V. cholerae* are introduced via a human vector (numbering in the trillions released by a cholera victim (300)), might they be able to gain a foothold against a diverse and locally adapted pre-existing community of *V. cholerae*. In such cases, competition still continues in reservoirs and may have downstream effects for invaders: Haiti for example, has had no new cholera cases for over a year (301), and this may be due in part to the extant environmental community reducing pandemic *V. cholerae* abundance.

Table S2.1: List of all isolates used in competition assays and effector-immunity (EI) combinations of aux-1, aux-2 and large cluster. Blank cells indicate no relevant metadata. Clonal complex (CC), *viuB*, and EI typing schemes listed in literature cited list. Slashes designate loci positions. Dashes denote gaps between genes. Uppercase letters indicate effector-immunity; lower case letters are orphan immunity genes. Numbers at the end of EI specify aux3-5 presence.

Species	Strain	CC*	*viuB* allele	T6SS Effector Immunity	Accession Number	Year + Source Isolated	References
Vibrio cholerae	OYP6F10	1	*viuB-39*	C/A/Ga	NMSZ00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP2E01	2	*viuB-64*	Cccc/E/B	NMTK00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP4G06	3	*viuB-30*	C/E/B	LBGH00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP2A12	4	*viuB-43*	Aaaa-c/BcD-caE/MKEcgaiDbjh/4	NMTN00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP4B01	5	*viuB-41*	C/D/C	NMTI00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP7G04	6	*viuB-61a*	Ccc/Ddd/Cga	QEAX00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP6E10	7	*viuB-60*	C/Ddd/LI	QEBI00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP6D09	8	*viuB-61b*	Ccc/Ddd/Ga4	QEBM00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP2D07	9	*viuB-62a*	Cccc/A/Ecga/5	NMTL00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP1G01	10	*viuB-29*	Ac/A/Li/4	NMTO00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP4H04	11	*viuB-49*	Ccccccc/A/Jh	QEBY00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP4D04	12	*viuB-62b*	Cccc/D/A	QECE00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP4C07	13	*viuB-34*	C/A/A	LBGE00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	OYP4E08	14	*viuB-53*	C/A/C/5	QECB00000000	2009 Oyster Pond, MA, USA	Orata et al. (2015)
Vibrio cholerae	V52		*viuB-73*	Ac/A/A	AAKJ00000000	1968 Sudan (Clinical)	Chun et al. (2009)
Escherichia coli	K12 substr. MG1655				U00096	1997 Wild-type lab strain	Blattner et al. (1997)

Literature Cited

Blattner FR, et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Sci. DOI:10.1126/science.277.5331.1453

Chun J, et al. 2009. Comparative genomics reveals mechanism for short-term and long-term clonal transitions in pandemic *Vibrio cholerae*. Proc Natl Acad Sci USA. DOI:106:15442-15447.

Kirchberger PC, et al. 2016. A small number of phylogenetically distinct clonal complexes dominate a coastal *Vibrio cholerae* population. Appl Environ Microbiol. DOI:10.1128/AEM.01177-16

Kirchberger PC, et al. 2017. Sequential displacement of Type VI secretion system effector genes leads to evolution of diverse immunity gene arrays in *Vibrio cholerae*. Sci Rep. DOI:10.1038/srep45133

Kirchberger PC, et al. 2020. Culture-independent tracking of *Vibrio cholerae* lineages reveals complex spatiotemporal dynamics in a natural population. Environ Microbiol. DOI:10.1111/1462-2920.14921

Orata FD, et al. 2015. The dynamics of genetic interactions between *Vibrio metoecus* and *Vibrio cholerae*, two close relatives co-occurring in the environment. Genome Biol Evol. DOI:10.1093/gbe/evv193.

Unterweger, D., et al. 2014. "The *Vibrio cholerae* type VI secretion system employs diverse effector modules for intraspecific competition." Nat Commun. DOI: 10.1038/ncomms4549

Chapter 3: What have we learned, and where could we go?

3.1 Extrapolating bench scale data to environmental outcomes

Interrogating how microbes respond in the environment requires constant modifications into both method and interpretation of laboratory protocols. This is specifically highlighted in the T6SS, as when initial findings on its role as virulence factor were discovered *in vitro*, it was later suggested that the T6SS is more involved in microbial competition in nature, with virulence towards hosts being a secondary trait (302). While the T6SS can indeed contribute to virulence towards eukaryotes, with the actin crosslinking domain found at some aux-1 loci (230), my results support the evolution of the T6SS as an important tool in bacterial warfare at species and sub-species scales. Environmentally relevant experimental designs may allow further interrogation of how the T6SS mediated interactions exist *in situ*.

