VIBRIO CORALLILYTYCUS RE22 TYPE VI SECRETION SYSTEMS CONTRIBUTE TO TEMPERATE CORAL LYSIS AND ENDOSYMBIONT DEATH

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VIBRIO CORALLIILYTICUS RE22 TYPE VI SECRETION SYSTEMS CONTRIBUTE TO TEMPERATE CORAL LYSIS AND ENDOSYMBIONT DEATH

BY

NATHANIEL AWKERMAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY

UNIVERSITY OF RHODE ISLAND

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MASTER OF SCIENCE THESIS

OF

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2021
ABSTRACT

*Astrangia poulata* is a facultatively symbiotic temperate coral that is being explored as a model system for studying the physiology and ecology of cnidarian-microbe symbiosis. *Vibrio coralliilyticus* is a known causative agent of a class of coral diseases called “white syndromes” that result in bleaching in tropical coral species. It is an effective pathogen due to a wide array of virulence factors including two Type 6 Secretion Systems (T6SS1 and T6SS2). In this study, we investigated the pathogenic potential of *V. coralliilyticus* RE22Sm in *A. poulata* and in cultures of its endosymbiont, *Breviolum psygmophilum*. To independently gauge the antagonistic effects of each of the two T6SSs, allelic exchange mutants of the *hcp* genes were utilized. In the *A. poulata* challenge, *V. coralliilyticus* RE22Sm caused tissue lysis in coral samples. Both aposymbiotic and symbiotic corals were susceptible to infection, and aposymbiotic corals displayed tissue lysis faster than symbiotic corals. Mutation of the T6SS1 *hcp1* gene resulted in the greatest attenuation of virulence in the coral system. Coral survival increased from 12% in the wild-type challenged samples, to 60% for those challenged with Δhcp1. Virulence was also attenuated in corals challenged with Δhcp2, with 30% survival. Similarly, *B. psygmophilum* challenged with the Δhcp1 strain had a 20% increase in both cell survival and chlorophyll a content, compared to cultures exposed to wild type *V. coralliilyticus* RE22Sm. An *hcp1 hcp2* double mutant resulted in minor attenuation of virulence in both coral and endosymbiont trials. Revertant strains with restored wild-type copies of the *hcp* genes displayed comparable virulence to wild-type *V. coralliilyticus* RE22Sm. These results suggest that Type 6 Secretion is a major component of pathogenesis against the
temperate coral *A. poculata* and *B. psygmophilum*. Heightened susceptibility of aposymbiotic coral samples to bacterial challenge is consistent with literature that suggests symbiotic *A. poculata* is more effective than aposymbiotic colonies at mitigating of environmental stress. The data are consistent with bacterial challenges in an oyster larval system, which indicate that T6SS1 is primarily involved in eukaryotic antagonism.
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DEDICATION

I dedicate this thesis to the people that have had to put up with my shenanigans the most over the course of my life, my parents Robert and Judith Awkerman. Thank you for always guiding me in the right direction and never giving up when I faltered. Thank you for pushing me to be the best that I could be in my education and career to prepare me for the future. Sometimes you never know how much a grounding influence helps until you need it and I can safely say I couldn’t have completed this endeavor without your support.
PREFACE

The following thesis has been prepared in manuscript format according to the guidelines of the Graduate School of the University of Rhode Island. This thesis contains a literature review and one manuscript.

The manuscript “*Vibrio coralliilyticus* RE22 Type VI Secretion Systems Contribute to Temperate Coral Lysis and Endosymbiont Death” has been formatted according to ASM guidelines and will be submitted to AEM.
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Literature Review
Introduction

Coral bleaching events have been increasing in magnitude and scope since the early 1980s [3], damaging both biodiversity associated with coral reef environments and commercial industries reliant on continued coral survival such as fisheries and tourism [1, 2]. Coral reef environments are essential for biodiversity with a conservative estimate of 2,594,000 unique reef associated species dependent on the environment [4]. Coral bleaching has a wide variety of potential causes due to the delicate symbiosis between coral and their dinoflagellate endosymbionts, which, for most tropical corals, is obligate in nature [5]. Ocean acidification and the gradual increase of ocean temperature have been shown to contribute to significant coral stress and loss of symbiosis [6, 7]; however, another cause of coral bleaching is bacterial pathogenesis. One prominent type of coral infection is referred to as White Syndrome (WS), occurring in a number of tropical coral species including Pocillopora damicornis, Montipora capitata, and Acropora cytherea [18, 21, 32]. Although the exact causes of WS have not been identified, WS often occurs in conjunction with an increase in levels of Vibrio species in infected coral microbiomes [22]. Vibrio species including Vibrio shiloi and Vibrio coralliilyticus have been demonstrated to cause bleaching and tissue lysis in coral samples under experimental conditions [17]. Because of the difficulties involved in prevention, avoidance, and treatment of coral reef infections long-term, a greater understanding of the mechanisms behind such widespread collapse is essential [35].
The pathogen *V. coralliilyticus* is the primary focus of this review due to its wide array of virulence factors and previously described interactions with coral hosts [30]. Virulence factors include multiple secretion systems including a type I, type II, type III, and 2 type VI secretion systems (T6SS) that are capable of translocation a wide variety of anti-eukaryotic and anti-bacterial effector compounds [60-64]. Additionally, *V. coralliilyticus* is capable of secreting 2 primary extracellular zinc-metalloproteases, VcpA and VcpB, the former of which has close evolutionary similarities to the EmpA protease found in *V. anguillarum* [57, 86]. High levels of proteolytic activity produced in high temperatures conditions within the host are primarily thought to be the main factors responsible for coral pathogenesis, likely due to a decline in photochemical efficiency of the coral endosymbiont when exposed to the pathogen [21]. However, recent studies have shown that bacterial knockout mutants of *V. coralliilyticus* affecting protease production can display a minimal effect on isolated cultures of coral zooxanthellae [68]. Genes commonly associated with Type VI Secretion (T6S) are also upregulated in such mutants potentially indicating the involvement of multiple virulence factors in coral pathogenesis [68]. While there are numerous strains of *V. coralliilyticus* currently categorized as coral pathogens [25], *V. coralliilyticus* RE22, a primary infectious agent of oyster aquaculture [83, 84], is relatively unexplored as a potentially infectious strain to coral despite having minimal differences between their virulence repertoires [30]. *V. coralliilyticus* RE22 has been shown capable of infecting *M. capitata* coral fragments but its pathogenicity against other coral species could indicate the ability to produce a broad-spectrum of virulence factors that necessitate further study [87].
Astrangia poculata is a facultatively symbiotic coral that is found in temperate waters along the North American coast [89]. It is currently being used to model the scleractinian coral response to stressors typically experienced by tropical coral without disturbing reef environments [108, 109]. A primary benefit to *A. poculata* as a model organism for bacterial challenge is the ability to examine the effects of stressors independent of endosymbiont density within coral tissue. Colonies can develop having either dense endosymbiont populations (symbiotic) or sparse endosymbiont populations (aposymbiotic) making them phenotypically white or “bleached” [95]. While other temperature facultatively symbiotic Cnidarian models have been used in the context of infection with *V. coralliilyticus* (e.g., *Exaiptasia pallida*) [102] *A. poculata* has the benefit of being a scleractinian coral species phylogenetically closer to other tropical corals. By examining the impact of various virulence factors on coral samples regardless of endosymbiont state, insights about the mechanistic action of the pathogen itself can be gleaned, establishing *A. poculata* as an ideal candidate for further coral testing while minimizing damage to reef ecosystems.
Main Body

Tropical coral bleaching and White Syndrome (WS)

Coral reefs have been significantly declining in health and structural integrity for the past 35 years [3]. While reef-associated tourism is estimated to be valued at 36 billion USD globally as of 2017 [1] and reef-associated fisheries are estimated to be valued at 6.8 billion USD globally [8], both industries are threatened with decline due to the current loss of reef environments. Additionally, there are a number of tropical communities largely reliant on tourism and goods derived from local coral reefs despite the declining population of reef associated species [9, 10]. Coral reef environments are host to an immense level of biodiversity concentrated in an area covering less than 1% of the ocean floor, creating a highly vulnerable environment currently threatened by increasing oceanic stressors [2]. Much of the biodiversity associated with coral reef environments is in sharp decline with as many as 75% of reef-associated fish species exhibiting as much as a 50% loss in abundance with marine reserves unable to perpetually insure the conservation of threatened species [26]. Primary coral stressors include ocean acidification, global warming, increased microplastic density, other pollutants like human sewage, and bacterial pathogens all of which have contributed to the declining health of coral reefs and have been increasing over time [11-13]. As these factors are not isolated in a natural environment, the decline in coral health reflects simultaneous exposure to several of these stress factors [14]. Additionally, stressors such as global warming also impact reef environment recovery due to the decline in larval viability making the issue of
maintaining reef health difficult to approach [15]. However, a factor that is somewhat understudied is the direct pathogenic potential of marine microbes as the cause of either primary or secondary infections of coral.

When approaching the issue of coral bleaching, it is important to consider that coral pathogens are numerous and produce a wide array of phenotypically different conditions based on the organism responsible, so specialized solutions will likely be necessary to combat each type of infection [16]. A bacterial infection of *Acropora palmata* referred to as white pox disease (WPD) is caused by the fecal bacterium *Serratia marcescens*. This is an example of a highly virulent coral pathogen, able to cause a high rate of tissue loss of approximately 2.5 cm² per day [33]. Originally, white pox disease was thought to be highly contagious as neighboring colonies were rapidly infected shortly after the initial infection; however, emerging research has demonstrated that spatial relationships *in situ* were not essential to disease progression and, instead, innate genomic susceptibility was a greater factor [34]. The emergence of WPD is attributed solely to the exposure of *A. palmata* colonies to high levels of human sewage containing the unique strain of *S. marcescens*, PDR60, as the strain commonly found in waste was isolated from infected colonies near offshore septic systems [88]. While not bacterial, fungal aspergillosis of soft gorgonian corals caused by *Aspergillus sydowii* is also a possible source of coral infection presenting another potential microbial threat in addition to those already affecting scleractinian coral [27]. Black band disease (BBD) is also a common coral disease primarily effecting scleractinian coral within the genera *Montastrea*, *Colpophyllia*, and *Diploria* which are typically categorized as boulder/brain coral [28]. Primarily caused by mixed
populations of cyanobacteria, sulfide-oxidizing bacteria, and sulfate-reducing bacteria, the tissue decay attributed to BBD is due to a sulfide-rich environment creating hypoxic conditions as well as the black coloration [29]. Additionally, BBD associated bacteria were found to be unrelated to any terrestrial bacteria indicating that tourism is not a likely a contributing stressor [28]. Yellow band disease (YBD) is a coral disease associated with high levels of *Vibrio* spp. colonizing pale yellow tissue lesions [39]. YBD infected coral samples often display a marked decrease in chlorophyll *a* and *c2* content likely indicating targeted degradation of the intracellular zooxanthellae causing coral tissue death through starvation [39]. *Vibrio* spp., primarily *V. natriegens* and *V. parahaemolyticus* are also the cause of *Porites* ulcerative white spot syndrome (PUWS) in *Porites cylindrica*. This coral species seems much more sensitive to infection than other coral species, as inocula of $1 \times 10^4$ CFU/mL were sufficient to achieve bleaching and tissue lysis under laboratory conditions [54].

