Chemotaxis in *Vibrio coralliilyticus*: deciphering the behavioral dynamics of a coral pathogen

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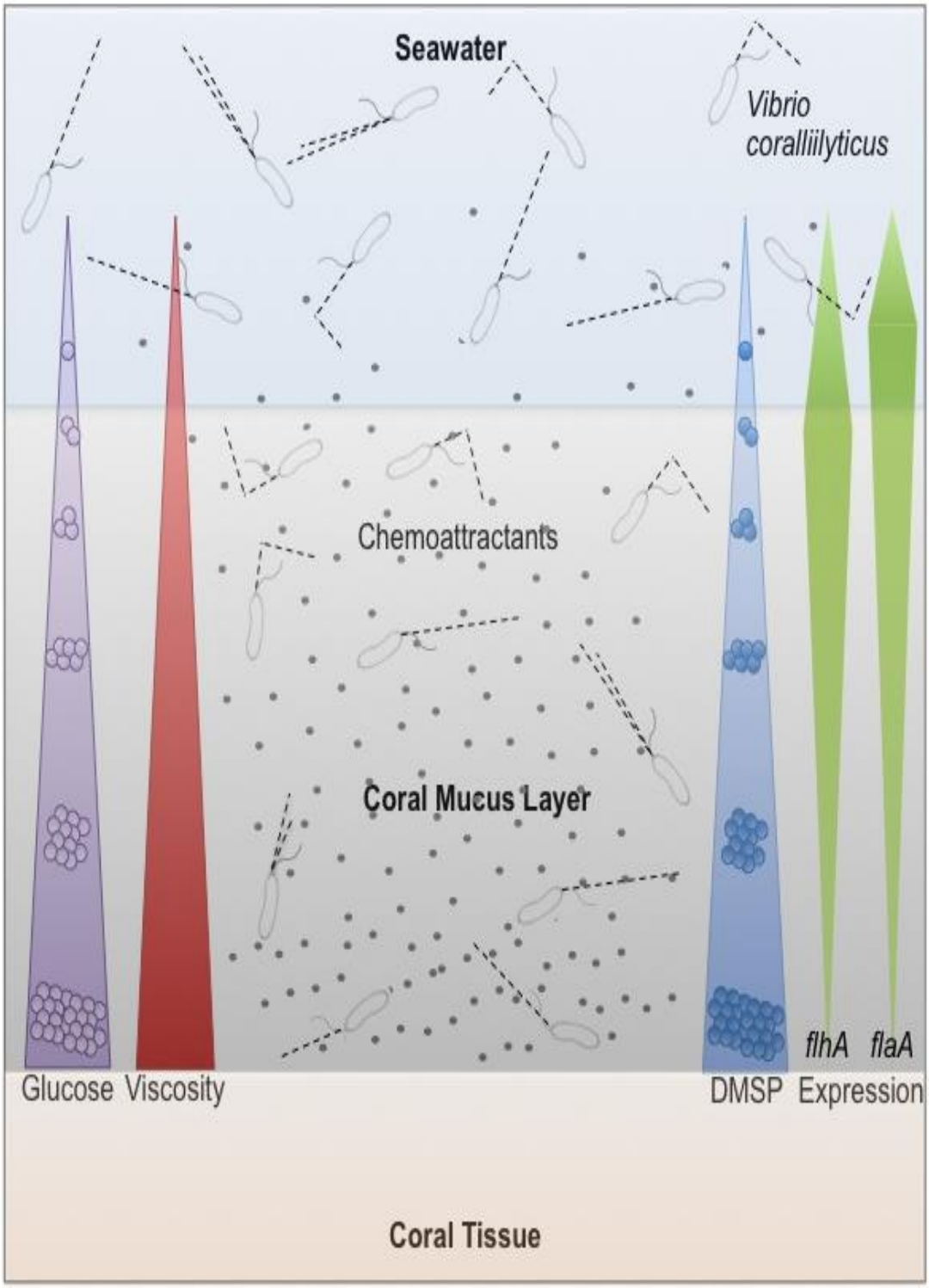
Seawater
Vibrio coralliilyticus
Chemoattractants
Coral Mucus Layer
flhA
flaA
Glucose
Viscosity
DMSP
Expression
Coral Tissue

Frontispiece Figure. The coral surface mucus layer microenvironment. **The coral surface mucus is full of gradients of chemoattractants that can influence the chemotactic patterns of *Vibrio coralliilyticus*. This microenvironment is discussed further in depth in chapter 6.**

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Summary

Microbes are linked to the fitness of coral and are integral players in the health of the entire coral reef ecosystem. Despite an increased awareness of the prevalence and influence microbes have on the decline of reef systems worldwide, there is limited information on the invasion mechanisms possessed by bacteria that initiate pathogenicity. Chemotactic motility and gene expression analysis is thought to yield the most informative and detailed view of bacterial capabilities, greatly increasing our ability to understand the methods, limitations and possibilities of infection. This thesis investigates the capabilities of *Vibrio coralliilyticus* to orientate through simulated regions of the natural coral holobiont. This determines the extent this pathogen remains motile irrespective of the fluctuations in the structural and chemical integrity of the coral mucus and surrounding seawater including viscosity variability and nutrient availability. The reoccurring theme of this thesis has been that *V. coralliilyticus's* chemotactic search paths altered following the viscosity and daily oxygen fluctuations present within the coral organism. Furthermore, this thesis demonstrated that *V. coralliilyticus's* 3-step flick search patterns showed a 14 times reduction when entering coral mucus regions presumably driven by the mechanical impediment viscosity has on the rotation of the flagellar motor. In addition, search patterns in the coral mucus exhibited an 11 times increase in energetic cost, in glucose molecules. This thesis provides detailed insight into the behavioral control *V. coralliilyticus* possess to manipulate and exploit nutrient and chemotactic regions by altering its search pattern run length to elevate its diffusivity values and for the first time, demonstrates that the gene expression of the *flaA* and *flhA* gene can be observed to correlate with chemotactic search pattern speed.

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Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Karina Margaret Winn

October 2015

Dedication

For my parents, Julie and Dennis Winn.

In loving memory of my late mother, Julie Winn, a kind and gentle woman and a wonderful and dedicated mother. I have missed you every single day since you left us.

Chapter 1

General Introduction

1.1 Coral reefs, a sea of diversity

Coral reefs represent biodiversity hotspots, supporting a myriad of marine organisms. Despite representing less than 1% of the ocean habitat, they provide a refuge for marine animals, supporting 32 out of the 34 recognised animal phyla compared to just 9 animal phyla found in the world's rainforests (Wilkinson 2002). Although numbers are conservative and can fluctuate widely, it has been suggested coral reef species may contain approximately 600,000 species worldwide (Knowlton 2001, Roberts *et al.* 2002). The productivity of coral reefs surpasses any other marine system with an estimated 7,000 g C m^2/yr (Reake-Kudla 1997, Roberts *et al.* 2002) making the study and preservation of this highly dynamic and fundamental ecosystem critical.

1.2 Coral reef microbiota

As with microbiota in all other ecosystems (Jernberg *et al.* 2007, Pérez *et al.* 2010, Zook 2010), the coral microbiota form the unseen essential building blocks for the coral reef ecosystem (Bourne and Munn 2005, Bourne *et al.* 2013, Krediet *et al.* 2013).

Beneficial symbionts

Corals house a group of diverse dinoflagellate algae symbionts of the genus *Symbiodinium* (Baker 2003). These dinoflagellates, commonly referred to as zooxanthellae, form a critical and essential photosynthetically active component of the coral reef ecosystem (Muscatine 1980). Up to 95% of the energy requirements of the coral host can be provided by these dinoflagellates (Muscatine 1990), and their loss through stress during coral bleaching events can be linked to the mortality of the

coral hosts (Baker 2003, Goulet 2006). In return, corals provide a protective home from predation and a habitat that is rich in inorganic nutrients (Muscatine and Porter 1977). These symbionts have been identified in other marine invertebrates including anemones, sea fans, jellyfish, clams and cockles (Baker 2003, Takabayashi *et al.* 2012).

Other symbiotic microbes have been identified which are responsible for the cycling of sulfur, fixing nitrogen (Moran *et al.* 2003, Howard *et al.* 2006, Lema *et al.* 2012, Lema *et al.* 2014), producing antimicrobial compounds, inhibiting cell-to-cell signalling and disrupting the virulence of opportunistic pathogens (Wilson *et al.* 2011, Bourne and Webster 2013, Krediet *et al*. 2013).

Opportunistic microbial pathogens

Numerous reports have also identified evidence of pathogenic bacteria, responsible for causing numerous disease outbreaks in coral species (Bourne 2005, Thompson *et al.* 2005, Sussman *et al*. 2006, Boyett *et al.* 2007, Sato *et al.* 2009, Willis *et al.* 2009, de O Santos *et al.* 2011). Over the last thirty years these disease outbreaks have been responsible for significant mortalities in tropical coral reefs of the Indo- Pacific and Caribbean region (Willis *et al.* 2004, Miller *et al.* 2006, Weil *et al.* 2009).

Approximately 18 diseases are recognised as key contributors to the overall health and decline of coral species (Krediet *et al.* 2013). Whilst the aetiological agents responsible for the diseases are largely unknown (Ben-Haim *et al.* 2003), there has been some evidence to suggest the influence or progression of disease may be linked to microbial agents (Rosenberg and Ben-Haim 2002, Rosenberg *et al.* 2007, Bourne *et al.* 2009). Some of these causative agents are aggressive with the

potential to kill several centimetres of coral tissue every day. At this rate, colony mortality can occur within days of the initial infection (Jordan-Garza *et al.* 2011).

Vibrio coralliilyticus is a relatively new aetiological agent, having only been isolated and recognised since 2002 (Ben- Haim *et al.* 2002, Ben-Haim *et al.* 2003). It is predominately a temperature dependant pathogen of *Pocillopora damicornis*, having been isolated from diseased corals from the Indian Ocean, Red Sea, Kent in the UK and Brazil (Ben-Haim *et al.* 2003). *Vibrio coralliilyticus* is a gram-negative, monoflagellated, rod shaped bacterium and is responsible for rapid coral tissue damage at temperatures above 25°C (Ben- Haim *et al.* 2003). Whilst it has also been implicated in the disease manifestation of bivalves during cooler seawater conditions, *V. coralliilyticus* has been found ubiquitously through the coral organism including the surface mucus layer (de O Santos *et al.* 2011).

Motile versus non-motile cells

Cell motility and chemotaxis is fundamental to a bacterium's ability to orientate themselves according to chemical and nutrient gradients, and is of fundamental importance in the initial step of infection (Fenchel 2002, Josenhans and Suerbaum 2002, Rosenberg and Ben-Haim 2002). The ability to chemotax and be motile are important adaptations that some bacteria possess. These bacteria are critical for the assemblages in the ocean and reef communities (Grossart *et al.* 2001). This combination of chemoreception and motility allows for motile bacteria to seek out and maintain beneficial habitats within changing environmental conditions (Adler 1973, Ottemann and Miller 1997, Josenhans and Suerbaum 2002, Stocker *et al.* 2008). *Vibrio shiloi*, a coral bleaching pathogen of *Oculina patagonica* chemotaxes and adheres to the coral surface microlayer (Toren *et al.* 1998, Rosenberg and

Falkovitz 2004). Once *V. shiloi's* adhesion to the microlayer has taken place, the motility search pattern speeds increase with viscosity. The significance of motility early on in the infection process is further evident in plant-based pathogens whereby non-flagellated mutants have reduced pathogenicity (Josenhans and Suerbaum 2002, Rosenberg and Ben-Haim 2002).

Flagella mediated motility and chemotaxis has been observed in a significant number of bacterial species including those associated with human hosts, where *Vibrio* pathogens exert a chemotactic response towards the intestinal mucus layer. This signifies that the bacteria are competent in penetrating the mucus within the intestine (Josenhans and Suerbaum 2002). Similarly, chemotaxis is exhibited towards several components within the mucus excreted by some fish (Banin *et al.* 2001). It appears that motility is reduced once adherence has taken place, and replaced by a cell invasion process (Josenhans and Suerbaum 2002). The progression of disease can be influenced by external environmental conditions such as temperature, which can initiate more extreme responses and infections if conditions are ideal for growth (Mitchell and Kogure 2006, Tunsjø *et al.* 2007, Miller *et al.* 2009).