3.1.2 A temperature dependent advantage in competition

Environmental conditions encompass a myriad of indescribable and dynamic parameters. The one I interrogated in Oyster Pond was arguably one of the simplest factors to test: temperature. Even then, the clear temperature dependent shift observed in competition is both incredibly interesting and ecologically important. Competition assays are generally performed at 37°C as in

several situations, the bacteria are involved in human disease. However, to understand bacterial interactions outside of pathogenicity, more environmentally relevant conditions should be considered. Ecological studies on macroorganisms are done in their natural habitat: the field of microbiology is slowly moving towards the same paradigm.

Our Oyster Pond dataset highlights an interesting dynamic with a noticeable shift in competition against the toxigenic *V. cholerae* V52 strain occurring when environmental strains have a small “home turf” advantage. Competitions in temperatures more reflective of conditions where the strains were harvested conferred a marked advantage in favour of Oyster Pond isolates. As environmental *V. cholerae* are thought to generally express the T6SS constitutively, and PG isolates are more strictly regulated (282), it is tempting to think that competitions in environmental parameters may change our view of toxigenic strains being inherently better competitors due to their EI array (263, 278, 280). Additional data from environmental isolates would allow further exploration into this phenomenon, as well as competitions expanding into more ecologically relevant conditions. As most aquatic environments are oligotrophic (115, 303), it would be interesting to run competitions on agar plates with minimal nutrients as a simple expansion to see if the outcomes I observe in chapter 2 hold. Salinity and temperature have both been shown to affect expression of the T6SS (265), competitions with gradients of both parameters may reflect seasonal

fluctuations in the environment and perhaps also affect population-level T6SS dynamics.

3.1.3 Small ponds, large EI diversity

Crozier's paradox states that genetic heterogeneity is expected to erode overtime, as larger cooperative groups will increase in size due to more frequent resource sharing, and overtake smaller groups (304). The T6SS EI arrays in a single location not only contrasts this concept, but also rivals the global scale EI diversity seen within a species (236). This mosaic diversity is due to HGT events allowing diversification within an environment, for both antagonistic and cooperative interactions. Both CC2 and CC3 are abundant in Oyster Pond (271), and perhaps some of their success in this ecosystem is due to their compatibility with each other. Similarly sampled reservoirs would allow comparisons of EI arrays within ecologically relevant populations. The Kirschner lab has obtained nearly 500 environmental isolates of *V. cholerae* from a Neusiedler See, a lake straddling Austria and Hungary rich in wildlife and human recreational activity (290). As we discovered a novel EI class in Oyster Pond (CC4 M-type), it would be incredibly interesting to see this EI diversity and competition dynamics is reflected across different geographical points in *V. cholerae*.

Within the scope of the T6SS, HGT may be facilitated through the antagonistic interactions of bacterial warfare. Recently, the *V. cholerae* T6SS has been demonstrated to be co-regulated with natural competency on chitin (275, 305). As discussed in section 1.4, chitin is the second most abundant biopolymer

produced globally (126), and forms the crustaceous shells of the aquatic copepods that *V. cholerae* attaches to in the environment (306). The stimuli, such as chitin attachment, that induces natural transformation are well elucidated in *Vibrio cholerae* (305, 307-312). The *V. cholerae* regulatory protein TfoX, which is translated in the presence of chitin and products of chitin degradation, induces competency (275). Upon the initial transcription of *tfoX*, structural components of the T6SS are upregulated. Having natural transformation and a mechanism to kill neighboring cells and expose DNA occurring synchronously, facilitates genetic integration driven by predatory processes (313). It has been recently shown that DNA taken up by *V. cholerae* is expressed prior to cell replication, and the incorporated gene and its by-products are unequally distributed during cell division (312). This results in two types of progeny: one with only the expressed gene product (such as a protein), and the other receiving both the transformed DNA and product it encodes. While Dalia et al. (312) demonstrate *V. cholerae* conferring antibiotic resistance to kin lacking the specific resistance gene in this manner, it could be posited to also occur with T6SS EI genes. Cells that acquire T6SS immunity genes may be able to provide protection to both forms of progeny as kin try to establish a niche in the environment.