A large number of coral bleaching diseases have been found to be caused by various bacteria within the genus *Vibrio* including *V. alginolyticus*, *V. shiloi*, and *V. coralliilyticus* among others [17]. Many of these coral pathogens are responsible for a class of diseases referred to as white syndrome (WS) or white band disease (WBD) due to the gradual outward progression of a band of bleached tissue to appear on affected coral in contrast to the black band of BBD [18]. White syndrome has been known to affect a variety of tropical corals but it is particularly devastating to reef environments composed of plate corals of the *Acropora* spp. with prevalence among colonies as high as 50% in some regions [18]. While multiple causative agents of WS have been explored including *Vibrio shiloi* and *Vibrio mediterranei* [23, 24], bacterial
isolates from diseased *Montipora aequituberculata* and other Indo-Pacific reef corals had 98% homology with *Vibrio coralliilyticus*, a member of the γ-Proteobacteria family *Vibrionaceae* [25]. However, this does not indicate that all instances of WS in *Acropora* spp. or other coral are directly related to involvement with strains of *V. coralliilyticus*, indicating other potential pathogens or contributing factors that act by weakening the coral and allowing *Vibrio* spp. to act as opportunistic pathogens [30, 48]. Thermal stress has been correlated with an increase in outbreaks of white syndrome indicating heightened susceptibility to disease [19]. While host-endosymbiont interaction in coral can be disrupted by increased environmental water temperatures, reducing endosymbiont density up to 60% in heat-treated *Pocillopora damicornis* [20], a number of identified coral pathogens also increase in number and virulence at heightened temperatures [21]. Additionally, it was found that increased seawater temperatures were capable of increasing innate *Vibrio* populations associated with samples of *P. damicornis* potentially indicating an increase in ocean temperatures resulted in both an increase in coral stress as well as an increase in potential for opportunistic infections [22]. An increase upwards of 4 orders of magnitude in *V. coralliilyticus* populations specifically associated with heat-treated samples of *P. damicornis* is indicative of impending infection, since *V. coralliilyticus* is a well-documented pathogen of *P. damicornis* [21, 22]. *V. coralliilyticus* associated WS occurs commonly in *Montipora capitata* (figure 1), *Pocillopora damicornis* and *Acropora cytherea* [21, 32], and can also occur as a co-infection of Stony Coral Tissue Loss Disease (SCTLD) affected *Montastraea cavernosa* and *Orbicella faveolata* [30]. This indicates that, though WS is not caused exclusively by *Vibrio* species [31], a
primary cause of coral disease is *V. coralliilyticus* necessitating further study on its interactions with its coral host.

![Image](image1.png)

**Figure 1.** *M. capitata* with aMWS. (A) A colony of *M. capitata* with acute tissue loss (black arrow) shown adjacent to a healthy *P. compressa* colony (white arrow). Bar, 10 cm. (B and C) Coral fragments from an infection trial at the time of inoculation (B) and 48 h after inoculation (C). The spacing of the plastic supports of the grids under the coral fragments is 1 cm by 1 cm [32].

Understanding the pathogens involved in coral disease is essential due to the limitations associated with management measures currently being used to protect coral environments. While some coral infections may be overcome due to selection pressures against genetic susceptibility such as white pox disease in *A. palmata* [34],
more direct measures may be necessary to combat WS. Currently, antibiotics such as amoxicillin are being tested as potential protective measures against coral bleaching in *M. cavernosa* colonies affected by SCTLD outbreaks [35]. While lesions are reduced by 95% in treated colonies, the introduction of antibiotics likely alters the composition of the coral microbiome, potentially weakening it and allowing for future re-infections [35]. Additionally, the introduction of antibiotics into the water will promote further antibiotic resistance making future infections harder to combat and this antibiotic solution largely temporary. Coral mucus-associated *Vibrio* species have been shown capable of rapid genetic exchange of antibiotic resistance genes increasing the spread of antibiotic resistance among potentially pathogenic populations [36]. Ocean environments serve as a collection of mobile antibiotic resistance genes (ARG) that has been steadily growing with overuse of antibiotics making it imperative to carefully consider the use of antibiotics for coral treatment to prevent further expansion of the oceanic resistome [49, 50]. Alternative solutions to managing the spread of coral pathogens include treating affected coral with potential probiotic organisms, but that requires knowledge concerning the effectiveness of the targeted probionts against infectious species. Putative novel probiotic organisms isolated from *P. damicornis* colonies and the surrounding water column have recently been tested to determine their effectiveness at limiting coral bleaching from both pathogenic and thermal stressors [37]. In experiments testing the protective effects of several innate *Pseudoalteromonas* spp. against *V. coralliilyticus* in *P. damicornis* it was found that probiotic organisms successfully stymie the progression of coral bleaching at 30°C, reducing the decline in photochemical efficiency (Fv/Fm) [37]. While probiotic
solutions to Vibrio-induced pathogenesis have been widely explored in other systems [38], use of probiotics for coral protection and disease prevention is still largely unexplored. Introduction of new probiotic organisms to specifically target bacterial coral pathogens could potentially be a long-term solution to the problem of coral bleaching. However, extensive information about the pathogen itself as well as its interactions with a prospective probiont are necessary to best make use of a probiotic option due to the extant extensive anti-microbial and antibiotic resistance of coral associated V. coralliilyticus [42].

**Vibrio coralliilyticus**

Among potential coral pathogens, *Vibrio coralliilyticus* is among the most commonly identified during isolation of bacteria associated with infected coral tissue [30, 40]. *V. coralliilyticus* is a gram-negative marine Gammaproteobacteria and a member of the genus *Vibrio* within the family Vibrionaceae [41]. Characteristics that are typically associated with *Vibrio* spp. include a high level of flagellar motility, a curved-rod like shape, and the ability to be facultatively anaerobic [43]. The curved *Vibrio* shape typically determined by the CrvA protein is primarily identified as an adaptation allowing for bacterial tunneling through mucus with minimal resistance [44]. *Vibrio* spp. are near ubiquitous in the ocean with the distribution area of infectious species enlarging with increased ocean temperatures [45]. Additionally, they can be found in a wide variety of habits and environments with some isolates exhibiting extremophilic characteristics and some in association with hosts as either pathogens or members of the core microbiome [46]. Because a large number of *Vibrio*
spp. are broad spectrum pathogens, a single strain may be capable of causing infection in multiple host organisms, marine or otherwise, or the strain could be entirely avirulent, or avirulent under certain conditions [52, 53]. Corals are vulnerable to infection from a wide array of *Vibrio* spp. including *V. alginolyticus*, *V. shiloi*, *V. coralliiylyticus*, *V. natriegens*, *V. parahaemolyticus*, and *V. harveyi*; however, most of these organisms are capable of causing infection in other systems [17, 54]. Other potential hosts of the listed coral pathogens include: fish (e.g. *V. alginolyticus* and *V. harveyi*) [46, 55], shrimp (e.g. *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*) [46], bivalves (e.g. *V. alginolyticus*, *V. coralliiylyticus*, *V. parahaemolyticus*, and *V. harveyi*) [53], and humans (e.g. *V. alginolyticus* and *V. parahaemolyticus*) [46]. Most pathogenic *Vibrio* spp. are capable of infecting a wide variety of hosts thanks to their immense array of virulence factors that function against both prokaryotic and eukaryotic prey organisms [56].

*Vibrio coralliiylyticus* displays a number of identified virulence factors consistent with those found among other pathogenic *Vibrio* spp. [57]. Multiple secretion systems have been shown to be characteristic of infectious *Vibrio* spp. and are often present in multiple copies [58, 59]. Virulence factors associated with type I secretion (T1S) include enterotoxins, cytotoxins such as the multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins, siderophores and adhesion factors [60, 61]. The type II secretion system (T2SS) and general-secretory pathway are associated with the production and secretion of lytic enzymes including proteases, lipases, chitinases, and hemolysins [60, 62]. Both type III secretion systems (T3SS) and type VI secretion systems (T6SS) are also frequently present in *Vibrio* species.
contributing to the ability to evade phagocytosis and promote translocation of targeted effector compounds respectively [58, 63, 64]. Additionally, *Vibrio* are robust biofilm formers due to the presence of multiple systems of pili including mannose-sensitive haemagglutinin type IV pili (MSHA), toxin co-regulated pili (TCP), and chitin-regulated pili (ChiRP) working in concert to promote cell-to-cell or cell-to-surface adhesion [65]. Regulation by ToxT decreases MSHA and increases TCP allowing MSHA to first promote monolayer biofilm formation before up-regulation of ToxT increases levels of TCP promote more pronounced colony formation and 3-dimensional growth after the colony is established [66]. While all of these virulence factors contribute to pathogenesis against most eukaryotic host organisms, the primary virulence factor thought to cause degradation of coral tissue by *V. coralliilyticus* is the extracellular zinc-metalloproteases VcpA and VcpB regulated by VcpR of the quorum-sensing system [67]. However, bacterial mutants of *V. coralliilyticus* deficient in VcpA production displayed no significant difference in pathogenesis against coral zooxanthellae but transcriptomics revealed an upregulation in other virulence factors including the Hcp component of the T6SS (figure 2) [68]. This result would indicate that T6S is potentially a virulence factor of interest in regards to coral-endosymbiont pathogenesis.
Figure 2. Photosystem II inactivation of *Symbiodinium* Clade C1 cultures by *V. coralliilyticus* wild-type and ΔvcpA strain supernatants. The error bars indicate the mean±s.d. [68].