Bacterial motility and chemotaxis is not limited by temperature as recognised by Rosenberg and Falkovitz (2004), as this behavior was observed when bacteria were grown at both 16˚C and 25˚C. However, temperature did prove to be critical for the adhesion of *V. shiloi* to the coral. No adhesion was seen when the coral was grown at low winter temperatures of 16 — 20˚C, whereas those corals grown at elevated summer temperatures ranging between 25 — 30˚C showed significant adherence to corals (Rosenberg and Falkovitz 2004). This indicates that elevated seawater temperatures like those currently recorded in tropical environments may allow for greater adherence of not only the bleaching pathogen, *V. shiloi,* but of

many other coral pathogenic bacteria worldwide (Rosenberg and Ben-Haim 2002). This thesis focused on the motility and chemotactic properties of *Vibrio coralliilyticus*. The particular focus examined how they traverse seawater and mucus layers of the coral holobiont.

Key functional motility genes

The beginning of pathogenesis and bacterial establishment requires a genetic response (Meron *et al.* 2009). Specific genes coding for the flagellar motor apparatus has been linked to the motility of some *Vibrio* strains (Milton *et al.* 1996, O'Toole *et al.* 1996, Macnab 1999, Ormonde *et al.* 2000, Berg 2003, Meron *et al.* 2009). The flagellin filament outer-sheath gene (*flaA*) and flagellar biosynthesis gene (*flhA*) are key components of the flagellar motor apparatus, which is the mechanism that facilitates motility (Carrillo *et al.* 2004, Kennedy *et al.* 2006, Millikan and Ruby 2004, Meron *et al.* 2009). These genes have also been demonstrated to contribute to the motility response of *V. coralliilyticus* (Meron *et al.* 2009). This chemotactic motility plays a fundamental role in motility dependent pathogenesis and virulence of this organism to actively invade and colonize host cells. For example, Meron *et al.* (2009) reported that a mutation in key flagella genes including *flhA*, results in non-motile *V. coralliilyticus* cells that lack the ability to infect coral tissues. Similarly, in other *Vibrio* strains a single mutation in the *flaA* gene results in strains lacking a flagellum therefore being non-motile and displaying defective adherence properties which prevents subsequent infections and reduced virulence (Freter and O'Brien 1981, Wassenaar *et al.* 1991, Klose and Mekalanos 1998, Millikan and Ruby 2004). Yao *et al.* (1994) demonstrated that motility but not flagellin A (*flaA)* is required for the invasion of *Campylobacter jejuni* cells. In other studies, Milton *et al.* (1996) and McGee *et al.* (1996) discovered that *flaA* is required for *Vibrio anguillarum* to cross

the fish integument and has been suggested to play a role in the virulence of the bacteria after invasion to the host has occurred. Here, the gene expression data of *flaA* and *flhA* was collected through qPCR and examined in relation to the variance of coral mucus and DMSP concentrations for any discernable trends.

Penetrating the coral surface mucus (CSM) microlayer

Disease can occur in one of two ways, where one occurs due to the resident bacteria in the coral mucus and the other is from the invasion of microorganisms from the surrounding seawater (Ritchie 2006, Bourne *et al.* 2008*,* Bourne *et al.* 2009, Bourne and Webster 2013). The latter process can lead to the bacteria found within the coral tissue forming a pathogenic alliance with the introduced species, resulting in disease. This alliance has been identified to occur in the instances of cholera and diphtheria infections in developing nations (Draper and Simon 1980, Grossart *et al.* 2001). The mode of invasion that leads to disease and death in coral species is yet to be elucidated. It is uncertain whether coral possess the pathogenic microorganisms themselves or whether all microbiota found within the coral tissue originated from the surrounding environment. Whilst the coral mucus layer has been shown to house several species of microbiota, it is not yet known whether the mucus layer acts in similar ways to that of human mucus, in the form of trapping bacteria and viruses to prevent them from entering the coral tissue itself (Costerton *et al.* 1999). Here, we examined the influence of the mucus layer on the motility search patterns of *V. coralliilyticus* and whether it presented a similar trapping effect identified in other mucus environments.

The microbiota existing within the marine environment require specific adaptations to pass through the overlying mucus barrier layer present on most coral

species (Schneider and Doetsch 1974; Richardson 1996; Costerton *et al.* 1999). This barrier layer, similar to that associated with gastrointestinal surfaces (Ottemann and Miller 1997), may influence the role and type of motility utilised by both beneficial and pathogenic microorganisms in the process of nutrient uptake and invasion. Anatomically, intestines are characterised by pits or crypts on the surface which is also lined with an epithelium that includes mucus-secreting cells. It is currently not known whether coral tissues and the mucus layer possess similar characterisations whereby it may be possible, as is seen in the gastrointestinal example, to observe actively motile *Vibrios* and bacteria swimming rapidly up and down the crypts (Ottemann and Miller 1997). The ability to remain motile irrespective of the changing surrounding conditions is key to growth and virulence of pathogenic bacterial species.

Motility and virulence are intimately linked by complex regulatory networks of genes and functional apparatus (Josenhans and Seurbaum 2002), with the virulence of pathogenic microbiota showing a positive correlation with an increase in motility (Meron *et al.* 2009). However, motility may only be necessary to initiate an infection whereby once establishment has taken place motility may not be needed for proliferation (Josenhans and Seurbaum 2002). There appears to be three fundamental bacterial cell functions that need to occur simultaneously or consecutively depending on the stage of infection. These are chemoreception, motility and virulence (Manson 1992, Lux and Shi 2004, Ramos *et al.* 2004, Gode-Potratz *et al.* 2011). This suggests that whilst it is well established that motility is required to infect tissue cells, motility may not actually influence or be required for the bacteria's establishment success (Harshey 2003, Nyholm and McFall-Ngai 2004).

To increase the understanding of these diseases, evaluation of the infection mechanisms is necessary. Microorganisms may act individually or in sync with others and potentially form invading groups, however this knowledge is still limited (Bourne *et al.* 2008). Furthermore, whether these microbial communities form purely passive associations with the water column bacteria or whether they have specific associations of ecological importance is in dispute (Rohwer *et al.* 2002, Hibbing *et al.* 2010). Within a host, numerous microorganisms co-exist (Bourne and Munn 2005; Bourne *et al.* 2008 *a*; Bourne et al. 2008 *b*), which may impact the virulence of the microorganisms as they could be of benefit or detriment to each other (Ben-Haim *et al.* 2003). Despite being submerged in seawater, hosts may house unique bacteria to that of the surrounding environment whereby there appears to be almost no overlap between the water column and coral holobiont bacterial communities (Rohwer *et al.* 2001, Frias-Lopez *et al.* 2002).

Even though coral organisms may house unique bacterial communities, cross contamination can occur as healthy coral cells have been shown to lyse within 2 - 4 days of being in direct contact with a diseased coral (Ben-Haim and Rosenberg 2002). The study by Ben-Haim and Rosenberg (2002) showed that as little as 30 pathogenic bacteria/m^2 was sufficient to infect and bleach the corals. Incubation and inoculation times are often dependant on seawater temperature as this can present a fine line between the limited and ideal growth conditions and this can slow the invasion growth conditions. Whereby, temperatures of 28 ±1 °C can created a rapid invasion event, whilst seawater temperatures of 25 °C presented no signs of invasion. This indicates that as little as one degree in temperature can influence the prevalence of disease and infection (Sutherland *et al.* 2004, Aeby and Santavy 2006, Rosenberg *et al.* 2007), with other environmental and abiotic factors including salinity, water

depth, sedimentation, nutrient imbalances and ultraviolet radiation equally sensitive factors (Kuta and Richardson 2002, Morens *et al.* 2004, Harvell *et al.* 2007).

1.3 Coral constituents as nutrient resources

Coral surface mucus is a nutrient and particle trap for organic matter. It also contains energy and nutrient rich components including proteins, triglycerides and wax esters (Wild *et al.* 2004). Mucus also traps zooxanthellae, filamentous algae and other microorganisms (Wild *et al.* 2004). Coral mucus contains numerous chemicals such as amino acids, water-soluble glycoproteins and metabolites (Brown and Bythell 2005). Combined with the trapped zooxanthellae and filamentous algae, the mucus provides chemotactic cues to bacteria, including *V. coralliilyticus* (Garren *et al.* 2014). A significant chemoattractant that enables bacteria to target the coral mucus is the metabolite dimethylsulfoniopropionate (DMSP) (Raina *et al.* 2010, Seymour *et al.* 2010, Garren *et al.* 2014), with corals significant reservoirs for DMSP (Broadbent *et al.* 2002). DMSP is a key element in the global sulphur cycle and has been identified as a primary chemical cue for not just macro-organisms but also microorganisms, including *V. coralliilyticus* (Raina *et al.* 2009, Raina *et al.* 2010, Garren *et al.* 2014).

The presence of DMSP, or its derivative dimethysulfide (DMS) within corals starts out as an osmolyte to compensate for the osmotic balances resulting from photosynthesis (Hill *et al.* 1995, Malin 1996, Zimmer-Faust *et al.* 1996, Kiene *et al.* 2000). The production of DMS from the reef through bleaching and stress events could potentially alter the local climate impacting on the health of the coral reefs (Hill *et al.* 1995). In conjunction with the potential for local climate changes, studies report that as well as being found in corals, zooxanthellae and dinoflagellates also

contain high amounts of DMSP, which could convey a potent chemotactic cue, directing the microorganism towards the coral reef ecosystem (Hill *et al.* 1995). Recorded concentrations of DMSP are identified to vary in the mucus of many coral species. DMSP concentrations can reach 3 orders of magnitude higher in the mucus with values between 1 – 62 μM, compared to the surrounding seawater with values between 6 – 11 nM (Broadbent and Jones 2004, Van Alstyne *et al.* 2006), this may create a chemotactic gradient that assists in drawing potentially pathogenic microorganisms towards the coral holobiont.

1.4 Thesis objective

The primary objectives of this thesis were to investigate the chemotactic search pattern responses of *V. coralliilyticus* under conditions mimicking those present within the native coral holobiont. By understanding how this pathogen responds to the fluctuations in nutrient and stimulus availability and structural integrity of surrounding solutions, and by examining the gene expression of genes involved in the motility structures, provides a thorough examination of this pathogen as a model organism for monoflagellated bacteria.

The first data chapter examined the influence of oxygen fluctuations on *V. coralliilyticus* search pattern motility. In corals oxygen levels are reduced at night (Kühl *et al.* 1995) and therefore invading microorganisms must be able to survive in fluctuating oxygen environments.

The second data chapter examined the influence of viscosity on the motility and chemotaxis of *V. coralliilyticus* due to the presence of the protective coral mucus boundary layer. This layer coats the surface of the coral to act as a preventative measure against desiccation and protection against microorganisms (Krupp 1984, Davies and Hawkins 1998, Brown and Bythell 2005, Shnit-Orland and Kushmaro

2009, Krediet *et al.* 2013). This protective mucus boundary layer was a key interest point in this chapter as it examined the ability of a pathogenic organism to penetrate this layer and regions of increasing viscosity.

In the third data chapter, *Vibrio coralliilyticus's* chemotactic search pattern responses and the gene expression of key motility genes, *flaA* and *flhA*, were examined with various concentrations of known chemoattractants, namely coral mucus and DMSP. This assessed whether there are peak concentrations that facilitate high search pattern speeds and a discernable trend in search pattern types. The gene expression of *flaA* and *flhA* was also examined for any trends with the variations in coral mucus and DMSP concentrations.

Lastly, the final data chapter used a modeling approach to calculate the energetic costs required for *V. coralliilyticus* to perform the three most prominent chemotactic search patterns utilized in the presence of altered viscosity and DMSP concentrations. The diffusivity of nutrients associated with chemotactic search pattern types may provide insight into *V. coralliilyticus's* capabilities of controlling their motility to make use of the surrounding conditions. This combined approach into the changes in the motility search pattern types and speed of the coral pathogen, *V. coralliilyticus*, coupled with the genetic responses of fundamental motility genes, in relation to the variations identified within the coral, identifies the route and potential difficulties detected by *V. coralliilyticus* in colonizing a coral holobiont.

Specifically the aims were:

1. To determine the changes to *Vibrio corallilyticus* search pattern behavior due to the presence of oxygen gradients observed within the surface and internal tissue of the coral organism. The search patterns were assessed to identify whether *V. coralliilyticus* was able to remain motile

even in low oxygen environments, which may provide insight to their strengths and capabilities as invading pathogens.

2. To determine the changes to *Vibrio coralliilyticus* search pattern types and speed due to the presence of the coral surface mucus layer on the surface of the coral holobiont. The search patterns of *V. coralliilyticus* were assessed to identify whether viscosity changes influenced the search pattern behavior or whether chemoattractants cause the search pattern differentiation.