Overall, these processes may reflect how environmental populations of *V. cholerae* are able kill non-kin strains and species on chitin, and uptake exposed DNA from dead cells for recombination purposes. This invites a wide variety of

possibilities in terms of EI combinations, which is reflected in natural populations of *V. cholerae* as shown in our single location of Oyster Pond.

3.1.4 Most roads lead to death (in a somewhat predictable manner)

Competition is generally simpler to evolve and more immediately advantageous for resource acquisition compared to cooperation with non-kin bacteria (314). This is highlighted in our sole two sets of coexistence versus the equivalent reduction of most partners (60%) in our pairwise outcomes in Oyster Pond. The phenomenon of local competitor inhibition is also reflected in other forms of bacterial warfare; genetically similar, or sympatric *Streptomyces* are much more likely to engage in antimicrobial warfare compared to competitions with allopatric *Streptomyces* due to overlapping niches within an environment (315). In Oyster Pond, orphan immunity genes conferred protection against strains in a mainly predictable manner, although there was also some reduction observed in strains expected to be compatible. While this initially came as a surprise, examining genetic relatedness of individual effector and immunity genes allowed further exploration into this paradigm and present the option that possessing compatible EI classes does not guarantee full compatibility in co-cultures.

3.1.5. The spear-shaped Oyster Pond hierarchy

Tracking HGT of effector and immunity genes allows us to speculate on the acquisition of these genes on evolutionary timescales. HGT events suggest that strains such as CC2 and CC3 were combatants until recently. In environments

with high recombination frequency among members, there is limited opportunity for distinct genotypic clusters to be stably maintained outside of the main population due to the ubiquitous nature of gene exchange (10). Our snapshot of an environmental population of *V. cholerae* is temporally dependent, and likely to be significantly different if resampled now.

To address how pairwise interactions were reflected on a population level, we constructed a hierarchy. Our current scheme does not demonstrate a typical pyramid shaped hierarchy. Ranking strains based on statistically significant win-loss ratios formed low tiers with multiple strains, tapering off to CC9 as the winner. However, CC7, a strain that is not reduced significantly in any competition and wins only once, initiated a re-evaluation of the win-loss hierarchy in favor of scoring system that awards three points for a win, and one for a draw. The ecological significance of a strain that competes well despite not significantly winning often is something to be explored in the more environmentally relevant assays suggested. This current model resembles more of a spear (perhaps fatefully, due to the T6SS spear analogies), and offers insight into individual outcomes on a global scale. The majority of the winners encode key immunity genes to defend against common effectors observed in our subset of Oyster Pond, denoting why these victors were fairly successful. *P. mirabilis* strains also assemble into a structured T6SS-mediated hierarchy, with stronger competitors able to swarm and kill weaker strains (316). The inclusion of motility in T6SS competition allows the victors to effectively box in losers, killing cells around the

edges as they close in, and leaving survivors (if any) surrounded by dead kin. Only three hierarchical transgressions occur with a lower strain outcompeting a better one, allowing us to examine extended RPS dynamics as shown by circular hierarchies. These RPS interactions facilitate the maintenance of genetic diversity, as stronger isolates can be infrequently killed by lower strains, allowing less fit genotypes to persist. As the T6SS competitions observed in the Oyster Pond hierarchy are ultimately dependent on cell-to-cell interactions, I created a card game (Appendix B) that I use during guest lectures and conferences to illustrate this concept. Allowing people to behave as individual genotypes in a mixed community let us to see how competition truly relied on the order of encountering others. We have observed a wide variation of outcomes depending on how the game is set up (see Appendix B for more details), such as a single genotype as a winner, pockets of coexistence or surviving individuals, or no survivors left. In some scenarios, we also incorporate eukaryotic predators that certain isolates are able to defend against if they encode the actin crosslinking domain, a eukaryotic specific toxin. While this is not necessarily reflective of clonally segregated, high cell density environments that ensure an entire genotype is not removed due to a single interaction, this may provide insight into how competition occurs at the edge of these incompatible groups or in well-mixed environments. This game does support the maintenance of biodiversity if dispersal among participants is limited (such as scenarios in a biofilm), and has been a great educational tool for both participants to understand the system and for myself to envision competitive dynamics.