The T6SS of *V. coralliilyticus* resembles an inverted T4 bacteriophage-like nanomachine and is primarily purposed for the cell contact-mediated translocation of effector molecules [69, 70] but may have a secondary role in quorum sensing [71]. The system is composed of several distinct structures assembled from thirteen conserved proteins (Figure 3) including a baseplate complex anchored to the inner membrane, a hollow needle-like structure composed of hexomeric hemolysin co-regulated protein (Hcp), a VipA/B contractile sheath surrounding the Hcp barrel coupled with a ClpV for reassembly of the contractile apparatus, and a valine-glycine repeat protein (VgrG) which has a hardened proline-alanine-alanine-arginine (PAAR) repeat designed to puncture eukaryotic or prokaryotic prey cell membranes [72]. Both
the Hcp barrel and baseplate are essential for functionality of the T6SS; the baseplate complex is essential due to its role in assembly while the Hcp barrel is essential for its role in delivery of effector compounds activated through contact. The baseplate complex itself, primarily designated as TssEFGK-VgrG, anchored to the inner membrane is necessary for the polymerization of the sheath complex prior to activation and regulated by sequential assembly of component subunits [76]. Effectors either decorate the PAAR motif and are released upon activation of the mechanism or are translocated via the Hcp needle structure with Hcp components potentially acting as chaperones [73, 74]. The *V. coralliilyticus* RE22 T6SS on chromosome 1 (T6SS1) has been suggested to have divergent but overlapping function with the T6SS on chromosome 2 (T6SS2), with T6SS1 showing vital function for pathogenesis in the oyster host, compared to the primarily anti-bacterial activity associated with T6SS2 [75].

While the role of T6S in coral pathogenesis is unknown, there are several possibilities as to how the virulence factor and various effectors contribute to coral tissue loss. Samples of *P. damicornis* treated with cell densities of *V. coralliilyticus* of approximately $1 \times 10^8$ CFU/mL exhibited an increase in caspase-3 like activity potentially indicating apoptosis of coral tissue due to infection [77]. Components of the T6SS in mammalian models can induce apoptosis in host tissues, but it is unknown if this is bacterial exploitation of existing pathways or a controlled typical innate immune response to infection [78, 79]. The multitude of different potential effector compounds translocated by T6S could also potentially be an explanation for the virulence against coral. By increasing invasion via adhesion, increasing intracellular
viability through innate immune regulation, inducing disruptions to the actin cytoskeleton, the anti-eukaryotic effectors associated with T6S [73] could induce a stress response of sufficient magnitude to contribute to intact coral cell expulsion that occurs under temperature stress [80].

As most of the virulence factors associated with coral pathogenesis are regulated by quorum sensing, a sufficient cell density may need to be reached before the progression of coral disease such as WS [53]. While quorum sensing associated transcripts are up-regulated in the presence of coral mucus, reliance on a significant cell density would potentially make *V. coralliilyticus* more effective as an opportunist than a primary pathogen due to its ability to rapidly populate a new niche [81, 30].
Figure 3. Schematic Representation of the Structure and Mechanism of the Type VI Secretion System (T6SS). (A) The extended or ‘primed to fire’ machinery is assembled from cytoplasmic and membrane components. The membrane complex, which may initiate T6SS assembly at the inner membrane, contains TssJ, TssL, and TssM, represented in yellow, red and orange respectively. A putative baseplate-like structure, formed by TssAEFGK and represented in brown, sits at the cytoplasmic face of the inner membrane. Upon VgrG, within the baseplate, an elongated tubular structure of Hcp hexamers (light blue) is built and extends into the cytoplasm, encompassed in a TssBC sheath (blue). (B) The second step, ‘firing’, corresponds to sheath contraction and propels the inner tube towards the target cell. PAAR and VgrG,
represented in pink and purple triangles respectively, form the puncturing device responsible for membrane perforation prior to effector delivery. (C) Once effectors (grey stars) are delivered into the target cell, the contracted sheath is disassembled by ClpV (green hexamers). Abbreviations: IM, inner membrane; OM, outer membrane; PG, peptidoglycan [111].

Figure 4. Virulence factors and secondary metabolites in nineteen pathogenic Vibrio species. Gene counts associated with virulence factors and the biosynthesis of secondary metabolites in *V. coralliilyticus* and other vibrios from corals, fireworms, isopods, and human-associated pathogens [30].
There are multiple currently characterized strains of *V. coralliilyticus* that are known as aggressive coral pathogens. Strain BAA-450, also known as strain YB1, is primarily identified as a pathogen of *P. damicornis* causing a rapid progression of bleaching and tissue lysis with symptoms fully apparent in as little as ten days but largely avirulent at temperatures under 22°C [21]. The primary strain responsible for *M. capitata* WS is identified as OCN008, which causes bleaching of coral fragments exposed to doses of $1 \times 10^8$ CFU/mL within as little as two days; however, OCN008 was unable to bleach other coral species such as *Porites compressa* likely indicating a specificity for *Montipora* spp. [32]. In *A. cytherea*, OCN014 is the primary identified strain of *V. coralliilyticus* causing WS causing widespread tissue loss at Palmyra Atoll in 2009, with as many as 25% of coral colonies being affected by the pathogen [82].

Previously classified as *Vibrio tubiashii* [40], *V. coralliilyticus* strains RE98 and RE22 are primarily identified as pathogens of bivalves causing vibriosis in both larval Pacific oyster *Crassostrea gigas* and eastern oyster *C. virginica* [75, 83, 84]. Bacterial challenge experiments using multiple strains including BAA-450, RE98, OCN008, and OCN014 against Pacific oyster larvae demonstrated that OCN014 displayed the greatest virulence indicating that some strains are capable of pathogenesis against multiple organisms while others like BAA-450, which displayed minimal oyster pathogenesis, are highly specific to their target organism [85]. This is interesting considering the close evolutionary and metabolic similarities between strains with only some specific virulence factors differing between them such as the lack of RTX toxin in strains OCN008 and BAA-450 (Figure 4) [30]. *V. coralliilyticus* strain RE22,
similarly to OCN014, possesses RTX toxin and is capable of infecting both oyster larvae and *M. capitata*, though it does not cause coral bleaching to a robust degree, only effecting 10% of treated *M. capitata* samples [30, 87]. As such, further testing on RE22 and its capacity to infect other potential coral hosts is necessary to determine the scope of the strain’s virulence repertoire.

*Astrangia poculata*

*Astrangia poculata* is a temperate non-reef forming scleractinian coral with a wide geographical range and a facultative relationship with its endosymbiont, *Breviolum psygmophilum*, making it a model organism of particular interest in studying the dynamics of coral death independent of the endosymbiont state [89]. Its habitat spans the majority of the shallows off the eastern coast of North America ranging down into parts of South America as well [90]. At the northern end of its geographic range, *A. poculata* can be found along the coast of Cape Cod with average summer water temperatures reaching peaks of 24˚C and at the southern-most end of its range it can be found along costal Venezuela with average water temperatures reaching 30.5˚C indicating strong thermo-tolerance [90, 91]. Like many temperate corals, *A. poculata* will undergo a quiescence response in winter months as temperatures dip below 8˚C that, while beneficial in that it conserves energy, can result in the loss of both coenosarc tissue and endosymbiont density which can contribute to the decline of colony health in conjunction with the accumulation of algal commensals leading to skeletal fouling [93, 94]. Expulsion of the endosymbiont is not fatal as it would be in tropical coral and *A. poculata* colonies occur naturally in
both low endosymbiont density (aposymbiotic) and high endosymbiont density (symbiotic) phenotypes [95]. Single colonies of *A. poculata* can also have neighboring polyps display different phenotypes indicating endosymbiont state is not universal throughout the colony [95]. Additional seasonal differences include changes in the microbiome, which fluctuates primarily with seasonal temperatures rather than changing with the endosymbiont state [96]. The facultative relationship with its endosymbiont means *A. poculata* relies more extensively on heterotrophic metabolism benefiting from its larger polyps compared to tropical corals [97]. As would be expected, dark respiration levels are elevated in aposymbiotic corals compared to symbiotic corals across all temperature conditions but most notably at temperatures around 26°C whereas the inverse was true regarding gross holobiont photosynthesis at similar temperatures [47]. However, while both gross photosynthesis and dark respiration rates peaked at 26°C, the photochemical efficiency of the coral endosymbiont *B. psygmophilum* was greatest at 18°C indicating greater thermo-tolerance of the host than the zooxanthellae [47, 51, 92]. Despite the differences in optimally functional temperature, the facultative host-symbiont relationship allows for examination of the role of *B. psygmophilum* in the regulation and suppression of normal coral functions.

Facultative host-symbiont relationships have been studied in other Anthozoan models such as *Aiptasia* spp. to great effect, providing important insights as to the functional benefits and detriments to colonization by intracellular zooxanthellae [98]. In typical tropical coral models, the coral host has an obligate relationship with its endosymbiont but that relationship is not without cost [99]. The innate immune system
in tropical corals is typically modulated by the symbiont in order to prevent coral tissues from rejecting the dinoflagellate endosymbiont or collapsing the intracellular symbiosome [100]. Similar patterns are observed in other facultative Anthozoans such as *Exaiptasia pallida* [101]. Transcripts governing innate immunity are significantly down-regulated in symbiotic samples of *E. pallida* when compared to aposymbiotic samples of *E. pallida* after challenge with *V. coralliilyticus* BAA-450 [102]. While this might indicate that aposymbiotic *A. poculata* are likely to respond more capably to exposure to pathogenic organisms, they lack the metabolic efficiency and photosynthetic yield of symbiotic fragments that would enable them to survive longer under bacterial antagonism [47]. The difference in coral health based on symbiont state can be observed in the process of wound recovery as symbiotic samples are capable of regenerating lost polyps or tentacles at a much greater pace than aposymbiotic samples [103]. Both endosymbiont state and heterotrophic metabolism are essential for wound recovery. Of 28 wounded samples for each condition, 0% of unfed aposymbiotic fragments displayed full recovery after 60 days and only 3.57% of fed aposymbiotic fragments displayed full recovery (Figure 5) [104]. In the unfed symbiotic samples, like in the fed aposymbiotic samples, only 3.57% of fragments were fully recovered and active by 60 days whereas 21.48% of fed symbiotic samples had recovered fully with the majority of samples in various states of partial recovery [104]. Further benefits of symbiosis in *A. poculata* include accelerated recovery of the microbiome in symbiotic corals with near complete recovery occurring only 2 weeks compared to partial recovery in aposymbiotic coral after exposure to antibiotics likely due to additional secreted metabolites from the endosymbiont [105]. Greater recovery
of the microbiota after disruption could also serve as a protective measure against potential re-infection after initial bacterial infections or may aid in stymieing the proliferation of opportunistic pathogens. The natural differences between aposymbiotic and symbiotic colonies provide ample opportunities to isolate the functional contributions and detriments of symbiosis.