3. To determine whether key motility genes are expressed in higher values in the presence of increasing concentrations of known chemoattractants of *V. coralliilyticus*, namely DMSP and coral mucus. The search patterns of *V. coralliilyticus* were assessed to identify if any trend or link was apparent with the gene expression values at identical chemoattractant values.

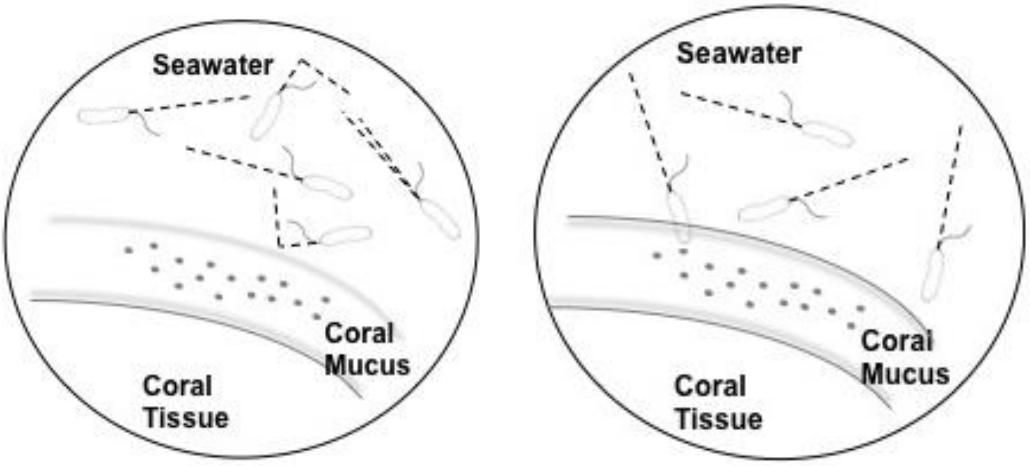
4. To provide calculations and estimates of the energy requirements and diffusivity values of *V. coralliilyticus* whilst utilizing specific chemotactic search patterns and with the variance in viscosity values associated with the coral mucus boundary layer. These values were also calculated and compared with other bacterial species to assess whether flagella orientation, cell shape and size may influence diffusivity values.

1.5 Thesis structure

This thesis is formatted in manuscript form for journal submission with each chapter addressing a specific aim. The results from Chapter 2 to 5 are either published in peer-reviewed journals, have been submitted for publication, or will be submitted for publication in the near future. Chapter 2 assesses the motility of *V. coralliilyticus* across an oxygen gradient mimicking the natural oxygen fluctuations that occur surrounding the coral tissue and was published in *PLoS* One, 8(7): 1-8, 2013. Chapter 3 examines the chemotactic search pattern motility of *V. coralliilyticus* in coral mucus and mucin controls, to investigate the effects of viscosity on cell search patterns and speed. This chapter has been submitted for review to the *Journal of Bacteriology*. Chapter 4 investigated the influence of various concentrations of coral mucus and DMSP on the gene expression of key motility genes including *flaA* and *flhA.* This manuscript has been submitted for publication in the *Journal of Bacteriology*. Chapter 5 calculates the energy requirements and diffusivity values of *V. coralliilyticus* in conjunction with viscous solutions, this manuscript has been submitted to the journal *MicrobiologyOpen*. The discussion and implications of these results form Chapter 6. A single reference list has been included at the end of this thesis that includes all literature cited throughout this thesis to reduce redundancy.

Chapter 2

Vibrio coralliilyticus search patterns across an oxygen gradient



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Chapter cover figure: A visual summary of the component influencing the search pattern response in chapter 2, (l – r) oxic conditions and anoxic conditions, which is further discussed in chapter 6.

2.0 Abstract

The coral pathogen, *Vibrio coralliilyticus* shows specific chemotactic search pattern preference for oxic and anoxic conditions, with the newly identified 3-step flick search pattern dominating the patterns used in oxic conditions. We analyzed motile *V. coralliilyticus* cells for behavioral changes with varying oxygen concentrations that mimicked the natural coral environment exhibited during light and dark conditions. Results showed that 3-step flicks were 1.4 times (P = 0.006) more likely to occur in oxic conditions than anoxic conditions with a mean value of 18 flicks (95% CI = 0.4, n = 53) identified in oxic regions compared to 13 (95% CI = 0.5, n = 38) at anoxic regions. In contrast, run-reverse search patterns were more frequent in anoxic regions with a mean value of 15 (95% CI = 0.7, n = 46), compared to a mean value of 10 (95% CI = 0.8, n = 29) at oxic regions. Straight swimming search patterns remained similar across oxic and anoxic regions with a mean value of 13 (95% CI = 0.7, n = oxic: 13; anoxic: 14). *V. coralliilyticus* remained motile in oxic and anoxic conditions, however, the 3-step flick search pattern occurred more frequently in oxic conditions. This result provides insight into the behavioral repertoire adopted by *V. coralliilyticus* and the implications this may have on adapting to a fluctuating environment.

2.1 Introduction

Bacteria use chemotaxis and motility search patterns to position themselves in chemical gradients, locate nutrient sources and initiate pathogenesis (Magariyama *et al.* 2005, Mitchell *et al.* 2005). These search patterns include run-reverse, run and tumble, straight swimming and the recently described, the 3-step run-reverse and

flick (Mitchell *et al.* 2005, Altindal *et al.* 2011, Berg and Brown 1972, Xie *et al.* 2011). The majority of evidence suggests that only one search strategy is adopted by a single bacterial species (Guerin 1999, Mitchel 2002), with marine bacteria suggested to adopt a 'back and forth' swimming behavior pattern, known as a run-reverse, utilizing turbulence-induced shear in the ocean (Luchsinger *et al.* 1999). While the interactions of the run-reverse pattern with its environment is clear, how the 3-step flick pattern, henceforth 'the flick', which is a cyclic motion of: forward, reverse, flick and repeat (Xie *et al.* 2011, Stocker 2011) interacts with the physical environment is less clear. What is known so far is that by introducing a consistent directional change, the flick allows species such as *V. alginolyticus* to access nutrient patches quicker than bacteria using the run and tumble strategy such as *Escherichia coli* (Xie *et al.* 2011). The key trait of the flick search strategy seems to be a combination of the run-reverse strategy, where the 180° backtracking gives bacteria traveling down a gradient an opportunity to re-exploit nutrient patches found moments earlier, with the run and tumble strategy which uses frequent reorientation to find new nutrient sources (Altindal *et al.* 2011).

Pathogenic and symbiotic *Vibrio* species, including the coral pathogen *V. coralliilyticus*, are reliant on chemotaxis to invade and colonize host species. Abundant in the coral mucus (Chimetto *et al.* 2008, Alves *et al.* 2010) and the microbial community of diseased corals (Bourne and Munn 2005, Bourne *et al.* 2008), *Vibrios* alter the microbial metabolism in corals (Thurber *et al.* 2009). *V. coralliilyticus* causes the bleaching and tissue lysis of corals at temperatures greater than 25°C, and is further implicated in the disease of other marine organisms including bivalves during the winter months when temperatures are lower (Ben-Haim *et al.* 2003). Meron *et al*. (2009) demonstrated that the flagellum in *V.*

coralliilyticus is critical for infection, including the adhesion to the corals and chemotaxis towards coral mucus.

During coral infections, *V. coralliilyticus* must move through the surrounding seawater, the coral mucus surface layer and into the coral tissue for establishment and growth. This infection route, indicative of most coral pathogens, may illustrate unique search pattern strategies to assist cells in moving through numerous environments of differing viscosity and nutrient complexity. Local marine environments and conditions are constantly changing due to turbulence (Stocker 2011). Oxygen conditions and nutrient concentrations change during the day and night (Kühl *et al.* 1995). Oxygen saturation levels are reduced in coral tissue after being exposed to the darkness, with records showing that coral tissues have been shown to have < 2% oxygen saturation after 5 minutes in the dark (Kühl *et al.* 1995). In contrast, during daylight conditions coral tissues exhibit up to 250% oxygen saturation (Kühl *et al.* 1995). Furthermore, work by Kühl *et al.* (1995) showed that oxygen levels remained steady in the surrounding water in both light and low-light conditions, however these levels have shown to differ in the 0.1 – 1 mm layer on the coral tissue surface as well as inside the coral tissue (Kühl *et al.* 1995). This study examined the chemotactic search pattern changes of *V. coralliilyticus* in response to fluctuating oxygen levels. This will provide insight into how *V. coralliilyticus* behaves in fluctuating oxygen levels in the surrounding seawater, surface mucus layer or boundary layer and the coral tissue.

2.2 Materials and methods

2.2.1 Bacterial Culture and Growth

The single, polar flagellated *V. coralliilyticus* type strain, LMG20984 was used for all experiments. Pure cultures were stored at -80°C in 15% glycerol stock solutions. Strains were grown on Thiosulphate citrate bile salts sucrose agar media (Sigma Aldrich) overnight at 28°C. Liquid cultures were prepared in 30 mL of Luria-Bertani broth (Sigma Aldrich) and incubated for 16 hrs overnight at 28°C with 160 rpm shaking, and harvested during exponential growth. The culture was diluted with LB to 1/10 to adjust cell density to OD 0.1 ± 0.01, and visualized within 1 minute.

2.2.2 Microscope Observation Chamber and Microscope Video Analysis

A volume of 50 μL of cell suspension was added to an observation chamber constructed from two glass coverslips on a glass slide and covered by a third coverslip (Fig. 2.1). The distance between the glass slide and coverslip was 0.17 mm, with a chamber width of 15 mm. Swimming cells were observed at the edge and center of the chamber, midway between the coverslip and the microscope slide. The oxygen distribution was approximated by modeling, which illustrated that oxygen levels began to deplete within millimeters from the edge of the observation chamber. Additionally, cells were also observed at the chamber surface. Within the microscope observation chamber, recordings were made within 2 mm of the coverslip edge and center (Fig. 2.1). Swimming *V. coralliilyticus* cells were observed under dark-field, 200x magnification video-microscopy (AxioCamMrc5 camera, Carl Zeiss AxioSkop microscope) with the field of view at mid-depth in the microscope slide chamber. The video was recorded continuously for 10 seconds at 2.4 frames s^{-1} using a time-

lapse module (Carl Zeiss AxioVision 4) (see chapter 6). Search strategies of *V. coralliilyticus* were recorded and plotted across spatial gradients. Cell swimming paths of *V. coralliilyticus* were measured frame-by-frame and traced off the computer screen using overhead transparencies. Cell paths were defined as in Barbara and Mitchell (1996) and classified according to the angle of the run, with speed and turn angles calculated according to Barbara and Mitchell (2003). To ensure that all patterns could potentially include a complete flick, only cells visible for at least 5 frames were used.

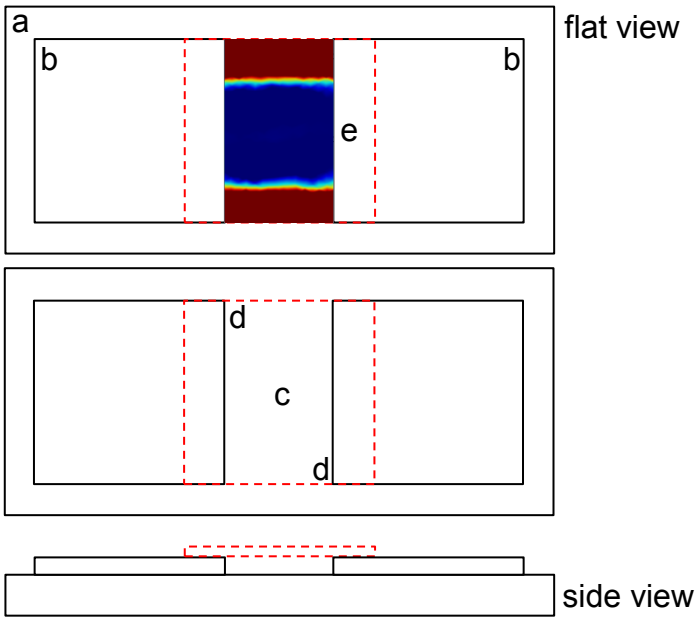


Figure 2.1. Measurement locations within the observation chamber. (a) Microscope slide, (b) cover slips, (c) center recordings, (d) edge recordings. A 3D numerical model depicts the oxygen diffusion from the top and the bottom of the coverslip labelled (e). Red depicts high oxygen concentrations with a vertical length of 5250 µm, whilst the blue depicts low oxygen concentrations. The chamber has a vertical length of 26,250 µm. The red dashed line illustrates the chamber cover, which sits on top of two cover slips.