3.1.6 On Conan the Barbarian's surprising performance

The interesting CC4 strain – affectionately termed Conan the Barbarian by us after Arnold Schwarzengger's portrayal – possesses nearly all the required immunity genes to survive the entirety of Oyster Pond *Vibrio cholerae* T6SS competition, while presenting unique effectors that are unable to be neutralized by any member. Not an underdog by any means based on its extensive EI modules, the incongruity between our experimental results and pairwise predictions is intriguing. Why didn't CC4 decimate its competition consistently? After analyzing its genome, we began to glean insights into its EI modules, and more importantly, its unusual genetic organization. The EI array of CC4 is unlike others in Oyster Pond – unknown genes are haphazardly presented (with inverted reading frame in some scenarios) within the aux-1 and aux-2 loci, leading to gaps and insertions between effector and immunity genes. Expression on a gene level is not something we tested aside from competitions with a T6SS deficient *E. coli* to ensure there was an actively expressed T6SS. Transcriptomic analysis of CC4 EI modules could further inform its overall wildtype expression in the environment and potentially determine why our champion is not as successful as its movie counterpart.

3.2 Ponderings beyond the bench: what is next?

Examining our data allows us to speculate on several attractive new options to explore: how do these competitions shift as we continue to work towards more in

situ conditions? Consideration into both abiotic and biotic factors may allow future research to reflect more ecologically pertinent approaches.

3.2.1 T6SS interactions in a biofilm

In many Gram-negative bacteria, T6SS expression and biofilm formation are correlated with one another, with the formation of mature biofilms requiring T6SS expression (317-320). Early stages of biofilm formation have been observed in *V. cholerae* colonization of the gut in animal models, with the T6SS hypothesized to play a role in outcompeting the host commensal microbiome upon infection (220, 236, 321). These structures have recently been shown to offer defensive capabilities in T6SS competition. The formation of exopolysaccharide (EPS) matrix, a secreted polymer involved in biofilm formation and surface adhesion, was also found to protect *V. cholerae* from external T6SS antagonism while allowing self-initiated T6SS attacks to occur (291). Endogenous attacks are not impeded by the EPS layer, suggesting that protection is not dependent on an increase in physical distance between neighbouring cells. Deletion of other biofilm-associated genes does not inhibit generation of the EPS armour, but mutations increasing EPS production allow elevated resistance to contact-dependent killing. Although this EPS-mediated protection against T6SS assaults have been shown to be independent of biofilm formation, in established environmental biofilms with complex secreted components interacting with one another, species-specific EPS may confer differing degrees of protection in microbial competition (142, 291). EPS production is favoured in sustained

resource patches fostering mature biofilms; *V. cholerae* mutants unable to produce EPS are reduced by over 80% in competition with EPS producing strains (322). In addition to the abiotic components of biofilms, social interactions also influence the constituent makeup. This thesis outlines population level structuring within a single species, but interspecific interactions are much more likely in the environment. *P. aeruginosa* expresses a genetically regulated T6SS that is activated upon nonkin attack (323). The counterattack is preferentially directed at adjacent members launching assaults, and thus results in a punishment against non co-operators. This socially dependent behavior encourages microbial cooperation and diversification of metabolic potential within a community, which synergistically increases bioproductivity (323, 324). Additionally, it has been demonstrated that T6SS deficient strains such as *E. coli* MG1655 can outcompete predatory *V. cholerae* provided the opportunity to initially form microcolonies to survive T6SS-mediated killing, indicating that sheer abundance can mitigate competition (325). Interestingly, a recent preprint has also suggested that T6SS antagonism is most effective during initial competition and its efficacy is reduced over time due to the build up of cell debris surrounding competitors, separating incompatible groups and facilitating coexistence by spatial segregation (326). Previous T6SS competitions I performed at low densities during optimization were unsuccessful and resulted in growth, not reduction of strains, potentially due to cells not encountering competitors. However, as the T6SS is expression is also regulated by quorum sensing within *V. cholerae* (327), this may be something to further explore. Overall, the

ubiquitous presence of biofilms and its function as high-density, diverse microcosms, suggests a profound role for contact-dependent population structuring in nature.

3.2.2 Death as a means, not an end

While the mechanism of death has been investigated in this research, the event of mortality itself as a factor of population structuring has not been addressed. In pairwise competition between slow and fast growing strains of soil bacteria (four species of *Pseudomonas* and one of *Enterobacter*), the faster growers outcompeted the slower ones when subjected to successive daily dilutions; a proxy for cell death due to the removal of cells (328). In contrast, under high cell density, and low dilution, slow growth species dominated, indicating a fitness trade-off between niche establishment and capacity to propagate. In addition to interactions within a biofilm, secreted antimicrobial compounds and intraspecific lifestyle may also influence the outcome of T6SS competition. Bacteriocins have been historically identified in *V. cholerae* that exhibit bactericidal or bacteriostatic effects that may provide an additional weapon during competition (329). Death releases new cell contents for homologous recombination (330) or a potential nutrient source (31), to reinforce the extracellular matrix in biofilms (331), and frees up physical space (151). As such, the effects of cell death must be considered in microbial ecology, and not only examined as the endpoint result.