Figure 5. Proportion of colonies in landmark recovery stages (full polyp, tentacle nubs, undifferentiated tissue, or no healing) after 60 days. Bars in all shades of gray collectively represent healing initiation, while bars in dark gray represent developmental healing success. Numbers in bars signify total number of colonies in each stage [104].
Unlike other currently explored facultative Cnidarian models, *A. poculata* is a calcifying scleractinian coral making it more applicable to mimic infection in tropical coral species [107]. Autotrophic and heterotrophic contributions to nutrient acquisition can be monitored through the use of N$^{15}$ isotope of dissolved inorganic nitrogen (DIN) further allowing analysis of the benefits of symbiosis [110]. Studies have been conducted measuring the response of *A. poculata* to increasing pCO$_2$ to mimic increasing CO$_2$ levels and ocean acidification [109]. While symbiont state appears to have minimal effect on calcification, with calcification lowering as pCO$_2$ increases forming carbonic acid in both conditions at 16˚C, female coral had 39% lower calcification than female control coral at 24˚C potentially due to energy expenditure necessary for spawning [109]. Additionally, the life cycle of the organism has been closed and successful spawning events have been induced in a laboratory environment and, should settlement trial be successful, could indicate cultivation of *A. poculata* increasing viability as a model organism [106, 107]. *A. poculata* can also be used as a bioindicator of microplastic pollution in local waters due to its robust rate of survival despite uptake and long-term retention of microplastics as non-nutritive prey or potential vectors for pathogenic infection [108]. While *A. poculata* preferentially ingested non-fouled microplastics rather than microplastics with a biofilm potentially indicating the ability to selectively filter potential contaminants, it also favored microplastics over typical nutritive food sources such as brine shrimp eggs [108]. Overall, *A. poculata* has been used to model the response of a temperate facultative coral to a variety of potential contributors to tropical coral stress and bleaching such as extreme temperature stress, microplastic consumption, and ocean acidification.
Currently, infection against *A. poculata* has not been observed in marine environments potentially due to the non-reef forming nature of the coral as well as the greater skeletal surface area, which could obscure disease with algal fouling. However, as *E. pallida*, another facultative Anthozoan, and multiple tropical scleractinian corals have been successfully infected with *V. coralliilyticus* [30, 32, 102], it is likely that *A. poculata* will be able to serve as a model for coral stress from *Vibrio* bacterial infection as well.

**Goals of this study**

The overall goal of this study was to examine the bacterial pathogenic potential of *V. coralliilyticus* RE22 against temperate coral species *A. poculata*. An emerging model organism, *A. poculata* could be beneficial in modeling coral disease progression typically associated with bleaching diseases such as White Syndromes (WS) [18] or Stony coral tissue loss disease (SCTLD) [30] while also minimizing environmental impact due to being a temperature non-reef forming scleractinian coral [47]. Additionally, while the role of *V. coralliilyticus* in tropical coral pathogenesis is partially understood, the role of many of its individual virulence factors remains unexplored necessitating further research to combat the spreading pathogen.

The first aim of this study was to establish a protocol for bacterial challenge of *A. poculata* to determine the novel coral’s susceptibility to infection. *A. poculata* samples and isolated cultures of its endosymbiont, *B. psygmophilum*, were obtained and challenged with *V. coralliilyticus* to observe comparable responses to infection as those observed in infected tropical coral samples. The effects of challenge on both
oral and endosymbiont samples were quantified by percent survival, while the effect on endosymbiont samples was additionally quantified by chlorophyll a concentrations normalized from initial experimental counts.

The second aim of this study was determining differential responses to infection in *A. poculata* samples exhibiting differing endosymbiont states. One of the primary hypotheses of bacteria-induced tropical coral bleaching is pathogenic targeting of the intracellular endosymbiont. Due to the nature of *A. poculata* possessing a facultative relationship with its endosymbiont, one major advantage of the established model system is the ability to potentially examine the impact of bacterial infection independent of the symbiont state. Survival rates among aposymbiotic coral and symbiotic coral were compared post-infection to determine if endosymbiont state had a significant impact on susceptibility to infection.

The third aim of this study was to characterize the involvement of the *V. coralliilyticus* T6SS in coral and dinoflagellate pathogenesis. Knockout mutants of several essential components of the T6SS were constructed and confirmed using PCR and protease testing to confirm expected phenotype. Mutants were then tested in both coral and endosymbiont challenge systems to examine any attenuation in virulence associated with removed or impeded T6S functionality. Focus was on the components of the T6SS essential for translocation of effectors and pathogenic potential rather than assembly and anchoring to the inner membrane.

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Title: *Vibrio coralliilyticus* RE22 Type VI Secretion Systems Contribute to Temperate Coral Lysis and Endosymbiont Death

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ABSTRACT:

*Astrangia poculata* is a facultatively symbiotic temperate coral that is being explored as a model system for studying the physiology and ecology of cnidarian-microbe symbiosis. *Vibrio coralliilyticus* is a known causative agent of a class of coral diseases called “white syndromes” that result in bleaching in tropical coral species. It is an effective pathogen due to a wide array of virulence factors including two Type 6 Secretion Systems (T6SS1 and T6SS2). In this study, we investigated the pathogenic potential of *V. coralliilyticus* RE22Sm in *A. poculata* and in cultures of its endosymbiont, *Breviolum psygmophilum*. To independently gauge the antagonistic effects of each of the two T6SSs, allelic exchange mutants of the *hcp* genes were utilized. In the *A. poculata* challenge, *V. coralliilyticus* RE22Sm caused tissue lysis in coral samples. Both aposymbiotic and symbiotic corals were susceptible to infection, and aposymbiotic corals displayed tissue lysis faster than symbiotic corals. Mutation of the T6SS1 *hcp1* gene resulted in the greatest attenuation of virulence in the coral system. Coral survival increased from 12% in the wild-type challenged samples, to 60% for those challenged with Δhcp1. Virulence was also attenuated in corals challenged with Δhcp2, with 30% survival. Similarly, *B. psygmophilum* challenged with the Δhcp1 strain had a 20% increase in both cell survival and chlorophyll a content, compared to cultures exposed to wild type *V. coralliilyticus* RE22Sm. An hcp1 hcp2 double mutant resulted in minor attenuation of virulence in both coral and endosymbiont trials. Revertant strains with restored wild-type copies of the *hcp* genes displayed comparable virulence to wild-type *V. coralliilyticus* RE22Sm. These results
suggest that Type 6 Secretion is a major component of pathogenesis against the temperate coral *A. poculata* and *B. psygmophilum*. Heightened susceptibility of aposymbiotic coral samples to bacterial challenge is consistent with literature that suggests symbiotic *A. poculata* is more effective than aposymbiotic colonies at mitigating of environmental stress. The data are consistent with bacterial challenges in an oyster larval system, which indicate that T6SS1 is primarily involved in eukaryotic antagonism.

**Importance:** The rapid decline of coral reefs is a grave threat to ocean biodiversity as well as tourism and the fisheries industry. Reef-forming coral populations have declined by at least 50% across species since 1995 but this decline is especially pronounced in branching and table corals. Understanding the primary pathogens and mechanisms of virulence involved in bacterial antagonism of coral is essential to design potential protective management tools such as microbial colonization of coral by probionts. This study additionally aims to establish *A. poculata* as a model organism for testing bacteria-induced coral bleaching and pathogenesis in a temperate host. Additional benefits to the *A. poculata* coral system include its widespread availability, which enables use and collection without further disrupting the declining tropical coral ecosystem and that it is facultatively symbiotic with *B. psygmophilum*.

**INTRODUCTION:**
The collapse of reef environments due to coral tissue bleaching and lysis has profound economic impacts on tourism and fishery industries while also contributing to a massive decline in biodiversity with the loss of reef associated species [1-2]. A conservative estimate of reef associated species would be 2,594,000 unique organisms [3] with Caribbean reef biodiversity alone accounting for ~8-9% of potential reef species. Coral populations have declined by approximately 50% since 1995 due to a wide array of factors including ocean acidification and temperature increase [15, 16], but another primary cause includes bacterial pathogenesis. One of the primary bacterial threats to reef health is a class of coral diseases called White Syndromes (WS) [17]. While multiple causative agents of WS have been explored including Vibrio shiloi and Vibrio mediterranei [4, 5, 8], other Vibrio spp. may be involved in pathogenesis. For example, several bacterial isolates from diseased Montipora aequituberculata and other Indo-Pacific reef corals showed 98% genomic homology with Vibrio coralliilyticus [6, 7]. Moreover, investigations into causative agents of a tropical coral bleaching disease known as “white syndrome” revealed that coral species including Pocillopora damicornis, Montipora capitata, and Acropora cytherea were increasingly affected by various strains of V. coralliilyticus [9-11].

The progression of coral bleaching in infected corals occurs rapidly causing noticeable tissue lysis and bleaching in tropical corals in as little as 5-10 days [9]. Proposed blanket treatments of diseased tropical coral such as antibiotics use does not necessarily prevent reinfection [12] as it can disrupt the innate coral microbiome and create greater opportunities for potentially opportunistic pathogens like V.
coralliilyticus [14]. As a result, it has become imperative to increase our understanding of coral pathogenesis to develop long-term management measures.

The strain used in this study, V. coralliilyticus RE22, is a broad-spectrum pathogen commonly associated with vibriosis in oyster species Crassostrea gigas and C. virginica [18-20] but has also been shown to cause bleaching in tropical coral M. capitata [21]. Vibrio coralliilyticus RE22Sm is a Gram-negative motile marine bacterium with a wide array of virulence factors including a Type I Secretion System (T1SS), a Type II Secretion System (T2SS), a Type III Secretion System (T3SS), two Type VI Secretion Systems (T6SS) and several extracellular zinc-metalloproteases [7, 20]. Little is known about the direct mechanistic effect of V. coralliilyticus strains on tropical corals and whether or not pathogenesis is toxin-mediated, contact-mediated, or both. It has previously been hypothesized that the zinc-metalloprotease produced by V. coralliilyticus is the most likely causative agent of endosymbiont death in tropical coral [6]; however, it has been shown that a knockout mutation of the primary protease gene, vcpA, has no significant effect on virulence against a clade C1 Symbiodinium culture isolated from Acropora tenuis [13]. Additionally, loss of vcpA leads to the up-regulation of other virulence factors including components of the T6SS [13]. This is a strong indicator of the involvement of additional virulence factors like type 6 secretion (T6S) in the progression of disease in A. tenuis.

The T6SS of V. coralliilyticus resembles an inverted T4 bacteriophage-like nanomachine and is primarily purposed for the cell contact mediated translocation of effector molecules [22, 23] but may have a secondary role in quorum sensing [24]. The system is composed of several distinct structures assembled from thirteen
conserved proteins including a baseplate complex anchored to the inner membrane, a hollow needle-like structure composed of hexomeric hemolysin co-regulated protein (Hcp), a VipA/B contractile sheath surrounding the Hcp barrel, and a valine-glycine repeat protein (VgrG) which has a hardened proline-alanine-alanine-arginine (PAAR) repeat designed to puncture eukaryotic or prokaryotic prey cell membranes [25]. Effectors either decorate the PAAR motif and are released upon activation of the mechanism or are translocated via the Hcp needle structure [26, 27]. The *V. coralliilyticus* RE22 T6SS on chromosome 1 (T6SS1) has been suggested to have divergent but overlapping function with the T6SS on chromosome 2 (T6SS2). T6SS1 has been shown to be vital for pathogenesis in eukaryotic oyster models compared to the primarily anti-bacterial activity associated with T6SS2 [20]. In this report, we focus on the involvement of the T6SS puncturing device in antagonism against a potential coral prey organism and its endosymbiont.