2.2.3 Transect line experiments from oxic to anoxic conditions

A volume of 50 μL of 1/10 dilution of overnight culture was added to the created observation chamber. Triplicate transects were collected ranging from the oxic edge to the anoxic center of the observation chamber. Each transect consisted of 40 square eyepiece quadrats measuring 172 by 172 μm, placed next to each other. The transect line spanned 6,880 μm from the oxic to anoxic regions across. At each quadrat a 10 second, 49-frame video was recorded. All distinguishable cell trajectory paths were classified into one of three search patterns based on the angles observed while tracking the bacteria's swimming path: run-reverses, straight swimming or 3-step flick (Supp. Fig. 1). Cells were considered distinguishable if they remained in focus for at least 5 frames. This is particularly the case for the flick events which can occur within milliseconds. Here, the potential for a flick search pattern was determined by any 90° reorientation of the cell at any one time. All distinguishable cell trajectories were classified into one of three search patterns: run-reverse, straight swimming or 3-step flick (Supp. Table 2.1). Each trajectory was then measured and the swimming speed was calculated.

2.2.4 Aerobic and Anaerobic Culture Control Samples

Cell cultures and the headspaces were bubbled with either pure air or nitrogen for 10 minutes, and grown in conditions as mentioned above. Methylene blue and resazurin indicators (Sigma-Aldrich) were used to confirm whether a solution was saturated throughout the experiment (Guerin 1999, Guerin *et al.* 2001, Høj *et al.* 2005, Karakashev *et al.* 2003). Methylene blue produced a blue/green color in oxygen-saturated cultures, where oxygen levels were above 0.5%, whereas resazurin

produced a bright pink color in nitrogen-saturated cultures, where oxygen levels were below 0.1%. At values between 0.1 and 0.5%, a blue/violet color was produced (Guerin 1999). For gas experiments, the slide preparation including the construction of observation chambers, the opening of culture bottles and the transfer of liquid culture onto the slides were all prepared within gas filled plastic bags to minimize any changes to saturated cultures. In addition, cell-free media broth used for dilutions was also bubbled with air or nitrogen. Slides were viewed immediately and video recordings were taken within 1-2 minutes after the removal from the gas-filled bag. Previous testing showed that cultures remained colored, either green-blue or pink for aerobic or anaerobic tests respectively, for an average time of 180 seconds. This was calculated by measuring how long the color held when viewing the cultures under the microscope. Ten replicates were measured with the color holding values ranging from 160 to 220 seconds. To remain certain that *V. coralliilyticus* was being viewed in oxic or anoxic conditions all videos were recorded well before the lower value in this time window.

2.2.5 *Vibrio coralliilyticus* Search Pattern Changes Over Time

Microscope videos in oxic and anoxic conditions were collected at 5 minute intervals between 0 and 25 mins to measure the behavioral changes of *V. coralliilyticus* over time. Slides were prepared as stated above. New observation chambers were prepared for each oxygen condition of the timed experiment which was run in triplicate (Supp. Table 2.2).

2.2.6 Mathematical Modeling

Numerical simulations of oxygen diffusion in the presence of bacteria were produced using the MatLab-derived COMSOL Multiphysics with the shown geometries using transport of dilute species with convection and diffusion models (Fig. 2.2). Previous studies have illustrated multiple ways in which this model has been used (Schneider *et al.* 2005, Cardenas 2008, Musielak *et al.* 2010). Dilute species transport was represented by:

$$\partial c / \partial t + \mathbf{u} \cdot \nabla c = \nabla \cdot (D \nabla c) + R \qquad (1)$$

where c is concentration (mol/m^3), D is the diffusion coefficient (m^2/s), R is the reaction rate for the species (mol/(m^{3}s)) and in this case is used to signify flux, **u** is the vector velocity (m/s). Low Reynolds number, incompressible flows are characterized by:

$$\nabla \cdot \mathbf{u} = 0 \qquad (2)$$

However, a modeled chamber depth of 0.2 mm and isothermal conditions damped convective flow and left only molecular diffusion. Equations (1) and (2) were coupled in the MatLab-derived COMSOL Multiphysics 4.0a as a finite element 3 dimensional, free tetrahedron mesh model. The minimum mesh size was 10^{-7} m.

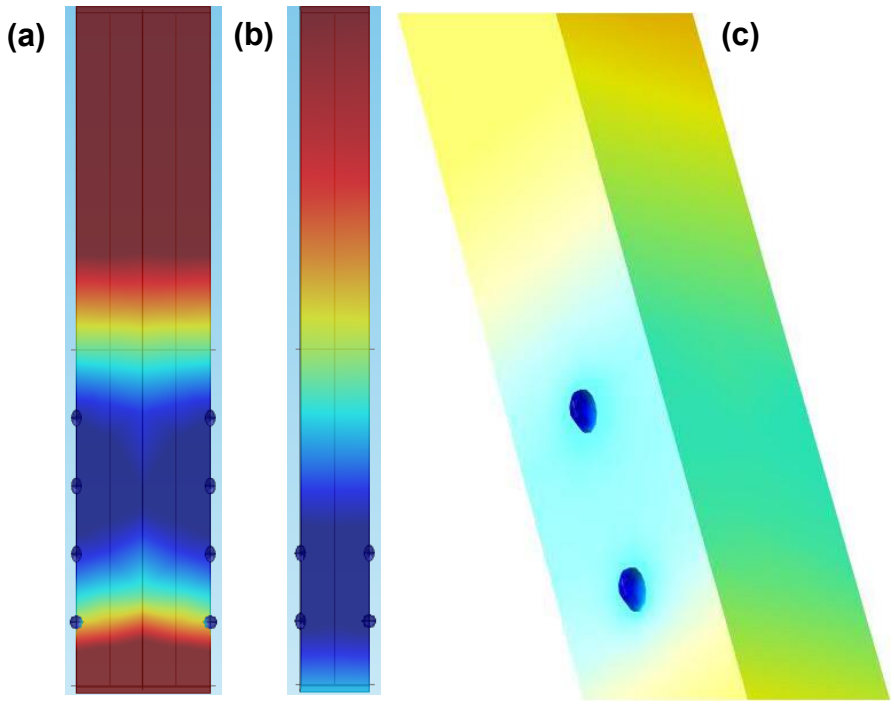


Figure 2.2. Three-dimensional numerical models of oxygen diffusion microscope slide chamber where oxygen diffuses from the top and the bottom. Blue dots represent bacteria that absorb oxygen as it diffuses past. The columns are 100 µm high (a and b). The relative concentration difference between blue and red is 30 mM. Orthogonalized spacings represent average cell distributions seen in the experiments with (a) for low cell density regions and (b) for cell cluster regions. Real distributions will be heterogenously rather than uniformly distributed. The diagonal in (c) is the rotated volume showing the z axis and the position of the bacteria in that direction.

2.2.7 Statistical Analysis

All cell trajectories were classified into search pattern types and the results were analyzed using SPSS Statistics (version 19.0). Independent sample t-tests were utilized to calculate P values to establish whether flick or non-flick search patterns

differed in mean numbers or speed across the two observation chamber locations. T-tests were also used to determine any statistical differences in search pattern behavior during the time series.

2.3 Results

2.3.1 *Vibrio coralliilyticus* Search Pattern Preferences in Oxic and Anoxic Conditions

V. coralliilyticus search paths were identified and classified into three search strategies according to the angle of the runs identified, namely straight swimming, run-reverse and the 3-step flick (Fig. 2.3). In oxic areas of the observation chamber, 17 flicks, 21 run-reverses and 13 straight swimming search patterns with mean speeds of 17 μm s^{-1} (95% Confidence Interval (CI) = 2.0, n = 17), 21 μm s^{-1} (95% CI = 4.1, n = 21) and 21 μm s^{-1} (95% CI = 8.4, n = 13) respectively, were observed (Table 2.1). In anoxic areas of the observation chamber, 38 flicks, 46 run-reverses and 14 straight swimming search patterns with mean speeds of 20 μm s^{-1} (95% CI = 2.6, n = 38), 20 μm s^{-1} (95% CI = 2.5, n = 46) and 18 μm s^{-1} (95% CI = 5.5, n = 14) respectively, were observed (Table 2.1). Flick search patterns in oxic areas of the observation viewing chamber, namely the edge, were 1.4 times more frequent in number than at anoxic areas, or the center of the viewing chamber (P = 0.006) (95% CI = 1.5, n = oxic: 53; anoxic: 38) and were significantly slower than the non-flick search patterns (P = 0.016) (95% CI = 2.9, n = flicks: 53; non-flicks: 42) seen in the oxic areas of the observation chamber. In anoxic conditions, run-reverse search patterns were 1.6 times more frequent than in oxic conditions (P = 0.040) (95% CI = 2.3, n = oxic: 29; anoxic: 46). In oxic or anoxic conditions there were no differences

in swimming search patterns in the chamber regions (P = 0.778) (95% CI = 1.4, n = oxic: 13; anoxic: 14).

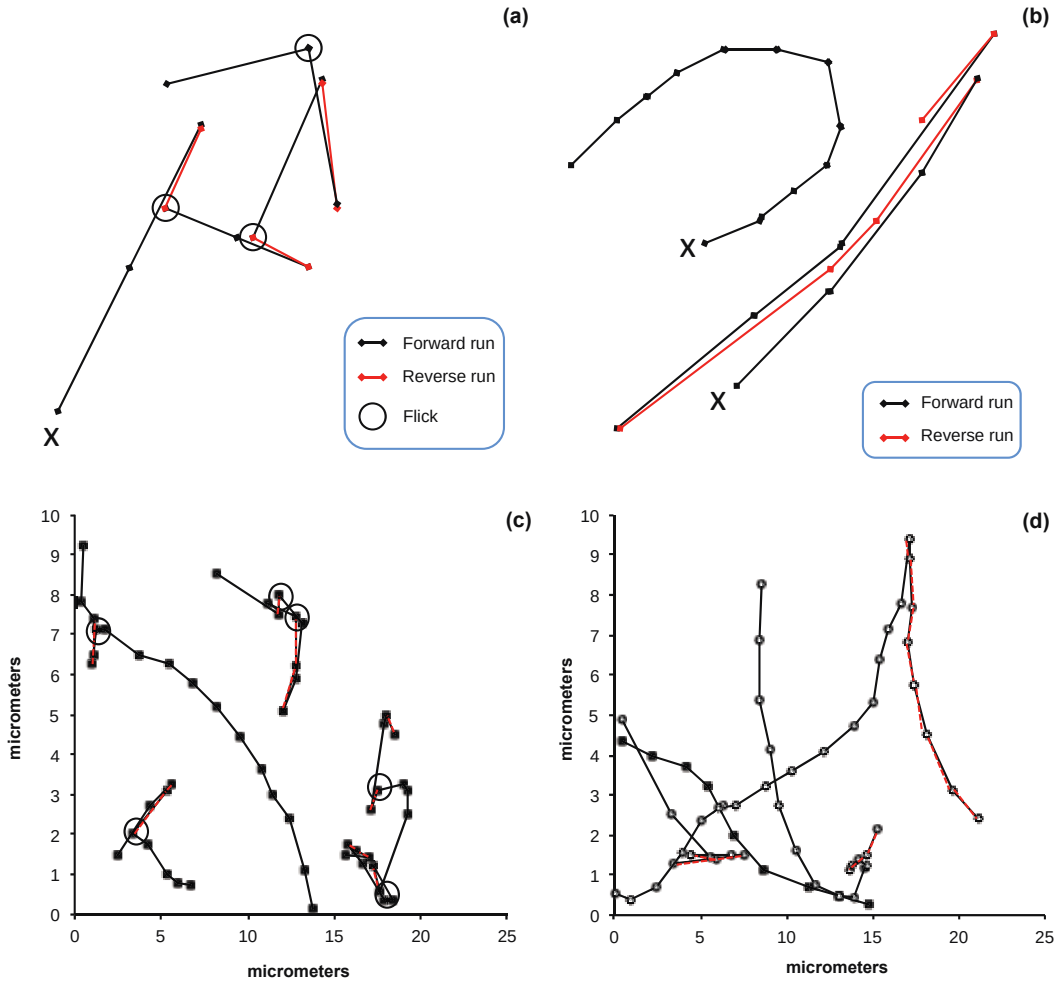


Figure 2.3. Schematic and observed chemotactic search patterns of *V. coralliilyticus*. The search pattern path starts at X; (a) cyclic 3-step flick search pattern, shown as a forward run, a reverse; seen as a 180° reorientation, and a 90° flick of the flagellum and repeat; (b) run-reverse and swimming search pattern. Run-reverse search patterns are characteristic of a 180° reorientation and reversal. Straight swimming search patterns shows no reversals; (c) 3-step flick search pattern trajectories (closed squares) collected from *V. coralliilyticus* overnight cultures collected from the oxic region of the observation chamber. For clarity, the open circles mark the

90° flicking events and the red-dashed lines indicate the 180° reversals; (d) search pattern trajectories collected from *V. coralliilyticus* overnight cultures collected from the anoxic region of the observation chamber, the search patterns exhibited here are run-reverses (open circles) and straight swimming (closed squares).