3.2.3 Towards an ecologically relevant framework to explore T6SS

The experimental design of Datta et al. (112) to examine chitin bead colonization with seawater as inoculum offers an attractive mechanism to explore T6SS interactions in more environmentally relevant conditions. *Vibrio* preferentially associates with chitin particles due to its ability to use the substrate as a nutrient source (332). Inoculation of strains in a concentration dependent manner would allow us to track colonization and pairwise and multistrain competitions in naturalized conditions. Chitin beads serve as individual microcosms in a tractable model to observe population dynamics. Photostable fluorescent dye bound to dead cells (333) may allow the aftermath of competitions to be visualized over the span of days in live/dead assays, in addition to *viuB* allele amplicon sequencing at sampled time points (63). Establishing spatially segregated patches on particles may allow non-isogenic strains to be maintained in a microcosm framework, which could be visualized and quantified through culture independent techniques.

Early stage colonization may offer insight into how competition may be effected by factors outside of the T6SS. Preliminary data from a small subset of Oyster Pond strains show evident differences in colonization rates on chitin beads (Figure 3.1), and the concentration of founder cells has been demonstrated to affect overall biofilm formation in other bacteria (150). CC2 and CC3 seem to degrade the chitin much more rapidly than CC4 or CC5 leading to an erosion of bead integrity (Figure 3.1). Perhaps this could be explored further as a potential

fast versus slow growth lifestyle as outlined in the previous section with work done by Abreu et al. (328). Motility has also been observed to be a factor in competition: Gude and colleagues (149) show that fast growing bacteria can displace highly motile competitors by occupying niches, while fast moving populations can impede fast growers by physically blocking expansion. Both scenarios are observed when each group is inoculated at low concentration, which is indicative of environmental distributions. Adding new competitors after the initial biofilm establishment on chitin beads may allow us to observe the resiliency of each microcosm in response to challengers.

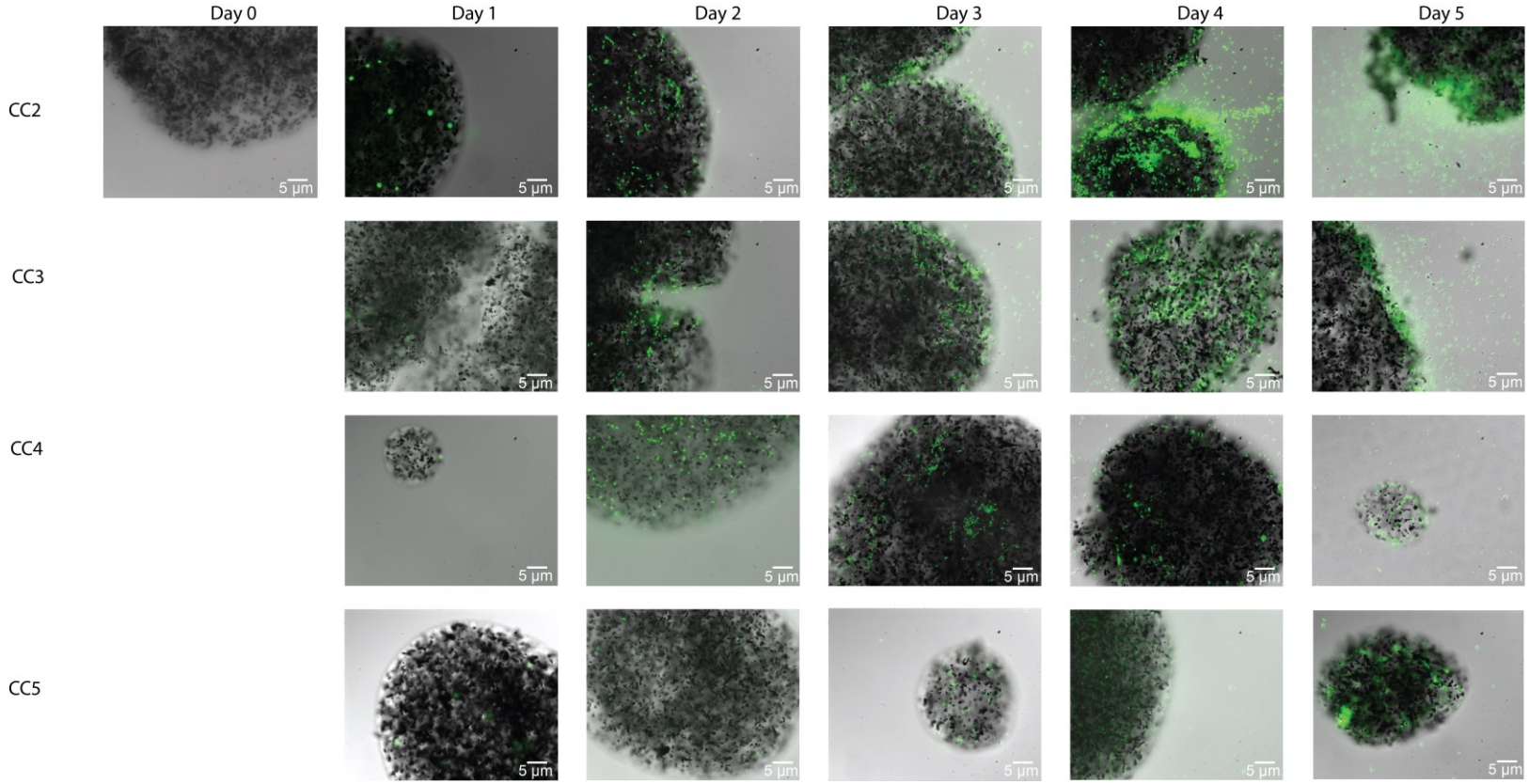
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
CC2
CC3
CC4
CC5
5 µm