The coral species used in this study is the emerging model organism temperate coral *Astrangia poculata*. This coral has an ability to tolerate a wide range of temperatures and a facultative relationship with its dinoflagellate endosymbiont, *Breviolum psygmophilum* [28-30]. Differences in *A. poculata* stress responses between symbiont states are already well characterized with densely colonized (symbiotic) fragments recovering from stress events and wounding at a greater rate than sparsely colonized (aposymbiotic) fragments [31]. The symbiont state in *A. poculata* also has an impact on innate immune gene expression. Suppression of genes associated with the innate immune response has been observed in symbiotic samples when compared to aposymbiotic samples [32]. Additionally, the composition of the *A. poculata*
mucosal microbiome has been shown to vary negligibly between coral fragments with differing symbiont states suggesting greater consistency in comparative experimental trials [33]. Establishing *A. poculata* as a model for tropical coral pathogenesis can minimize the impact of research on the tenuous state of reef environments while also providing opportunities to study host-symbiont interactions and their role in pathogenesis. The distinct differences between colonies will allow investigation into the impacts of bacterial challenge on coral health independent of the symbiont state, further elucidating the role of the endosymbiont in the progression of *Vibrio* associated coral disease.

**RESULTS:**

*V. coralliilyticus* causes differing rates of tissue lysis in *A. poculata* by symbiont state

Killing potential of *V. coralliilyticus* RE22Sm against *A. poculata* was first examined by exposing isolated symbiotic coral samples to *V. coralliilyticus* at cell densities ranging from $1 \times 10^5$ to $1 \times 10^8$ CFU/mL. Coral fragments exposed to cell densities below $1 \times 10^7$ CFU/mL displayed 100% survival identical to no treatment control conditions (Fig. 1c-d). At cell densities of $1 \times 10^7$ (Fig. 1e) and $1 \times 10^8$ CFU/mL (Fig. 1f) coral fragment survival was reduced to 80% and 10% respectively. This would indicate an effective LD50 of approximately $5 \times 10^7$ CFU/mL for *V. coralliilyticus* RE22 against symbiotic *A. poculata*. In diseased coral fragments, tissue lysis was observed within 5-10 days of primary exposure to *V. coralliilyticus* RE22Sm with
concurrent bleaching rather than bleaching before tissue decay (Fig. 1b). Progression of ill-health in coral began with a decrease in polyp activity coupled with an overproduction of coral mucus which became clouded in the water column. V. coralliilyticus RE22Sm was present in high densities within clouded mucus samples. Within 2-4 days of preliminary pathogenesis, coral tissue began to develop a pale color and dissociate from the skeleton upon disturbance of the water column. This indicated that coral bleaching and tissue lysis of the polyps were happening concurrently rather than sequentially. No-treatment control coral samples (Fig. 1a) exhibited high activity levels including polyp extension and responsiveness to feeding, consistently scoring 4-6 on the activity scale, that remained stable over the 20 day experiment. In contrast, A. poculata samples exposed to the pathogen became inactive within 5 days and largely remained inactive for the duration of the experiment regardless of their survival.

In experiments comparing the responses of aposymbiotic and densely symbiotic coral fragments to infection with a high dosage (1×10⁸ CFU/ml) of V. coralliilyticus, a difference in the rate of death was observed. Within the first 5 days of the experiment 79.4% of all symbiotic coral samples were still alive, albeit mostly quiescent, compared to a survival of 37.8% in treated aposymbiotic samples (Fig. 2). This difference was found to be significant (P < 0.02) only at the 5-day measure as survival of densely symbiotic fragments dropped by day 10 to 47.6%, which was not significantly different from the aposymbiotic survival rate of 31.1%. This trend continued for the remainder of the experiment with both aposymbiotic and symbiotic samples dying at comparable rates after day 10. All measures for both aposymbiotic
and densely symbiotic samples were significantly different from the negative control after 0 days.

**T6SS mutations attenuate virulence of *V. corallilyticus* against coral host *A. poculata***

To assess the contributions of the T6SSs towards the coral pathogenicity of *V. corallilyticus* RE22Sm, coral challenge assays were performed using RE22Sm bacterial mutants deficient in major structural components of the T6SS. Coral treated with wild-type RE22Sm declined in health over 20 days until only 15.6% of fragments survived, which was significantly different (*P* < 0.001) from the 94.9% survival observed in the no treatment control (Fig. S1). Increased survival was observed in coral fragments treated with bacterial mutants of the *hcp1* and *hcp2* T6S components. Survival increased significantly (*P* < 0.05) from 15.6% in the wild-type treated samples to 66.7% in the coral samples exposed to the Δ*hcp1* strain (Fig. 3e).

Bleaching but not tissue lysis was observed in 2 samples treated with Δ*hcp1* within 10 days, which were counted among surviving fragments (Fig. 3b). Bleached coral fragments were only observed in Δ*hcp1* treated samples. While virulence against coral was moderately attenuated in the Δ*hcp2* strain with survival increasing to 40%, the difference was not found to be significant to wild type (Fig. 3g). Treatment of coral samples with revertant strains of either the Δ*hcp1* and Δ*hcp2* mutant resulted in restored levels of virulence and were not significantly different from the wild-type treated positive control samples (Fig. 3f, 3h). Double mutant strains of T6SS genes *hcp1* and *hcp2* were also used to test whether or not activity from both T6SS1 and
T6SS2 contributed towards an additive effect in regards to virulence. Surprisingly, inactivation of both *hcp1* and *hcp2* in double mutant strain RE22 Δ*hcp2* pDM5::*hcp1* resulted in increased virulence with reduced survival of coral fragments treated with this strain compared to either of the single mutant strains with a 0% survival observed after 20 days (Fig. 3j). A double mutant strain for *vgrG1* and *vgrG2* was also used and, comparably, survival of treated coral samples reached only 20% (Fig. 3i). Inactivity of coral polyps was observed consistently in all samples treated with RE22 regardless of strain (Fig. 3d). Treated polyps were deeply retracted into the coral skeleton with minimal coenosarc visible and lacked response to stimuli.

**B. psygmophilum is impacted by exposure to *V. coralliilyticus* RE22**

The experiments described above showed coral bleaching prior to coral death, suggesting the possibility that *V. coralliilyticus* RE22Sm could directly affect the *B. psygmophilum* endosymbiont of *A. pociulata*. To examine this, a culture of *B. psygmophilum* was challenged with *V. coralliilyticus* in a direct exposure assay at a multiplicity of infection (MOI) = 25 for 144 h. This was to keep the RE22Sm dosage of $1 \times 10^8$ consistent across both coral and endosymbiont challenge experiments. Across all *V. coralliilyticus* strains tested, *B. psygmophilum* demonstrated sensitivity to bacterial challenge with RE22 (Fig. 4). A 14.5% increase (114.5% of T = 0 *B. psygmophilum* density of $4 \times 10^6$ cell/ml) in cell density was observed in the no treatment control (treated with 3% sterile ASW) samples after 144 h, which was significantly different ($P < 0.005$) from the decline in cell density of 59.3% observed in samples treated with RE22Sm. The drop from $3.05 \times 10^6$ cells/mL at T = 0 to $1.24 \times$
$10^6$ cells/mL at $T = 144$ h after treatment with $1 \times 10^8$ CFU/mL \textit{V. coralliilyticus} RE22Sm was 59.3\% so the dosage was kept consistent for subsequent experiments as an approximate LD50. \textit{V. coralliilyticus} RE22Sm was capable of inducing a reduction in membrane stability and discoloration in treated \textit{B. psygmophilum} (Fig. 5). Additionally the presence of regions of high auto-fluorescence, hypothesized to be chloroplasts, decreased in quantity and fluorescent intensity by 96 hours after exposure to RE22Sm.

Several previously described bacterial mutants deficient in production of the Hcp and VgrG proteins of T6SS1 and T6SS2 were also tested for their effects on both \textit{B. psygmophilum} cell density and chlorophyll a levels. When \textit{B. psygmophilum} cells were challenged with RE22Sm \textit{Δhcp1} mutant cells for 144 h, the \textit{B. psygmophilum} cell density declined by ~31.8\% (Fig. 4) ($T=0$ h: $2.88 \times 10^6$ cells/mL to $T=144$ h: $1.97 \times 10^6$ cells/mL). This result was significantly different from both the cell density of the no treatment control ($P < 0.05$) and the cell density of the RE22Sm WT- treated culture ($P < 0.05$). \textit{B. psygmophilum} cultures treated with the RE22Sm \textit{Δhcp2} mutant exhibited a decline in cell density of 45.1\% (Fig. 4) ($T=0$ h: $3.33 \times 10^6$ cells/mL to $T=144$ h: $1.83 \times 10^6$ cells/mL), which is significantly different from the no treatment control ($P < 0.05$) but not the RE22Sm. Decline in cell density was less in samples treated with the \textit{Δhcp2 pDM5::hcp1} double mutant than in samples treated with either \textit{hcp} single mutant. Cell density decreased from $2.98 \times 10^6$ cells/mL to $2.09 \times 10^6$ cells/mL after 144 hours, which constitutes a 22.4\% decline. Cell density after 144 hours in samples treated with the RE22Sm \textit{ΔvgrG1 pDM5::vgrG2} declined by 35.3\%.
The hcp and vgrG double mutants were significantly different from both the no treatment control and the RE22Sm treated samples ($P < 0.05$).

Chlorophyll was also extracted and quantified for each sample and time point, normalized by initial $B. psygmophilum$ cell density for each condition and represented as pg/cell (Fig. 6). Comparable to the initial $B. psygmophilum$ enumeration experiments, chlorophyll a increased by 22.65% in no treatment control samples and declined from 1.68 pg/cell to 1.11 pg/cell (34.4%) in $B. psygmophilum$ cultures treated with RE22Sm at $T = 144$ h. This difference was also found to be significant ($P < 0.001$). Additionally, chlorophyll a levels declined by only 11.2% at 144 h in $\Delta hcp1$ treated samples. This was only significantly different from the RE22Sm wild type treated $B. psygmophilum$ chlorophyll a levels ($P < 0.05$). $B. psygmophilum$ cultures treated with the RE22Sm $\Delta hcp2$ mutant exhibited a decline of 10.2% in chlorophyll a per cell. However, despite observing results comparable to those derived from the $\Delta hcp1$ treated samples, greater variance was observed in $\Delta hcp2$ treated samples resulting in a significant difference against only the no treatment control cell density ($P < 0.05$). Treatment with the RE22Sm $\Delta hcp2$ pDM5::hcp1 double mutant strain resulted in a decline of 20.50% in chlorophyll a per cell which was greater than the 11.9% and 13.2% declines observed in the single $\Delta hcp1$ and $\Delta hcp2$ mutants, respectively. While the change in chlorophyll a concentration in $B. psygmophilum$ treated with hcp double mutant was significantly different from the change seen in $B. psygmophilum$ treated with RE22Sm ($P < 0.05$), the change in chlorophyll a concentration was not significantly different from those observed in either the no treatment control or the single mutants. Chlorophyll a per cell was reduced from 1.73
pg/cell to 1.22 pg/cell (29. 9% decline) in samples treated with RE22Sm AvgrGl pDM5::vgrG2 indicating higher virulence than any tested bacterial mutant of the hcp component.