2.3.2 *Vibrio coralliilyticus* Search Patterns Change across Oxic to Anoxic Transect Line Experiments

The chemotactic search pattern behavior of *V. coralliilyticus* changed across the oxic-anoxic interface, identified across a microscope transect line. Three-step flick search pattern numbers decreased 8-fold (P = 0.048), from a mean value of 8 flicks (95% CI = 0.4; n = 24) in oxic environments to a mean value of 0.5 flicks (95% CI = 0.6; n = 3) in anoxic environments. Run-reverse search patterns decreased 5-fold (P = 0.048), from a mean value of 5 run-reverse search patterns (95% CI = 0.51; n = 15) in oxic conditions through to 1 run-reverse (95% CI = 1.13; n = 3) in anoxic conditions. Straight swimming search pattern numbers increased from a mean value of 5.7 straight swimming search patterns (95% CI = 1.2; n = 17) in oxic conditions to a mean value of 9 straight swimming search patterns (95% CI = 0.8; n = 27) in anoxic conditions (Fig. 2.4a).

Search pattern speeds were shown to decrease 2-fold from oxic to anoxic environments (P = 0.002), from 63.2 μm s^{-1} (95% CI = 2.0, n = 58) to 21.9 μm s^{-1} (95% CI = 3.4, n = 32) respectively, although the error bars identify large variations in search pattern speed across the run-reverse and 3-step flick search patterns (Fig. 2.4b). Search pattern speeds remained steady at 23.2 μm s^{-1} from the 2000 μm distance location onwards (P = 0.578, 95% CI = 0.6, n = 400). Speeds did not differ

significantly between the three types of search patterns identified, namely the flick, run-reverse and straight swimming (Fig. 2.4b).

2.3.3 Aerobic and Anaerobic Controls

Motile cells of *V. coralliilyticus* were observed in cultures grown under both aerobic and anaerobic conditions. Immediate visualization of these cell cultures showed that in aerobically grown cultures, 38 flicks, 13 run-reverses and 9 straight swimming patterns with mean speeds of 45 μm s^{-1} (95% CI = 5.6, n = 38), 45 μm s^{-1} (95% CI = 8.4, n = 13) and 32 μm s^{-1} (95% CI = 0.7, n = 9) respectively, were observed (Table 2.1). Cultures grown under anaerobic conditions identified 5 flicks, 6 run-reverses and 40 straight swimming search patterns with mean speeds of 46 μm s^{-1} (95% CI = 7.0, n = 5), 48 μm s^{-1} (95% CI = 6.9, n = 6) and 46 μm s^{-1} (95% CI = 3.5, n = 40) respectively (Table 2.1). In aerobic conditions, flick search patterns were 7.6 times greater than in anaerobic cultures (P = 0.024). In addition, a 2.2 fold increase in run-reverse search patterns (P = 0.010, 95% CI = 8.4, n = 13) and a 4.4 fold decrease (P = 0.003, 95% CI = 6.6, n = 9) in straight swimming search patterns were seen in aerobic conditions compared to anaerobic conditions. Search patterns speeds did not differ across aerobic or anaerobically grown cultures (P = $\geq$ 0.05, 95% CI = 2.6, n = 111) (Table 2.1).

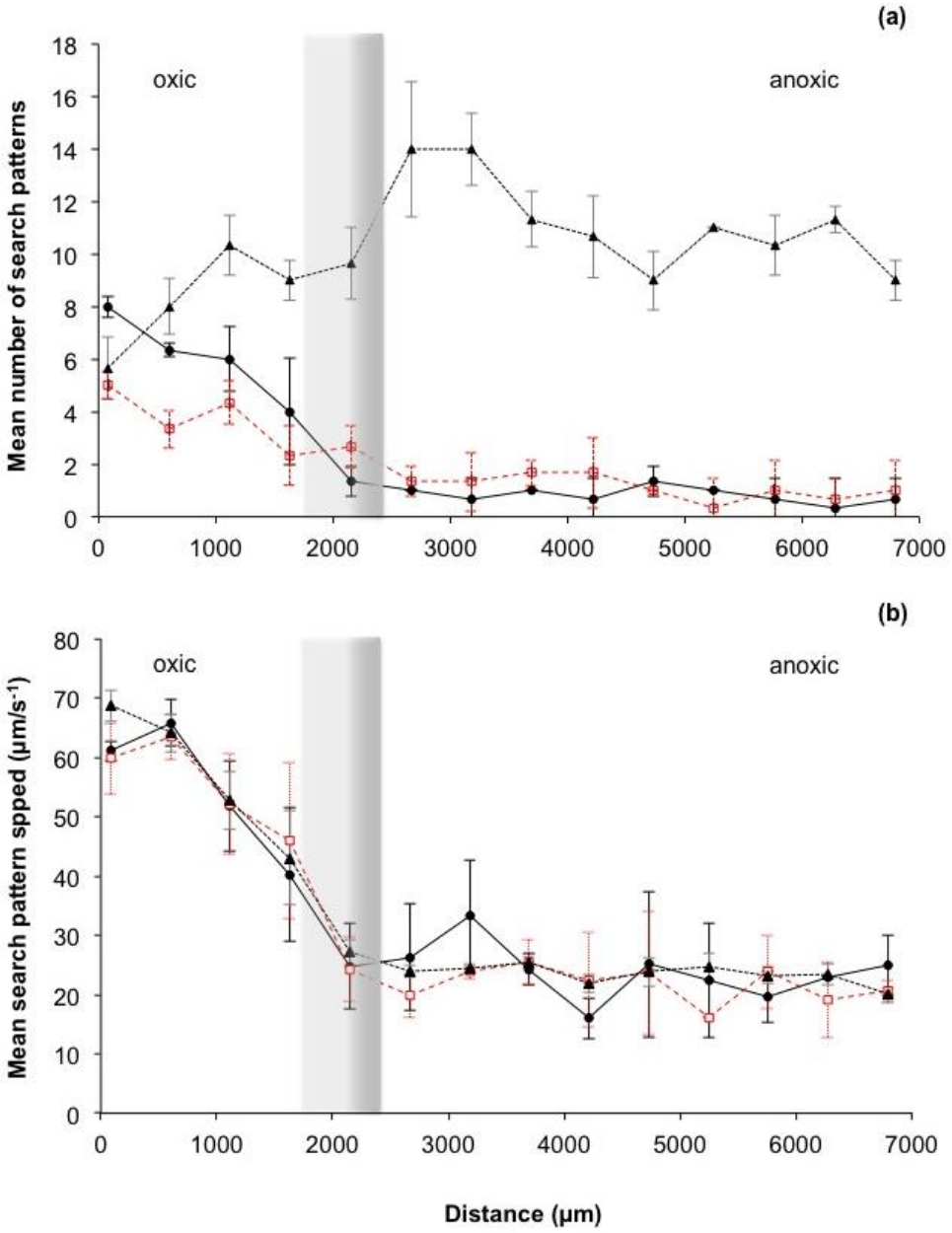


Figure 2.4. *Vibrio coralliilyticus* search patterns along an oxygen gradient. (a) **The mean total number of search patterns seen along the transect line consisting of 40 quadrat boxes (error bars = 95% CI); (b) Mean velocity (µm s^{-1}) of search patterns across the transect line (error bars = 95% CI). The location of the grey gradient lines represents the location where the oxygen transition occurs in Fig. 2.1 (orange and green area). The circles and solid line represents the 3-step flick search patterns, the square and dashed line represents the run-reverse search patterns and the triangle and dotted line represents the straight swimming search patterns.**

2.3.4 *Vibrio coralliilyticus* Search Patterns Change Over Time

During a 25-minute time series experiment, the search pattern numbers in anoxic conditions levelled off by the 5-minute mark, whereas in oxic conditions, search pattern numbers began to level off from 10 minutes onwards. In anoxic conditions, flick search patterns decreased by 1.9 times ($P = 0.022$, 95% CI = 8.0, $n = 38$), whilst non-flick patterns increased by 2.1 times (95% CI = 3.4, $n = 26$) over the 25-minute experiment, though this result was not significant ($P = 0.230$) (Fig. 2.5a). Straight swimming search patterns remained constant ($P = 0.333$, 95% CI = 3.1, $n = 203$). Similarly, in oxic conditions, flick search patterns decreased 1.8 times ($P = 0.014$, 95% CI = 2.5, $n = 120$), whilst non-flick search patterns were variable and demonstrated no significant trend ($P = \geq 0.05$, 95% CI = 3.4, $n = 122$). All search pattern speeds except the straight swimming patterns remained relatively constant at oxic and anoxic conditions over time (Fig. 2.5b).

Table 2.1. Maximum, and mean search pattern speeds (μm s^{-1}) and total search pattern numbers (n) identified in oxic and anoxic regions of the observation viewing chamber

Search pattern	Observation chamber region				Aerobically grown cultures	Anaerobically grown cultures
	Edge (Oxic)		Center (Anoxic)			
	Chamber	Surface	Chamber	Surface		
Straight swimming	56*, 21 (n = 13)	18*, 14 (n = 7)	46*, 18 (n = 14)	21*, 14 (n = 22)	47*, 32 (n = 9)	65*, 46 (n = 40)
Run-reverse	47*, 21 (n = 21)	35*, 22 (n = 12)	49*, 20 (n = 46)	29*, 15 (n = 9)	76*, 45 (n = 13)	59*, 48 (n = 6)
Combined non-flicks	57*, 25 (n = 25)	35*, 19 (n = 19)	49*, 19 (n = 60)	29*, 14 (n = 31)	76*, 40 (n = 22)	61*, 46 (n = 46)
3-step flicks	37*, 17 (n = 17)	41*, 25 (n = 21)	43*, 20 (n = 38)	25*, 17 (n = 10)	79*, 45 (n = 38)	56*, 46 (n = 5)

* = maximum search pattern speed

n = sample size (number of recordings)

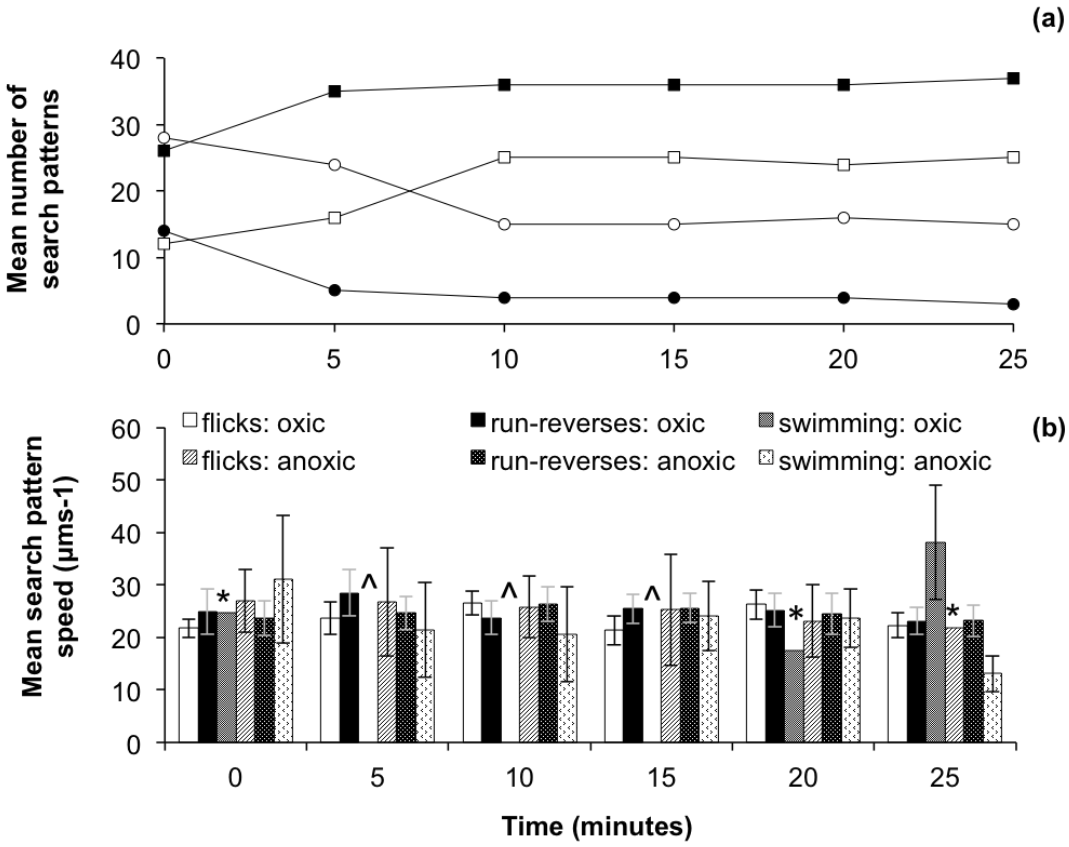


Figure 2.5. *Vibrio coralliilyticus* search patterns over a 25-minute time series. (a) The mean total number of search patterns seen at the edge of the cover slip and center of the cover slip over a 25-minute time series. The solid squares indicate the number of non-flick search patterns at an anoxic area of the chamber, open squares indicate the number of non-flick search patterns at an oxic area of the chamber, closed circles indicate the number of flicks search patterns at an anoxic area of the chamber, open circles indicate the number of flick search patterns at an oxic area of the chamber. Run-reverses and straight swims were combined here due to the limited number of straight swims identified (Supp. Table 2.2); (b) The mean search pattern velocities seen at oxic and anoxic regions of the cover slip chamber over a 25 minute time experiment (error bars = 95% CI). * = only 1 search pattern observed so no 95% CI value could be calculated; ^ = no search pattern was identified.