Figure 3.1. Biofilm progression of selected Oyster Pond strains on chitin coated beads. 1000 cells/ml were added to M9 minimal media without glucose, with approximately chitin beads added to wells. Aliquots for each strain were collected and imaged daily. Bead degradation is a product of chitin metabolism. Day 0 is added for CC2 as a starting reference for all chitin beads. Scale bars equal 5 μm. Further methods are available in Appendix A.

3.3 Conclusions

Bridging the gap between experimental observations and ecologically meaningful outcomes is an ongoing task in the field of environmental microbiology. *V. cholerae* maintains its niche in the environment prior to causing disease after being ingested in regions where cholera is endemic (231). How it competes against the bacteria inhabiting environmental reservoirs is important to consider in addition understanding the dynamics underpinning natural populations. Representing parameters reflected in nature such as temperature in experiments will allow a more holistic framework to ask these questions. I believe my research has helped to raise some of these ecological questions in *V. cholerae* in the context of the T6SS, and offer opportunities for inquiries to begin to be addressed.

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Appendix A: Materials and methods for chitin bead biofilm progression

Strain selection and growth

Vibrio cholerae CC2, CC3, CC4, and CC5 were grown in Luria-Bertani broth (Difco) overnight. Cells were pelleted and washed in M9 minimal media (Cold Spring Harbor) modified without glucose three times to remove additional nutrients and ensure chitin was the only carbon source available to cells. All cultures were diluted to $1x10^3$ cells/ml through OD_{600} measurement.

Biofilm assay

A small scoop of chitin coated magnetic beads (New England Biolabs) were added to the bottom of 24 well plates. 1 ml of each strain was added to wells, with six replicates. The lid was replaced and the plate was left to incubate at room temperature (approximately 25°C). Aliquots were pipetted onto microscope slides to prepare for wet mount preparation each day. Wells were sacrificed for imagining per strain.

Imaging

Images were obtained using Zeiss Axio Scope A1 at, mounted with an Optronics digital camera at 1000x magnification and visualized with PictureFrame Software Ver 2.3. Epifluorescence microscopy was used to stain nucleic acids with SYBR-Green 1 (Thermo Fischer Scientific). Beads were stained with SYBR-Green 1

and left to incubate in the dark according to manufacturer's instructions for 10 minutes before being imaged. Images were acquired simultaneously with two different channels and superimposed using Zen 2 Blue Edition software. Differential interference contract (DIC) channel, em: 590nm) and SYBR-Green 1 (green – ex: 497 nm, em: 520 nm). Images were collected and processed through Adobe Illustrator afterwards.