In both chlorophyll a measures and cell density measures, B. psygmophilum cells treated with either V. coralliilyticus RE22Sm hcp1 or hcp2 revertants were significantly different from the no treatment control cells ($P < 0.05$), but not from the wild type RE22Sm-treated samples indicating restored virulence. As a whole, these results suggest that V. coralliilyticus is an effective pathogen against both A. poculata and its endosymbiont B. psygmophilum and that T6S contributes to the anti-coral pathogenic activity of V. coralliilyticus RE22Sm.

**DISCUSSION:**

The broad-spectrum antagonistic activity of V. coralliilyticus makes it an effective primary and opportunistic pathogen against a large variety of scleractinian coral species [9, 10]. While current research has described potential virulence mechanisms active against tropical coral species through disruption of the standard coral-dinoflagellate symbiosis [13, 46], the involvement of T6S is largely unexplored. Prior work has demonstrated that exposure to the zinc-metalloproteases produced by V. coralliilyticus has a pronounced effect on the survival of the endosymbiont within coral tissues [46]; however, direct exposure assays to the supernatant of a VcpA deficient mutant V. coralliilyticus produced negligible differences in photoinactivation [13]. With the data, we demonstrate that V. coralliilyticus is capable of
infecting A. poculata at high doses and that aposymbiotic samples are more susceptible to infection than symbiotic samples. Additionally, we found that T6S is involved in pathogenesis against both A. poculata and its endosymbiont B. psygmophilum, with the V. coralliilyticus RE22 T6SS1 having greater involvement in pathogenesis than T6SS2. Our results have significant implications by: 1) expanding potential host systems for modeling coral bacterial infection to include the non-endangered, facultative, temperate coral A. poculata and 2) characterizing the impact of a largely unexplored virulence mechanism in coral-endosymbiont pathogenesis.

The data presented provide evidence that A. poculata and its endosymbiont are both vulnerable to the pathogenic activity of marine bacteria. Dosage sensitivity ranging from $1 \times 10^7$ and $1 \times 10^8$ CFU/mL (Fig. 1e) is consistent with cell densities used in tropical coral challenges across the literature [9, 40]. However, the cell density necessary for RE22Sm to exhibit vibriosis in oyster larval models is $1 \times 10^4$ CFU/mL, $10^{4}$-fold of the infectious dose in coral systems [38]. A proposed explanation is that despite the involvement of several virulence factors in cross-species pathogenesis, infections in coral systems require a greater virulence repertoire than infections in the oyster system [39]. However, temperature and cell density also influence the production of virulence factors associated with both pathogenesis in larval C. gigas and in coral. The role of temperature in production of virulence factors by Vibrio spp. was examined in Ben-Haim et al. [9] and suggested that the extracellular zinc-metalloprotease production was greatly influenced by growth temperature of the organism. V. coralliilyticus also relies on quorum-sensing for the regulation of a large number of its virulence factors, and Kimes et al. [41] demonstrate that quorum-sensing
can also be regulated by growth temperature. Since our experimental temperatures were set to 25-26°C, slightly below the optimal growth temperature of 27°C for the bacterial pathogen, a higher initial quorum may be necessary to establish virulence; however, the effect on growth is likely minimal as *V. coralliilyticus* is still pathogenic in the range of 24 – 28°C [9]. As virulence in *V. coralliilyticus* is tied to cell density due to quorum sensing mechanisms up-regulating several primary virulence factors, a higher dosage would be necessary to achieve optimal infectious potential. An additional factor that could contribute to the difference in susceptibility between adult *A. poculata* and larval *C. virginica* is the maturity of the host systems as oyster larvae have an immature immune response compared to adult oyster, which are resistant to infection, or coral [59].

The observed response to bacterial challenge in the *A. poculata* host system was also seemingly dependent not only on dosage of the pathogen but also the density of the endosymbiont population associated with the coral tissue. Coral fragments that exhibited an initially dense *B. psygmophilum* population were more resistant to infection than aposymbiotic samples in the beginning stages of coral infection. This is counterintuitive considering the observed immunomodulation used to selectively suppress genes of the innate immune system to accommodate endosymbiont in symbiotic samples compared to aposymbiotic samples [32]. Additionally, not only does *B. psygmophilum* have a greater sensitivity to temperature than its coral host [32], but also the endosymbiont population is thought to be the primary target of anti-coral *Vibrio* pathogenesis [42]. One potential explanation is the increase in metabolite availability in densely symbiotic coral that allows them to recover from initial
bacterial antagonism. *A. poculata* with high endosymbiont density have been shown to recover from wounding at a much greater rate than aposymbiotic samples [31]. Considering significantly greater photochemical efficiency was observed in symbiotic corals compared to aposymbiotic corals [43] and that consistent consumption of heterotrophic food sources can increase the rate of photosynthesis in tropical coral *Stylopora pistilla* [44], it is likely that symbiotic *A. poculata* fragment have greater energy availability than aposymbiotic fragments. While suppression of the innate immune system is a primary result of facultative Anthozoan host-symbiont association, caspase-family inducers of apoptosis are up-regulated in aposymbiotic samples of facultative model anemone *Exaiptasia pallida* after infection with *V. coralliilyticus* indicating a greater defensive response to infection but potentially a greater vulnerability to pathogen induced cell death as well [45]. Interactions between *A. poculata* and its endosymbiont may negatively affect survival during prolonged infection due to suppression of the immune system; however, our data suggest that during primary exposure to pathogenic bacteria, aposymbiotic samples are more likely to exhibit signs of infection.

Our data also indicate that the two T6SS present in the RE22Sm genome contribute heavily to eukaryotic antagonism in the coral model system. In both *A. poculata* (Fig. 3h) and *B. psygmophilum* bacterial challenge experiments (Figs. 4 and 5), RE22 mutants deficient for production of the *hcp* component of T6SS1 exhibited significantly attenuated virulence compared to the wild-type. Consistent with the observations of Schuttert et al [20], deletion mutations of *hcp1* or *vgrG1* of the T6SS1 have significantly greater effects on the virulence of RE22Sm against eukaryotic
oyster larvae than do deletion mutations of hcp2 or vgrG2 of the T6SS2. While the Δhcp2 knockout mutant demonstrated minimal attenuation and had a non-significant impact on survival in challenges against adult A. poculata and isolated cultures of B. psygmophilum, increased survival of the corals and endosymbionts was still observed compared to the samples treated with RE22Sm. Some overlapping but still specialized functionality is a feature of other organisms with multiple T6SSs such as P. aeruginosa, which uses the sigma factor RpoN (σ54) to induce activity of one T6SS while suppressing the other [49]. It was surprising that both double mutant strains for the Hcp (hcp1 hcp2) and VgrG (vgrG1 vgrG2) components of the two T6SSs were more virulent in both coral and endosymbiont systems when compared to the tested single mutants. This could be due to some compensatory up-regulation of alternative virulence factors [13] or potential alternative interactions of the secretion apparatus resulting in an altered virulence profile when two or more genes are rendered non-functional [50]. Additionally, coral fragments challenged with RE22 displayed an inactive quiescence phenotype consistent with the phenotype described in A. poculata samples immersed in cold-water conditions of 6-8°C which is typical of a general stress response [47]. This could be due to increased stress from bacterial infection causing a change in activity level as has been previously observed in tropical corals affected by SCTLD [12]. V. coralliilyticus is highly motile and attracted to coral mucus [55] so a retraction of the polyps is a well characterized defense to minimize production of chemo-attractants and intake of pathogenic bacterial from the water column [54].
It is not known whether or not Vibrio-induced coral pathogenesis is contact mediated or toxin mediated, but our data suggest that the T6SS, a virulence factor that relies on direct contact to translocate toxins, is a primary component in coral pathogenesis. It is known that *V. coralliilyticus* is capable of invading tropical coral tissue providing the opportunity for cell-to-cell contact and subsequent activation of contact mediated virulence factors [9]. Work in other eukaryotic models reveal the ability for components and effectors of the T6SS to induce apoptosis or autophagy in prey cells [51, 52], but as this work was done primarily in mammalian models its applications to marine pathogenesis may be limited. Additionally, little is known about the effectors translocated by T6S in *V. coralliilyticus*. While the up-regulation of apoptotic mechanisms in response to *V. coralliilyticus* infection has been observed in tropical corals [40] and facultative anthozoans [45], it is unknown if RE22 is capable of exploiting or regulating apoptosis to exacerbate infection. An alternative could be that T6S plays a broad role in pathogenesis due to its wide array of secreted effector proteins rather than a single specialized function. By increasing invasion via adhesion, increasing intracellular viability through innate immune regulation, inducing disruptions to the actin cytoskeleton, the anti-eukaryotic effectors associated with T6S [26] could induce a stress response of sufficient magnitude to lead to intact coral cell expulsion that occurs under temperature stress [56]. Among the many anti-eukaryotic effectors translocated through T6S are those that allow for the evasion of eukaryote innate immune response potentially making the difference between symbiotic and aposymbiotic regulation of immunity negligible [48]. *V. shiloi* adhesion and penetration of tropical coral tissue has been previously characterized [53], but *V.*
coralliilyticus adhesion and accumulation around coral tissue occurs minimally and largely around the polyp pharynx rather than the coenosarc suggesting intake through the coral gastrovascular cavity [54]. However, it is still possible that V. coralliilyticus is still able to penetrate coral tissue and become intracellular potentially providing the opportunity for the pathogen to disrupt the integrity of the symbiosome.

B. psygmophilum survival was reduced due to exposure to V. coralliilyticus, but pathogenic activity against B. psygmophilum was attenuated in all of the V. coralliilyticus T6SS mutants. Despite T6SS mutants producing a measurable increase in endosymbiont survival, the involvement of the RE22Sm zinc-metalloproteases is unexplored in our system and would need to be tested in further research to assess which virulence factor has a more pronounced impact on survival. Both cell density and chlorophyll a content declined after bacterial challenge; however, chlorophyll a declined at a slower rate than cell density suggesting that chlorophyll a is potentially a delayed measure of cell survival due to natural degradation [57]. The pigment itself would be unaffected by pathogen exposure and, therefore, more established measures of endosymbiont health such as photochemical efficiency should be examined to support the data presented.