2.4 Discussion

Oxygen concentrations can differ between the surrounding water layer and the various microbiomes present in coral organisms, namely the surface mucus layer and coral tissue (Kühl *et al.* 1995). During the night corals can experience complete hypoxia (Morrow *et al.* 2011, Nilsson *et al.* 2004, Wijgerde *et al.* 2012). As such, coral microorganisms must be able to survive in changing oxygen conditions (Thurber 2009). We provide evidence that the coral pathogen, *V. coralliilyticus* is capable of utilizing chemotactic search patterns in oxic and anoxic conditions whilst maintaining search pattern speeds across these conditions. This indicates that regardless of oxygen concentrations *V. coralliilyticus* are capable of remaining motile.

Our results indicate that *V. coralliilyticus* uses the newly discovered cyclic flick search pattern, first found in *V. alginolyticus*, which is morphologically distinct from the run-reverse or run and tumble patterns adopted by *E. coli* (Luchsinger *et al.* 1999, Thurber *et al.* 2009, Xie *et al.* 2011). The search pattern behavior identified in oxic and anoxic conditions in *V. coralliilyticus* may be the result of saturated oxic conditions, similar to that seen in *Salmonella typhimurium* and *Escherichia coli* (Shioi *et al.* 1987), whereby in high concentrations, dissolved oxygen is a repellent and in low conditions (0.25 mM), an attractant (Shioi *et al.* 1987). Whilst it is known that the 3-step flick allows for a 90° directional change to relocate to a more suitable nutrient environment (Thurber 2009, Xie *et al.* 2011), it is unknown whether bacteria may use the 90° flick in this search pattern to quickly move away from undesired nutrient patches as often the initial flick is $\geq$ 5 μm in length and continues for a distance of ~ 15 μm (Xie *et al.* 2011). Our work adds to this by showing that the 3-step flick is present in oxygenated environments and not in deoxygenated

environments. Determining the critical oxygen concentration for turning off the flick, and why oxygen might be needed provides pathways for further investigating the 3-step flick.

Changes in search pattern behavior of *V. coralliilyticus* in oxic and anoxic conditions may not be a response to the oxygen concentrations directly, or lack thereof. Instead, as suggested by Armitage (1997) bacteria can monitor changes in the electron transfer rate using redox sensors, which are signaled through sensory pathways. This sensory system converts environmental signals into a rotational change of the flagellar motor (Armitage 1999) allowing for cell relocation or revisiting older nutrient patches (Stocker *et al.* 2008). All cells possess an ability to sense oxygen and activate these adaptive sensory processes, which are fundamental to survival when oxygen becomes limited (Stocker *et al.* 2008). The observation chamber, which is open on two sides, creates an oxygen gradient from the interplay of microbial oxygen consumption and diffusion from the edges (Fig. 2.2). This simulates the diurnal and nocturnal changes that occur in the surface mucus layer and coral epithelial tissue highlighted in Kühl *et al.* (1995). The behavioral responses arising from this oxygen gradient, illustrates the behavioral complexities of a *V. coralliilyticus* infection, where *V. coralliilyticus* cells remain motile in both oxic and anoxic conditions. With *V. coralliilyticus's* motility fundamentally linked to infection and establishment in corals (Guerin *et al.* 2001), knowledge of its behavioral characteristics could provide greater insight into infection rates and mechanisms.

2.5 Acknowledgments

We'd like to thank the Australian Research Council and the Centre for Marine Microbiology and Genetics at the Australian Institute of Marine Science for funding support for this research.

Supplementary Table 2.1.

Experimental number of recordings (n) for *V. coralliilyticus* search pattern preference from oxic to anoxic conditions for figure 2.4

Search pattern type	Number of recordings	
	Oxic	Anoxic
3-step flick	24	3
Run-reverse	15	3
Straight swimming	17	27

Supplementary Table 2.2.

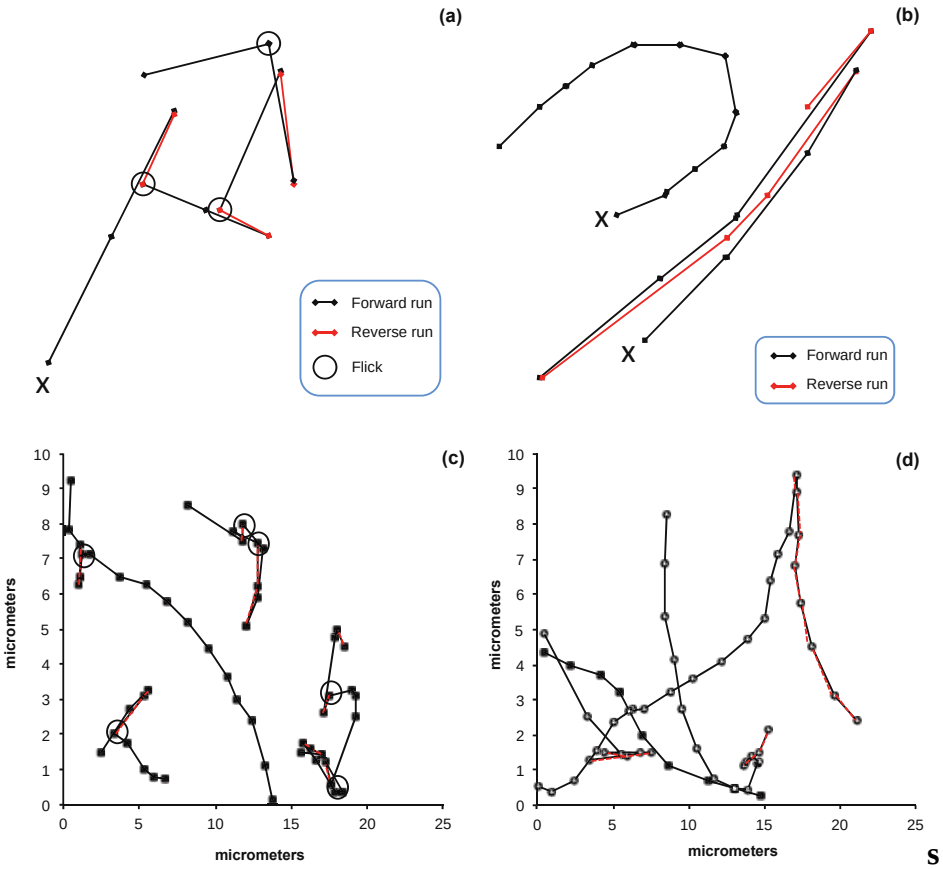
Experimental number of recordings (n) for *V. coralliilyticus* search pattern changes over time for figure 2.5

Time (mins)	Number of recordings					
	Oxic			Anoxic		
	3-step flick	Run-reverse	Straight swimming	3-step flick	Run-reverse	Straight swimming
0	28	11	1	14	22	4
5	25	16	0	9	25	7
10	18	23	0	6	29	5
15	15	25	0	4	25	11
20	18	21	1	4	17	19
25	16	21	3	1	30	9

2.6 Corrigendum

This corrigendum was included as the figure on the following page was adjusted so that the x-axis and y-axis were equal to give an accurate representation of the bacterial cell search paths (figure B) compared to the figure previously included in this earlier manuscript of this thesis (figure A). The following figure (figure B) also outlines the movements made by *V. coralliilyticus* and how they were characterized into search pattern types throughout this thesis.

A


(a)
(b)
X
X
X
Forward run
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Flick
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(c)
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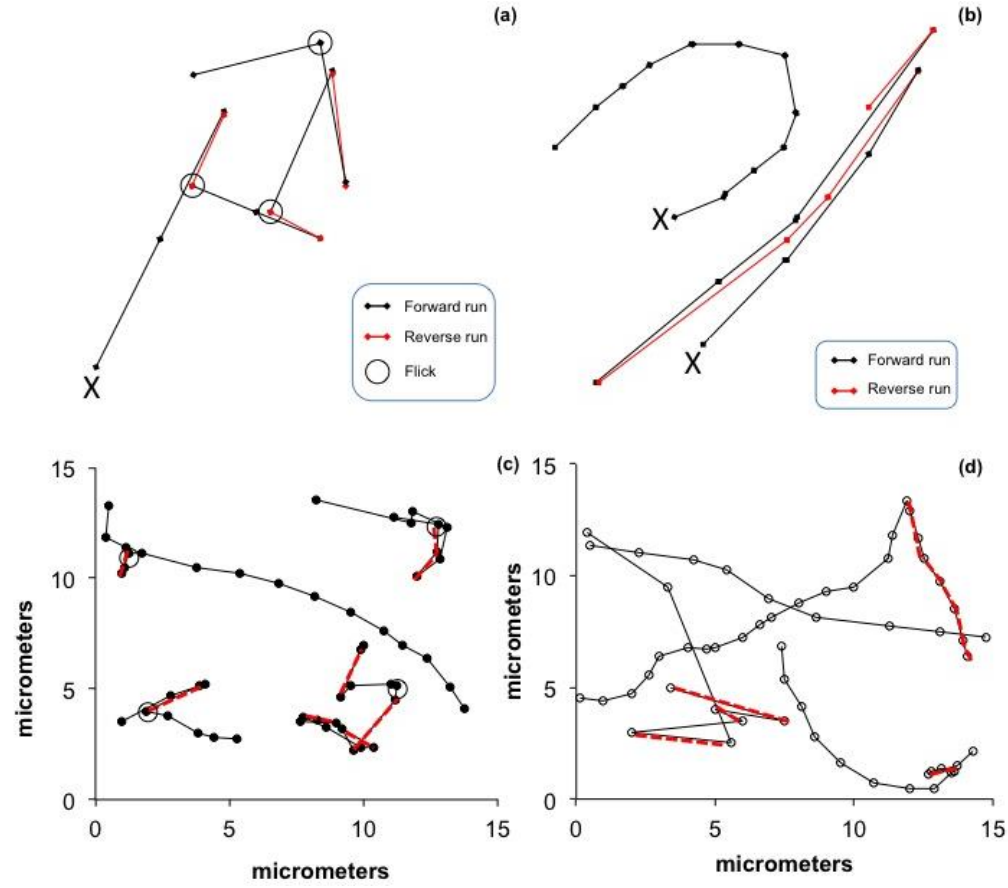
(a)
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Forward run
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Flick
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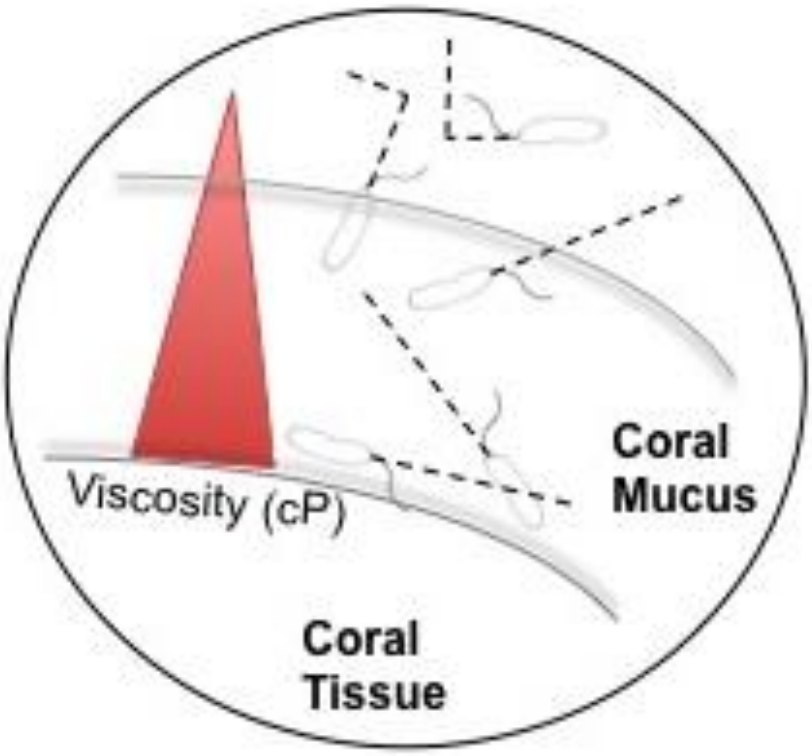
Chapter 3

Increased drag stops chemotactic flicks in *Vibrio coralliilyticus*



Submitted as:

Winn KM., Bourne D., Mitchell JG (2015). Increased drag stops chemotactic flicks in *Vibrio coralliilyticus*. Journal of Bacteriology (In Review).