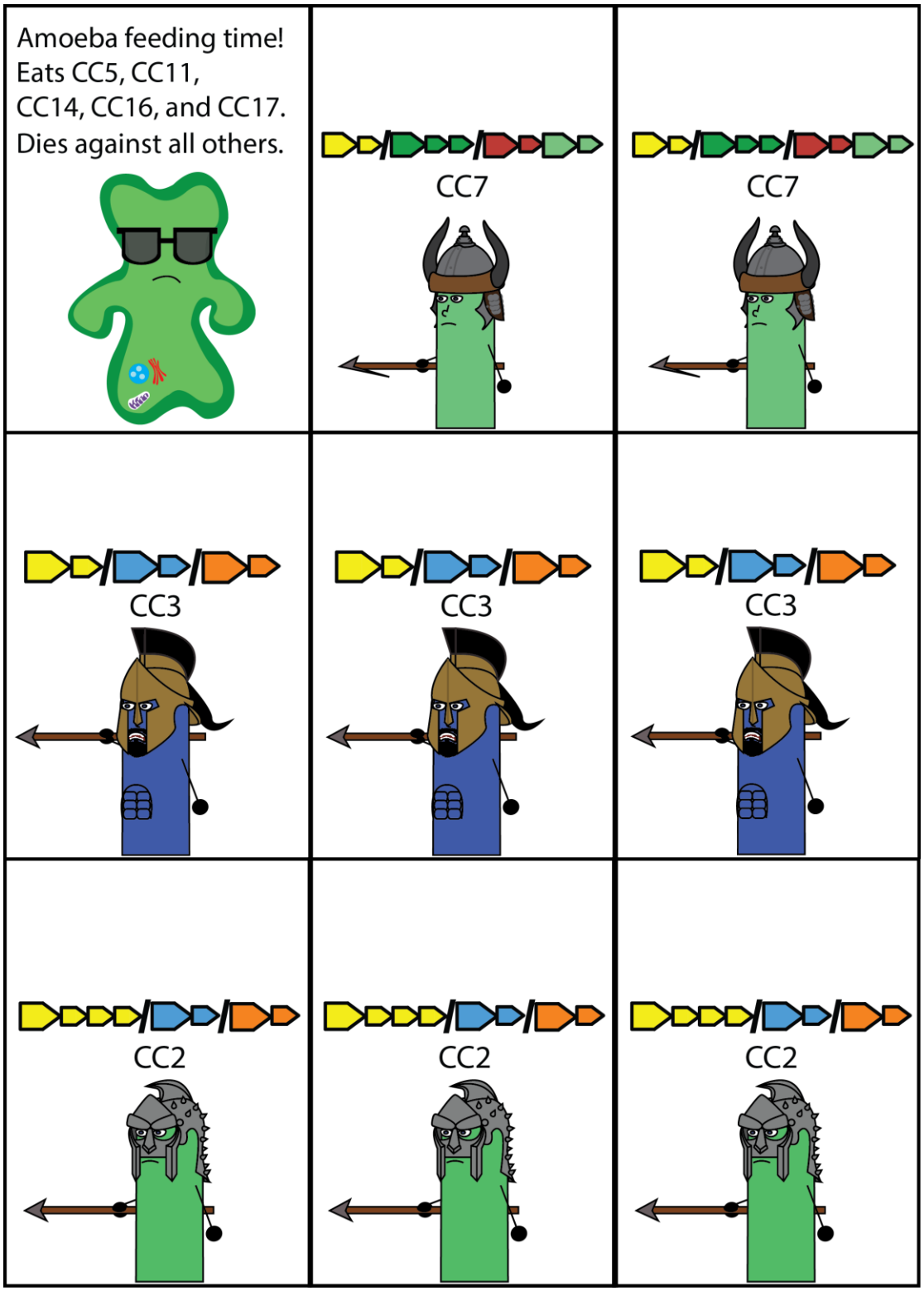
Appendix B: Example Oyster Pond Gladiator T6SS Card Game

Instructions: All *Vibrio cholerae* gladiators have a combination of large arrows (toxin) and small arrows (immunity), separated by slashes into three groups. Cut out cards and shuffle them. After picking a card, match colors grouped in each slash against your opponents. To survive, you must have the same small arrow color to **block each of your competitor's large arrows at every group (**i.e. a yellow immunity in the first group cannot protect against a yellow toxin in the third group). Amoebas can only eat a specific type of *V. cholerae*, and will die against other groups that can defend themselves with toxins that work against them. Continue on meeting others until you die, or are left as the winner.