While great strides have been made in the progression of coral research within the last decade, there are still numerous gaps in the literature regarding exact mechanisms behind bacterial-induced coral tissue lysis and collapse of the host-symbiont interaction. Elucidating the role of the V. coralliilyticus T6SS in coral virulence within an emerging model organism will help to develop potential protective measures to reduce the further degradation of already vulnerable reef environments.
METHODS:

**Bacterial strains, plasmids and growth conditions:** *V. coralliilyticus* RE22 strains (Table 1) were cultured in yeast peptone broth plus 3% Instant Ocean© sea salt (mYP30), supplemented with the appropriate antibiotic(s) and allowed to incubate in a shaking water bath (200 RPM) at 27°C. *Escherichia coli* SM10 strains (Table 1) were cultured in LB20 [58] supplemented with the appropriate antibiotic and allowed to incubate in a shaking 37°C dry incubator (200 RPM). Before experimental use, overnight cultures of *V. coralliilyticus* were centrifuged at 8000 × g for 10 minutes at 4°C in order to collect cells for experimental use. Cultures were washed twice and re-suspended in either sterile Nine Salt Solution (NSS) or 3% sterile Artificial Sea Water (ASW) depending on the experiment. Antibiotics were used at the following concentrations: streptomycin, 200 µg/mL (Sm200); chloramphenicol, 5 µg/mL (Cm5); kanamycin, 50 µg/mL (Km50) for *V. coralliilyticus* in liquid, and solid, media and chloramphenicol, 20 µg/mL (Cm20) for *E. coli* in liquid and solid media. Agar plates were prepared using Difco Bacto© agar at 1.6%.

**Merodiploid mutagenesis:** Construction of allelic exchange mutants adheres to protocols previously described by Schuttert et al. [20]. Briefly, this study utilized a pDM4 plasmid modified with a kanamycin resistance gene (Kmr), pDM5, and linearized at a SacI restriction site within the multicloning region and constructed with overlapping 5’ and 3’ target gene fragments using the Gibson Assembly Reaction [34].
Competent *E. coli* cells were transformed via electroporation with the BioRad Gene Pulser II in a 2 mm cuvette (2.5 kV; 25 µF; 200 Ω) after addition of ligation mixture. The plasmid was then conjugated from *E. coli* into *V. coralliilyticus* RE22Sm [35]. Transconjugates were selected for on mYP30Sm\(^{200}\)Cm\(^5\) due to the chloramphenicol resistance conveyed by pDM5.

**Coral husbandry and tank conditions:** Samples of *A. poculata* were obtained from Fort Wetherill State Park (Jamestown, RI, USA) via manual fragmentation and transported in sea water before being placed in an aerated holding tank of 3% ASW at temperatures ranging from 13 – 20°C to mimic local seasonal water temperatures. A 50% water change was performed every 2 weeks to ensure continued coral health. Fragments were further divided into fragments no larger than 4 cm\(^2\) in size and allowed to recover for ten days before water temperatures were gradually increased by 0.5°C per day up to 25 - 26°C to acclimate samples to experimental conditions. Salinity and pH were checked every five days to confirm optimal water conditions. Fragments were fed with homogenized frozen brine shrimp every five days.

**Bacterial coral challenges:** Coral fragments were placed in individual aerated 1 L glass beakers containing 750 mL of sterile 3% ASW which sat in a water bath at 25 - 26°C to mimic summer conditions. Light conditions were set to 14:10 hour light:dark with 2 hours of low intensity light at the beginning and end of the 14-hour period. Each experimental condition had 5 samples in individual beakers. Samples were then fed with 1-3 mL of homogenized frozen brine shrimp depending on the number of
polyps and photographed. Negative control samples were treated with 37 mL of 3% ASW while experimental samples were treated with re-suspended cultures of corresponding *V. coralliilyticus* strains for a final cell density of $1 \times 10^8$ CFU/mL.

Coral fragments were fed every 5 days, tested for tissue lysis via gentle aspiration of water over retracted polyps, and imaged post feeding. 10% water changes were performed every 7 days. Coral polyp activity was measured and recorded every 2 days using the visual observation scale established by Burmester et al. [36]. Activity was measured on a scale of 0 – 6 with a 6 indicating full polyp activity and extension of tentacles and 0 representing fully retracted non-responsive polyps. Bacterial cell density was measured every 48 hours via serial dilution and spot plating onto Difco Thiosulfate-citrate-bile salts-sucrose agar (TCBS) supplemented with streptomycin, 200 µg/mL (Sm$^{200}$), to select for *Vibrio* species associated with experimental treatment.

**Bacterial endosymbiont challenges:** Samples of *B. psygmophilum* from *A. poculata* isolated at Roger Williams University were cultured in sterile F/2 media to a cell density of $2.5 – 5 \times 10^6$ before use in experimental trials. In 6-well plates, 5 mL of dinoflagellate culture were placed into each well and treated with 0.5 mL sterile 3% ASW for negative control conditions and 0.5 mL of appropriate *V. coralliilyticus* culture for that level of treatment re-suspended in sterile 3% ASW and diluted to $1 \times 10^9$ CFU/mL for a final dosage of $1 \times 10^8$ CFU/mL. Plates were incubated at 27°C without shaking in minimal light for 144 hours. Each treatment had 3 technical replicates. Cell enumeration was performed every 48 hours by collecting 0.6 mL of culture from the appropriate well and counting cells present in 4 1mm squares on a
Petroff-Hausser counting chamber. This was performed in duplicate per replicate. Total chlorophyll was extracted every 48 hours via centrifugation of 0.6 mL of culture per replicate at 4°C 2,408 × g for 10 minutes which was then re-suspended in 3% sterile ASW. Culture was then centrifuged at 4°C 13,870 × g for 30 seconds and re-suspended in 100% acetone and allowed to extract for 24 hours in the dark at 4°C. Absorbance of samples was measured via spectrophotometry in 1mL glass cuvettes at 750, 663, and 630 nm and used to calculate chlorophyll a and chlorophyll c2 content of the sample [37].

**Statistical analysis:** Two-tailed Student’s t tests assuming unequal variance were used for all statistical analyses for all experiments. Two – Way Analysis of Variance (ANOVA) were also used to determine significance for endosymbiont challenge data. P values of < 0.05 were considered to be statistically significant.

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**Table 1.** Bacterial strains and plasmids used in this study

| Strain                 | Description                                                                 | Resistance | Reference            |
|------------------------|-----------------------------------------------------------------------------|------------|----------------------|
| V. coralliilyticus      |                                                                             |            |                      |
| RE22                   | Wild-type isolate from oyster larvae                                        |            | Estes et al, 2004    |
| RE22Sm                 | Spontaneous Sm\(^r\) mutant of RE22                                         | Sm\(^r\)   | Zhao et al, 2016     |
| RE22 pDM5::hcp1        | Sm\(^r\) Cm\(^r\); insertional merodiploid mutation of \(hcp1\) using pDM5 | Sm\(^r\) Cm\(^r\) Km\(^r\) | This study           |
| RE22 pDM5::hcp2        | Sm\(^r\) Cm\(^r\); insertional merodiploid mutation of \(hcp2\) using pDM5 | Sm\(^r\) Cm\(^r\) Km\(^r\) | This study           |
| RE22 Δhcp1             | Sm\(^r\) Km\(^r\); Allelic exchange mutant deficient in \(hcp1\)           | Sm\(^r\) Km\(^r\) | Schuttert et al, 2021|
| RE22 Δhcp2             | Sm\(^r\) Km\(^r\); Allelic exchange mutant deficient in \(hcp2\)           | Sm\(^r\) Km\(^r\) | Schuttert et al, 2021|
| RE22 Δhcp2 pDM5::hcp1  | Sm\(^r\) Cm\(^r\); Allelic exchange mutation of \(hcp2\) and insertional merodiploid mutation of \(hcp1\) using pDM5 | Sm\(^r\) Cm\(^r\) Km\(^r\) | Schuttert et al, 2021|
| RE22 ΔvgrG1            | Sm\(^r\) Cm\(^r\); Allelic exchange mutant                                | Sm\(^r\) Cm\(^r\) | Schuttert et al, 2021|
| **pDM5::vgrG2** | Insertion of vgrG1 and insertional merodiploid mutation of vgrG2 using pDM5 | Km\(^r\) | 2021 |
|-----------------|--------------------------------------------------------------------------------|--------|-----|
| **RE22 \(\Delta hcp1\) revertant** | Sm\(^R\); In cis complementation from insertional deletion of hcp1 due to a 2\(^r\) recombination event | Sm\(^r\) Km\(^r\) | Schuttert et al, 2021 |
| **RE22 \(\Delta hcp2\) revertant** | Sm\(^R\); In cis complementation from insertional deletion of hcp2 due to a 2\(^r\) recombination event | Sm\(^r\) Km\(^r\) | Schuttert et al, 2021 |

**E. coli**

| Sm10 | Thi thr leu tonA lacY supE recA RP4-2 Tc::Mu::Km (\(\lambda\)) | Km\(^r\) | Simon et al, 1983 |
| CS01 | Sm10 harboring pDM5-hcp1 | Km\(^r\) Cm\(^r\) | This study |
| CS02 | Sm10 harboring pDM5-hcp2 | Km\(^r\) Cm\(^r\) | This study |

**Plasmids**

| pDM5 | Cm\(^r\) Km\(^r\); suicide vector with R6K origin and sacB | Cm\(^r\) Km\(^r\) | This study |

\(\Delta\) indicates allelic exchange deletion mutation

:: indicates insertional – merodiploid - mutant
Table 2. Primers used in this study

| Primer | Sequence (5’ to 3’, underlined sequences are engineered for Gibson Assembly sites in pDM5) Schuttert et al. [20]. | Description |
|--------|-------------------------------------------------------------------------------------------------|-------------|
| PmH37  | tgtggaatccggagagctCAATGTGAACAGACTATTCAAAC                                                    | For *hcp1* insertional mutation, 5’ forward |
| PmH38  | tgtgcaacacCGTAAAGGCACAGCAGAC                                                                 | For *hcp1* insertional mutation, 5’ reverse |
| PmH39  | tgctttacgGTGTTCACACATTTGAAG                                                                  | For *hcp1* insertional mutation, 3’ forward |
| PmH40  | gcctcggtataacctgagctCAGATCGTCTTTACATTG                                                        | For *hcp1* insertional mutation, 3’ reverse |
| Pmh41  | tgtggaatccggagagctCAGCAGTCGAAGTAACTTTC                                                        | For *hcp2* insertional mutation, 5’ |
| Pmh42 | `aacgacctgCGAATTTCTCTCTAACC` | For hcp insertional mutation, 5’ reverse |
|-------|-----------------------------|----------------------------------------|
| Pmh43 | `gaaagagtcGGTCAGCGTTTTGCCTAC` | For hcp2 insertional mutation, 3’ forward |
| Pmh44 | `gcatgcggtaacctgatGAAGGAGATCAACATGGCTTC` | For hcp2 insertional mutation, 3’ reverse |
**Figure 1.** Representative photographs of *A. poculata* fragments over time in the bacterial coral challenge system with A) no treatment control samples surviving and maintaining high activity to 20 days while B) samples treated with $1 \times 10^8$ CFU/mL *V. coralliilyticus* RE22Sm begin to display tissue lysis by day 10 of the experiment preceding complete tissue loss by day 20. C-F) Kaplan-Meir survival curve of *A. poculata* fragments exposed to different cell densities of *V. coralliilyticus* RE22Sm. C) The dashed line with open squares represents a dosage of $1 \times 10^5$ CFU/mL; D) the solid line with open triangles represents a dosage of $1 \times 10^6$ CFU/mL; E) the solid line with closed squares represents a dosage of $1 \times 10^7$ CFU/mL; F) the dashed line with closed triangles represents a dosage of $1 \times 10^8$ CFU/mL. For all curves the dotted line with open circles represents fragments treated with sterile 3% ASW. The data represents n=10 independent replicates for each treatment.