Chapter cover figure: A visual summary of the component influencing the search pattern response in chapter 3, chemotactic motility through the coral mucus layer, which is further discussed in chapter 6.

3.0 Abstract

Chemotaxis of the coral pathogen, *Vibrio coralliilyticus* was investigated across a transect line spanning from the culture media to coral mucus. This transect line identified viscosity and oxygen gradients between these microenvironments. Three-step flick search patterns ('flicks') occurred with greater frequency in the culture medium with an average value of 0.73 flicks s^{-1} observed (SEM = 0.07, n = 98), compared to the mucus region, with 0.07 flicks s^{-1} (SEM = 0.01, n = 197). Straight swimming search patterns differed significantly across the culture to mucus interface (P = 0.028) with an average value of 0.64 (SEM = 0.05, n = 347) straight swimming paths s^{-1} and 0.71 (SEM = 0.05, n = 306) straight swimming paths s^{-1} respectively. Similarly, significant differences were observed (P = 0.013) in the average number of run-reverse search patterns across the transect with average values ranging from 0.29 (SEM = 0.04, n = 145) run-reverses s^{-1} in the culture to 0.09 (SEM = 0.03, n = 126) run-reverses s^{-1} in the mucus region. Tracking experiments that assessed the search patterns of *V. coralliilyticus* in solutions of differing viscosities demonstrated that straight swimming patterns were 14-fold more likely to occur in liquids with viscosities greater than 28 centipoise (cP) than 3-step flick search patterns. Flick search patterns also appeared less frequently in low viscosity media ranging between 1.3 – 2.7 cP, with a 15-fold decrease observed between the culture medium and 2.7 cP mucin control. Results from this integrated assessment of motility behavior demonstrate that *V. coralliilyticus's* motility response is dependent on microenvironments, which exhibit fluctuations in oxygen and viscosity. This suggests that the search patterns of monopolar bacteria are limited by the surrounding liquid medium. From this we infer that one defensive function of coral

mucus is the reduction of the reorientation capability of monotrichous bacteria, which may reduce the likelihood of infection.

3.1 Introduction

Bacteria utilize specific search patterns to move through liquids in response to chemicals or nutrients patches (Adler 1966, Mcnab and Koshland 1972, Mitchell 1991, Magariyama *et al.* 2005). For pathogenic microorganisms, chemotaxing to a host is the important first step in an infection (Tomich *et al.* 2002, Meron *et al.* 2009). Past studies have explored how corals respond to pathogenic microorganisms (Ben-Haim and Rosenberg 2002, Koren and Rosenberg 2006), however, few studies have examined the motility response of pathogenic microbes to coral mucus cues. Studying these responses is particularly timely because many bacterial pathogens utilize specific chemotactic search patterns (Berg and Brown 1972, Schnitzer *et al.* 1990, Winn *et al.* 2013). These patterns act to re-orientate the cells in an effort to seek out and utilize available nutrient patches. The distinction between the search patterns is the result of the counter-clock wise and clockwise flagellar motor rotation creating a forward or reverse movement (Silverman and Simon 1974, Khan and Macnab 1980). The buckling of the motor presumably due to the cellular speed, allows for a 90° reorientation flick event (Stocker 2011, Son *et al.* 2013). *V. coralliilyticus's* search pattern changes with increases in viscosity demonstrates the adaptability of chemotaxis in this species and potentially in many other species. These results suggest that viscous mucus boundaries do not trap all bacteria, but require that bacteria modify their behavior to traverse through the mucus.

In the marine environment, many invertebrate and vertebrate species possess viscous mucus layers. Fish have measured surface mucus viscosities from 18 to 1454 cP compared to the 1 to 1.8 cP of the surrounding seawater (Bordas *et al.* 1998, O'Toole *et al.* 1999, Larsen *et al.* 2006). Previously, the exact viscosity of coral mucus has been tentative, in part due to the mixing with the seawater (Brown and Bythell 2005). Past studies have highlighted that mucus provides a barrier against infection (Bordas *et al.* 1998, Deplancke and Gaskins 2001, Shnit-Orland and Kushmaro 2009), potentially through slowing down the cell's forward movement due to the viscosity of the liquid, which may prevent infection of the underlying tissue (Berg 1974, Atsumi *et al.* 1992, Xing *et al.* 2006, Chen *et al.* 2008). However, in other studies, mucus layers have been identified to provide *Vibrios* a surface which assists in aiding the formation of biofilms (Nyholm *et al.* 2002, Banin *et al.* 2006). Furthermore, some bacteria may show chemotaxis to the mucus as it contains chemoattractants (Bordas *et al.* 1998, Nithyanand *et al.* 2011). The mucus layers are also vital for preventing desiccation of the coral (Krupp 1984, Davis and Hawkins 1998, O'Toole *et al.* 1999, Brown and Bythell 2005, Krediet *et al.* 2013). Bacteria slow at greater than 2 – 5 cP due to increased drag (Schneider and Doetsch 1974, Shigematsu *et al.* 1998), which may act to decrease the flagella rotation (Schneider and Doetsch 1974, Kaiser and Doetsch 1975). *Campylobacter jejuni* possesses a single, polar flagellum and maintains speeds of 60 – 100 μm s^{-1} at greater than 40 cP. This adaptation is a competitive advantage over other bacteria because it permits infiltration of intestinal mucus layers, which subsequently leads to infection and greater access to nutrients (Ferrero and Lee 1988).

Chemotaxis in *Vibrio coralliilyticus* is believed to be a critical factor involved in pathogenesis (Hada *et al*. 1984, Ben-Haim and Rosenberg 2002, Ben-Haim *et al.*

2003, Austin *et al.* 2005, Sussman *et al.* 2008, Pollock *et al.* 2010). Meron *et al.* (2009) demonstrated that non-motile *V. coralliilyticus* mutants were not able to adhere to the coral tissue or cause infection. To further investigate the influence of the mucus boundary layer on *V. coralliilyticus's* motility, we investigated the search pattern trajectories under increasing viscosity conditions using a culture media and coral mucus boundary interface to simulate the natural seawater to coral mucus interface.

3.2 Materials and Methods

3.2.1 Bacterial culture and growth

The type strain of *V. coralliilyticus*, LMG20984 isolated from *Pocillopora damicornis* was used for all experiments (Ben-Haim *et al.* 2003). Isolated cultures were stored at -80°C in 15% glycerol stock solutions and grown on thiosulphate citrate bile salts sucrose (TCBS) agar media (Sigma Aldrich) overnight at 28°C. Liquid cultures were prepared in 30mls of Luria-Bertani (LB) broth (Sigma Aldrich) and incubated overnight at 28°C with 160 rpm shaking.

3.2.2 Sterile artificial and native coral mucus controls

Due to the presence of mucus surface layers in corals and the potential for this mucus to influence flagella behavior of motile bacterial cells, we studied the effect of viscosity on search pattern tracking behavior. Sterile methocel and mucin formed the controls to assess the influence of viscosity on search pattern tracking behavior. This was to confirm whether the motility responses observed in the culture media – mucus

transect, were also present in non-chemotactic sterile viscosity controls. Coral mucus thickness is a visible and immediate response to changing environmental conditions (Jatkar *et al.* 2010). The protection and lubricating properties of mucus are largely governed by the polymeric high molecular weight mucin which is responsible for the gel-forming properties of the mucus (Jatkar *et al.* 2010). The changes in the rheological properties of mucus such as film formation, adhesiveness and gelation (Schrager 1970) can greatly affect the protective and lubricating function of coral mucus (Jatkar *et al.* 2010) and the physical properties are intimately associated with the biological function of mucus (Schrager 1970). Coral mucus as a non-Newtonian fluid shows a disproportionate increase in viscosity at low flow velocities (Jüngst *et al.* 2001) where changes in viscosity occur in response to changing rates of shear (Curt and Pringle 1969).

Methocel (Sigma-Aldrich; 3000 – 5500 cP), diluted to 0.5% (28 cP) and 1% (66 cP) was used to provide a high viscosity sterile medium to measure the chemotactic search patterns in *V. coralliilyticus*. This media provided a sterile equivalent to mimic the higher natural viscosity conditions of native coral mucus. Twenty cells were tracked for each experiment and experiments performed in triplicate.

The kinematic viscosity of the 1% methocel was confirmed using a DIN Ford viscosity cup (Gardo) using the flow method by gravity, where the rate of flow is proportional to the kinematic viscosity expressed in centistokes (cSt). This implies that the longer the liquid takes to flow through the cup, the higher the viscosity of the liquid. Dynamic viscosity was then calculated by using the equation:

$$\mu = vx\rho \quad (1)$$

where μ is dynamic viscosity, v is kinematic viscosity and ρ is the density. The viscosity was measured 10 times using the viscosity cup, and the values averaged. The efflux time was converted to kinematic viscosity (cSt) using the equation;

$$v = 3.85(t - 4.49) \quad (2)$$

Where *t* is the efflux time and *v* is the kinematic viscosity (ASTM #D 1200 Standard, Cannon Instrument Company, Pennsylvania).

Porcine gastric mucin type III (Sigma Aldrich) at 5 µg mL^{-1} was used to artificially simulate diluted coral mucus. Low mucin viscosity controls ranging between 1 and 2 cP were also tested for this experiment due to reports that the mixing of mucus with surrounding seawater has been shown to reduce the viscosity and in some cases made it difficult to discern from the viscosity of water (Garren and Azam 2010, Garren and Azam 2011). The mucin was dissolved in 0.1 M acetate buffer at pH 5.0 and adjusted to pH of 7.0 with sodium hydroxide to final concentrations of 0.1, 0.5, 1.0 and 2.0 %. The viscosity of all mucin solutions was measured using a Zahn viscosity cup using the method previous described for the methocel control.

A 10X dilution stock of *V. coralliilyticus* was created from the overnight LB culture, with fresh sterile LB used for the dilutions. 50 µL of diluted culture was added to 0.5 mL of mucin and gently inverted to mix prior to transferring into a slide observation chamber and viewed immediately. Videos were recorded for 10 s at 4.9 fps. All videos were analyzed and distinguishable cells were tracked and their search pattern paths collected. Cells were determined to be distinguishable if they remained in the field of view and in focus for at least 5 frames to allow for a potential flick

search pattern to occur. Three replicates of twenty trajectory search paths were tracked for each mucin and methocel experiment.

We additionally assessed the motility behavior of any unknown bacteria in the native coral mucus collected. Coral mucus was collected from *Acropora millipora* colonies maintained in 300 L aquaria holding tanks at the Australian Institute of Marine Science. Mucus was collected by removal of the coral from the aquarium and removal of residual seawater by gentle shaking and then exposing the coral colony to air for two to four minutes. The colonies released substantial quantities of mucus, with 5 mL collected by balancing the coral nubbins over a sterile 50 mL falcon tube. Mucus 'flocs' or 'ropes' were gently pipetted off using disposable plastic Pasteur pipettes that had 1 cm of the tip cut off to minimize structural changes to the mucus through pipetting. The coral mucus was used immediately or stored at 4°C and used within 4 hrs of collection.