Variations:

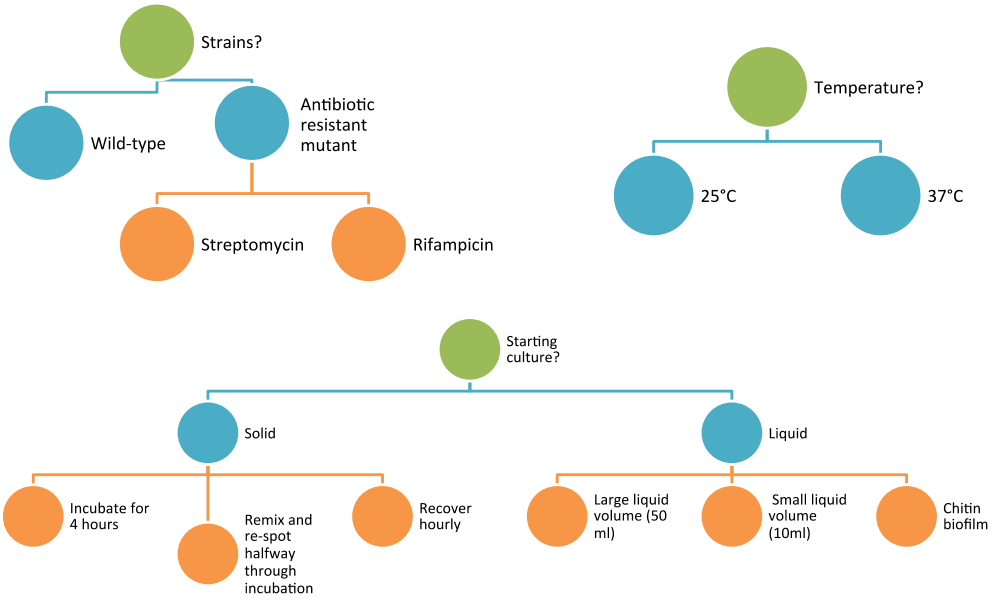
1) Have participants seated, and only interact with those directly beside them, to mimic a biofilm where movement is restricted.

2) The variation of Oyster Pond isolates can be switched and expanded on – in some cases V52 is added. In most circumstances, strains are added in similar proportions as they were environmentally present in, and gladiators are colored according to the CC color scheme outlined in Kirchberger et al. (271).


Amoeba feeding time!
Eats CC5, CC11,
CC14, CC16, and CC17.
Dies against all others.
CC7
CC7
CC3
CC3
CC3
CC2
CC2
CC2

CC11
CC9
CC4
CC11
CC9
CC4
CC11
CC9
CC4

Appendix C: T6SS Optimization Steps



This figure outlines the multiple variations attempted during my optimization of the T6SS competition assays. I will briefly outline these alternatives I explored during my time spent modifying my experiment for potential future reference.

I created rifampicin resistant strains to test against wild-type isolates, as I found that spontaneously generated streptomycin mutants underperformed in every experiment. Resistance to both streptomycin and rifampicin are facilitated through single point mutations (334, 335), however, I found that the streptomycin mutant consistently was recovered in lower proportions each time in competition.

This led me to generate rifampicin mutants, which did not have the same fitness deficit in competition.

The liquid starting cultures (both large and small volumes) were difficult to concentrate to the 10^7 CFU/ml concentration needed for assays. There were not enough cells in overnight cultures within a single tube, leading to the requirement of multiple tubes for growth, centrifugation to pellet cells, and re-suspension before examining cell density. This introduces more routes for contamination compared to single agar plates in high cell density, in addition to a much longer length of preparation before competition. Liquid starting cultures also had a biofilm above the liquid portion representing the majority of the cells, which was difficult to homogenize completely resulting in inconsistent optical density estimates. For these reasons, we decided to go with solid, petri plate, starting cultures.

Recovering hourly isolates and remixing half way were attempted to test whether there was an increase in reduction over time, or to see if we could initiate more killing by removing spatial boundaries set by the T6SS by homogenizing strains again, respectively. I did not find any conclusive results on the hourly recovery experiment; however, there have been recent preliminary results from other authors suggesting that T6SS-killing slows after three hours due to cell debris accumulating and physically separating attackers (326). The remix experiment actually resulted in growth, not reduction of strains. This may be due to cells that

were reduced and spatially separated, to expand into gaps after being re-suspended and spotted out. As this did not measure competition but rather growth, we did not pursue either of these routes further.

Experiments were performed at 25°C and 37°C, and for 4 hours as outlined in the thesis, with further discussion in the second chapter. The chitin biofilm preliminary data was outlined in the conclusion chapter.