**Figure 2.** Survival of *A. poculata* during bacterial challenge using *V. coralliilyticus* RE22Sm at $1 \times 10^8$ CFU/mL. Percent survival by endosymbiont state is modeled to demonstrate differing susceptibility to infection based on presence or absence of high *B. psygmophilum* density in host tissue. Average of at least 3 biological replicates; represents n=5 replicates per treatment per experiment; error bars indicate ±1 SD. P < 0.05 = *

**Figure 3.** Survival of *A. poculata* 20 d after challenge with *V. coralliilyticus* RE22 wild type or mutant strains at $1 \times 10^8$ CFU/mL. A) Representative photo of coral before exposure to T6SS mutant Δhcp1 and B) the same fragment 10 days post-
inoculation demonstrating a bleached phenotype. C) Representative photo of coral before exposure to T6SS mutant Δhcp2 and D) the same fragment 10 days post-inoculation demonstrating reduced polyp activity. E-J) Kaplan-Meir survival curve of *A. poculata* fragments exposed to different bacterial mutants of *V. coralliilyticus* RE22. E) the dashed line with open triangles represents fragments exposed to mutant strain Δhcp1; F) the dashed line with closed diamonds represents fragments exposed to the in-cis revertant strain for Δhcp1; G) the solid line with open diamonds represents fragments exposed to mutant strain Δhcp2; H) the dashed line with closed circles represents fragments exposed to the in-cis revertant strain for Δhcp2; I) the solid line with closed triangles represents fragments exposed to double mutant strain ΔvgrG1 pDM5::vrgG2; J) the solid line with closed squares represents fragments exposed to double mutant strain Δhcp2 pDM5::hcp1. For all curves the dotted line with open circles represents fragments treated with sterile 3% artificial seawater and the dashed line with open squares represents fragments treated with 1 × 10^8 CFU/mL of RE22Sm. These data represent at least 10 replicates for each treatment.

**Figure 4.** *B. psygmophilum* cell survival during bacterial challenge (MOI = 25, 27°C, 144 h, no light) with *V. coralliilyticus* RE22Sm wild type and mutant strains. The data represent enumerations of *B. psygmophilum* density at T = 0 h and T = 144 h. Average of at least 3 biological replicates; error bars indicate ±1 SD; different letters indicate statistical differences among groups from pairwise comparisons. A – B: P < 0.05, A – C: P < 0.01, B – C: P < 0.05 (Two – way ANOVA P < 0.05)
**Figure 5.** Images of *B. psygmophilum* treated with either sterile seawater (Control) or $1 \times 10^8$ CFU/mL *V. coralliilyticus* (RE22Sm) taken with phase contrast microscopy and fluorescent microscopy to capture auto-fluorescence at 100× magnification. A) phase contrast images of no treatment control *B. psygmophilum*; B) fluorescent images of no treatment control *B. psygmophilum*; C) phase contrast images of RE22Sm treated *B. psygmophilum*; D) fluorescent image of RE22Sm treated *B. psygmophilum*. Fluorescent images were taken at 445nm, 525nm, and 605nm and merged to represent gross auto-fluorescence. Images were taken every 48 hours until *B. psygmophilum* cells had become malformed and no longer displayed a high degree of auto-fluorescence.

**Figure 6.** Changes *B. psygmophilum* chlorophyll a concentration during bacterial challenge (MOI = 25, 27°C, 144 h, no light) by *V. coralliilyticus* RE22 wild type and mutant strains. Chlorophyll a measurements were normalized to the initial T0 density of *B. psygmophilum* cells in 1 mL of media. Strains tested include RE22Sm (wild-type), and bacterial mutants Δhcp1, Δhcp2, ΔvgrG1 pDM5::vgrG2, Δhcp2 pDM5::hcp1, and revertant strains of Δhcp1 and Δhcp2. Average of at least 3 biological replicates; error bars indicate ±1 SD; different letters indicate statistical differences among groups from pairwise comparisons. A: P < 0.05, B: P < 0.05, C: P < 0.001 (Two – way ANOVA P < 0.05)
Figure 1. Representative photographs of A. poculata fragments over time in the bacterial coral challenge system with A) no treatment control samples surviving and maintaining high activity to 20 days while B) samples treated with $1 \times 10^8$ CFU/mL *V. coralliilyticus* RE22Sm beginning to display tissue lysis by day 10 of the experiment preceding complete tissue loss by day 20. C-F) Kaplan-Meir survival curve of A. poculata fragments exposed to different cell densities of *V. coralliilyticus* RE22Sm. C)
The dashed line with open squares represents a dosage of $1 \times 10^5$ CFU/mL; D) the solid line with open triangles represents a dosage of $1 \times 10^6$ CFU/mL; E) the solid line with closed squares represents a dosage of $1 \times 10^7$ CFU/mL; F) the dashed line with closed triangles represents a dosage of $1 \times 10^8$ CFU/mL. For all curves the dotted line with open circles represents fragments treated with sterile 3% ASW. The data represents n=10 independent replicates for each treatment.
Figure 2. Survival of *A. poculata* during bacterial challenge using *V. coralliilyticus* RE22Sm at $1 \times 10^8$ CFU/mL. Percent survival by endosymbiont state is modeled to demonstrate differing susceptibility to infection based on presence or absence of high *B. psygmophilum* density in host tissue. Average of at least 3 biological replicates; represents n=5 replicates per treatment per experiment; error bars indicate ±1 SD. P < 0.05 = *
Figure 3. Survival of *A. poculata* 20 d after challenge with *V. coralliilyticus* RE22 wild type or mutant strains at $1 \times 10^8$ CFU/mL. A) Representative photo of coral before exposure to T6SS mutant Δhep1 and B) the same fragment 10 days post-inoculation demonstrating a bleached phenotype. C) Representative photo of coral
before exposure to T6SS mutant Δhcp2 and D) the same fragment 10 days post-
inoculation demonstrating reduced polyp activity. E-J) Kaplan-Meir survival curve of
*A. poculata* fragments exposed to different bacterial mutants of *V. coralliilyticus* RE22.
E) the dashed line with open triangles represents fragments exposed to mutant strain
Δhcp1; F) the dashed line with closed diamonds represents fragments exposed to the
in-cis revertant strain for Δhcp1; G) the solid line with open diamonds represents
fragments exposed to mutant strain Δhcp2; H) the dashed line with closed circles
represents fragments exposed to the in-cis revertant strain for Δhcp2; I) the solid line
with closed triangles represents fragments exposed to double mutant strain ΔvgrG1
pDM5::vrgG2; J) the solid line with closed squares represents fragments exposed to
double mutant strain Δhcp2 pDM5::hcp1. For all curves the dotted line with open
circles represents fragments treated with sterile 3% artificial seawater and the dashed
line with open squares represents fragments treated with $1 \times 10^8$ CFU/mL of RE22Sm.
These data represent at least 10 replicates for each treatment.
Figure 4. *B. psygmophilum* cell survival during bacterial challenge (MOI = 25, 27°C, 144 h, no light) with *V. coralliilyticus* RE22Sm wild type and mutant strains. The data represent enumerations of *B. psygmophilum* density at T = 0 h and T = 144 h. Average of at least 3 biological replicates; error bars indicate ±1 SD; different letters indicate statistical differences among groups from pairwise comparisons. A – B: P < 0.05, A – C: P < 0.01, B – C: P < 0.05 (Two – way ANOVA P < 0.05)
Figure 5. Images of *B. psygmophilum* treated with either sterile seawater (Control) or $1 \times 10^8$ CFU/mL *V. coralliilyticus* (RE22Sm) taken with phase contrast microscopy and fluorescent microscopy to capture auto-fluorescence at 100× magnification. A) phase contrast images of no treatment control *B. psygmophilum*; B) fluorescent images of no treatment control *B. psygmophilum*; C) phase contrast images of RE22Sm treated *B. psygmophilum*; D) fluorescent image of RE22Sm treated *B. psygmophilum*. Fluorescent images were taken at 445nm, 525nm, and 605nm and merged to represent gross auto-fluorescence. Images were taken every 48 hours until *B. psygmophilum* cells had become malformed and no longer displayed a high degree of auto-fluorescence.
Figure 6. Changes *B. psygmophilum* chlorophyll a concentration during bacterial challenge (MOI = 25, 27°C, 144 h, no light) by *V. coralliilyticus* RE22 wild type and mutant strains. Chlorophyll a measurements were normalized to the initial T0 density of *B. psygmophilum* cells in 1 mL of media. Strains tested include RE22Sm (wild-type), and bacterial mutants Δhcp1, Δhcp2, ΔvgrG1 pDM5::vgrG2, Δhcp2 pDM5::hcp1, and revertant strains of Δhcp1 and Δhcp2. Average of at least 3 biological replicates; error bars indicate ±1 SD; different letters indicate statistical differences among groups from pairwise comparisons. A: P < 0.05, B: P < 0.05, C: P < 0.001 (Two – way ANOVA P < 0.05)
Supplemental Figure 1. Kaplan-Meir survival curve of *A. poculata* fragments exposed to *V. coralliilyticus* RE22Sm over 20 days. Data is an aggregate of all previous trials where a negative no treatment control and positive RE22Sm control were included. The dotted line with open circles represents fragments treated with sterile 3% artificial seawater and the dashed line with open squares represents fragments treated with $1 \times 10^8$ CFU/mL of RE22Sm. These data represent 45 replicates for each treatment.