Native mucus was used without filtration or autoclaving to minimize physical or chemical changes that could have occurred due to the introduction of heat or gravitational force. Ten video replicates of 10 s duration were recorded of the native coral mucus and if any motile bacteria were observed, these were counted and analyzed. The native bacterial cells were observed in terms of cell shape, speed and cell presence in reference to the number of frames which captured the cell, this was in an effort to distinguish the native bacteria observed from the culturally grown *V. coralliilyticus.*

LB broth media was used as a low viscosity control. A separate 50 mL sample of coral mucus was collected from multiple colonies for measuring its' viscosity. The kinematic viscosity of the coral mucus and 0.5% methocel solution

was measured using a Zahn viscosity cup (2210/1 Elcometer), using the flow method by gravity. A different viscosity cup was used for the coral mucus and 0.5% methocel solution due to the differing viscosity requirements of each cup. The cup was filled separately with the collected mucus and 0.5% methocel solution and the length of time for the cup to run empty was timed using a stopwatch. Ten recordings were taken and the values averaged. The efflux time was converted to kinematic viscosity (cSt) using the equation;

$$v = 1.1(t - 29) \quad (3)$$

Where *t* is the efflux time and *v* is the kinematic viscosity (ASTM #D 4212 Standard, Cannon Instrument Company, Pennsylvania)..

3.2.3 Microscope observation chamber and transect line experiments

The slide observation chamber was constructed from two glass coverslips on a glass slide and covered with a third coverslip to form a channel 0.17 mm high and 15 mm wide (Fig. 3.1). A volume of 25 μl of diluted cell culture was added to one side of the observation chamber and 25 μl of coral mucus was added to the other side of the observation chamber. Motile cells were observed within the observation chamber rather than the surface, which was used to observe swimming cells without wall effects (Winn *et al.* 2013). Twelve replicate transect lines were collected with each transect line consisting of 14 adjacent square eyepiece quadrats measuring 172 by 172 μm. The transect quadrats spanned the culture medium through the medium-mucus interface and into the coral mucus covering distance of 2,400 μm. The

interface spanned between 690 to 860 µm in distance (Fig. 3.1). At each quadrat a 10 second, 49-frame video was recorded. All distinguishable cells in each eyepiece quadrant video were tracked and analyzed. All distinguishable cell trajectory paths were classified into one of three search patterns based on the angles of the runs observed while tracking the bacteria's swimming path: namely run-reverses, straight swimming or 3-step flick (Supp. Fig. 3.1). This is particularly the case for the flick events, which can occur within milliseconds. Here, the potential for a flick search pattern was determined by any 90° reorientation of the cell at any one time. Each search pattern trajectory was then measured and the swimming speed calculated.

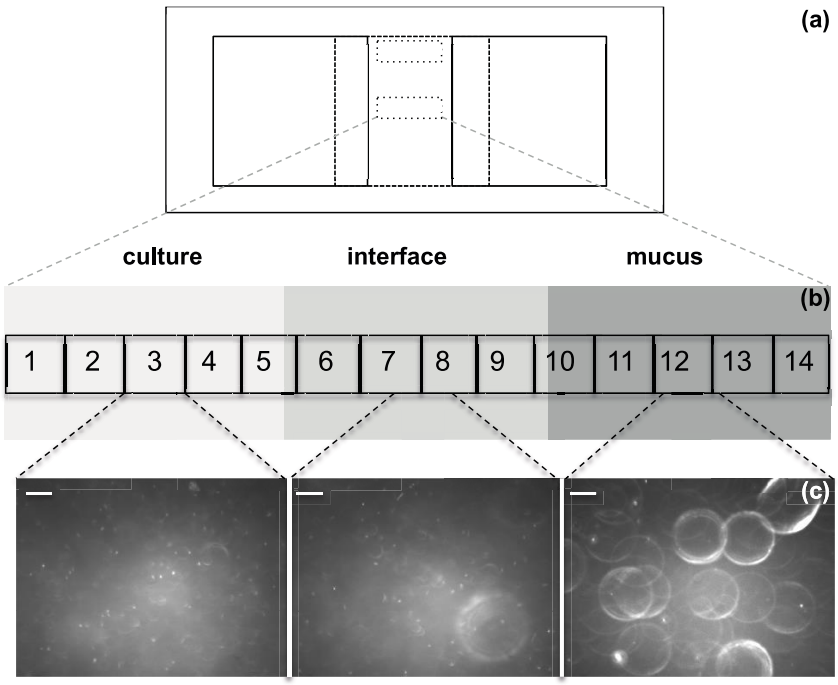


Figure 3.1. The observation viewing chamber set-up. (a) The observation viewing chamber created using cover slips. The dashed rectangles indicate the transect positions, (b) close up schematic of the transect line and individual quadrats, which were 2408 µm and 172 µm in length respectively. (c) Micrographs of the culture, interface and mucus. The diffraction rings indicate particulate matter in the mucus. Scale bar = 20 µm.

3.2.4 Statistical analysis

A *t*-test was used to analyze whether significant differences in the flagella energy usage were identified across chemotactic search patterns and across differing viscosities. Standard error of the means was used for all replicate data collected to examine the error estimates for all data. The data replicates for the mucin and methocel experiments were obtained by analyzing twenty cells and performed in triplicate. A chow test was used to statistically analyze whether the coefficients in two linear regressions on different data sets were equal (Toyoda 1974, Wilson 1978, Prescott 1986).

3.2.5 Energy Budget Estimates

Numerical estimates of the motility energy budget in differing viscosities in differing viscosities were calculated by:

$$P = 6\pi\eta a v^2 \quad (4)$$

Where P is power, η is dynamic viscosity, a is cell radius and v is cell velocity. All calculations used cgs units (Mitchell 1991).

3.2.6 Search pattern analysis

All cell trajectories were classified into search patterns and the results were analyzed using SPSS Statistics (version 19.0). Independent sample t-tests were used to calculate P values to establish whether search pattern types differed in average

number or speed across the culture medium, the medium – mucus interface and the mucus regions. Independent sample *t*-tests were used to analyze whether significant differences in search pattern type existed between mucin and methocel concentrations. The *t*-tests were also used to examine any significant results between search pattern speeds and the mucin and methocel concentrations.

3.3 Results

3.3.1 Culture media – mucus interfaces influence search pattern behavior of *V. coralliilyticus*

The chemotactic search pattern behavior of *V. coralliilyticus* changed spanning from the culture medium to mucus identified using microscopic transects. The average numbers of 3-step flick search patterns decreased along the transect line (P = 0.007), from 0.73 s^{-1} (SEM = 0.07, n = 98) in the culture region to 0.02 s^{-1} (SEM = 0.01, n = 197) in the mucus region (Fig. 3.2). The average numbers of run-reverse search patterns decreased (P = 0.006) from 0.29 s^{-1} (SEM = 0.04, n = 98) in the culture region, to 0.09 s^{-1} (SEM = 0.03, n = 197) in the mucus region. This decrease fluctuated and there was no linear decline as observed with the flick search patterns. Straight swimming search patterns occurred in high frequency in both the culture and mucus region (P = 0.028), with an average of 0.64 s^{-1} (SEM = 0.05, n = 98) straight swimming search patterns observed in the culture region and an average of 0.71 s^{-1} (SEM = 0.05, n = 197) straight swimming search patterns in the mucus regions (Fig. 3.2). There was no significant difference observed between straight swimming search patterns across the culture – mucus interface (P = 0.287).

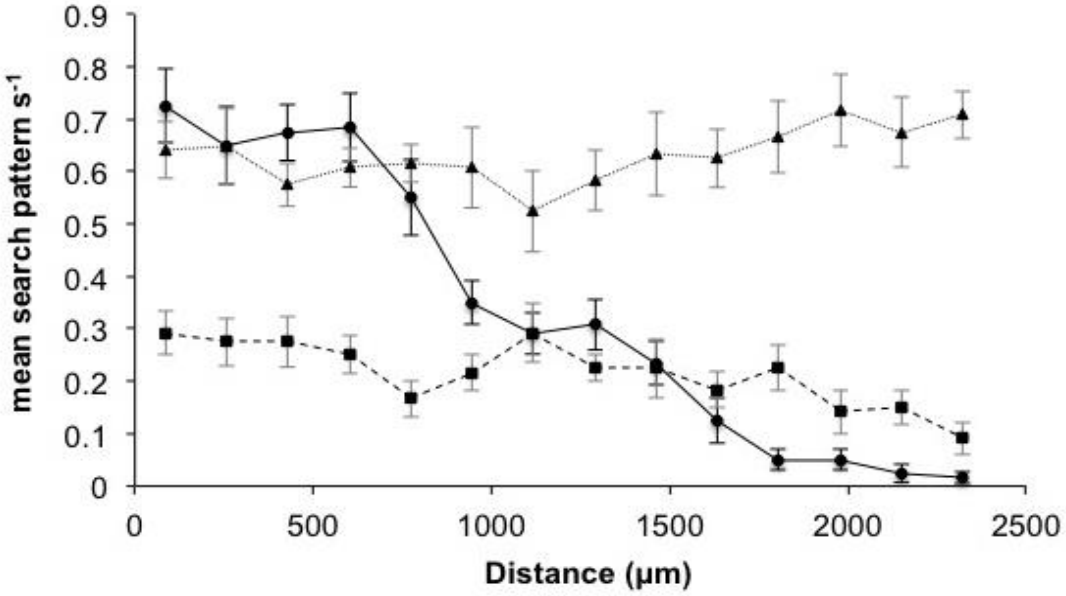


Figure 3.2. Chemotactic search patterns identified across a culture-mucus interface. **The average numbers of search patterns identified across a culture media to mucus interface. The circles and solid line represents the 3-step flick search patterns, the square and dashed line represents the run-reverse search patterns and the triangle and dotted line represents the straight swimming search patterns (error bars = SEM).**

Search pattern speed increased from the culture region to mucus region with 3-step flick speeds increasing from an average of 43 µm s^{-1} (SEM = 2.4, n = 98) in the culture region to 54 µm s^{-1} (SEM = 3.3, n = 197) in the mucus region (P = 0.01). Run-reverse search patterns increased from 47 µm s^{-1} (SEM = 2.8, n = 98) in the

culture region to 62 µm s^{-1} (SEM = 5.4, n = 197) in the mucus region (P = 0.032) and straight swimming search patterns increased from 48 µm s^{-1} (SEM = 3.8, n = 98) in the culture region to 56 µm s^{-1} (SEM = 3.8, n = 197) in the mucus region (P = 0.024) (Fig. 3.3). In the culture region there was a difference of 4.6 µm s^{-1} between all chemotactic search pattern averages, compared to 7.8 µm s^{-1} in the mucus region.

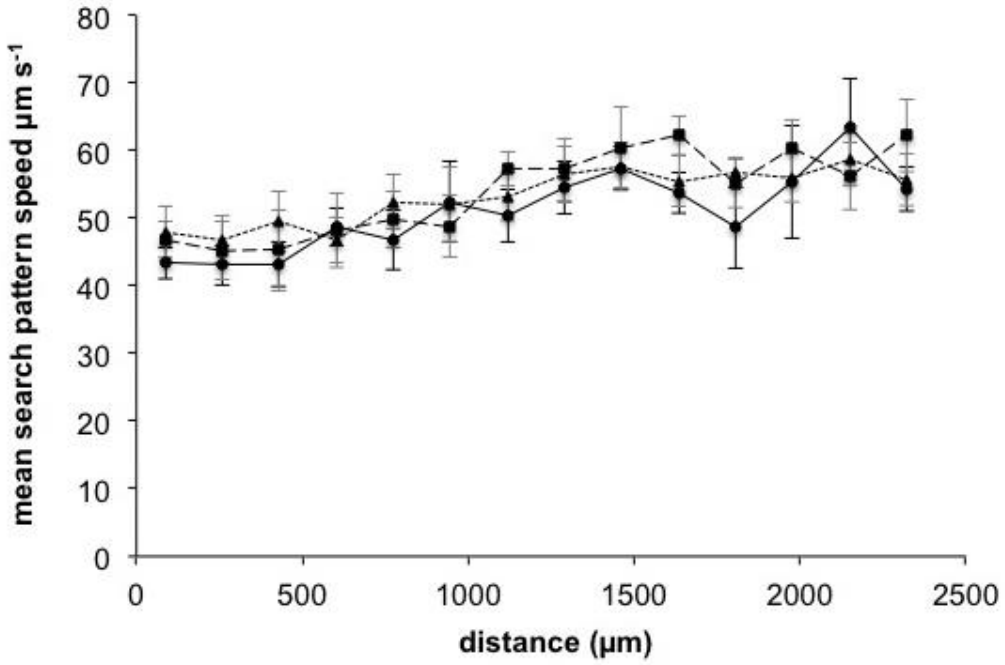


Figure 3.3. Chemotactic search pattern speeds identified across a culture-mucus interface. **The mean search pattern speeds identified across a culture media to mucus interface. The circles and solid line represents the 3-step flick search patterns, the square and dashed line represents the run-reverse search patterns and the triangle and dotted line represents the straight swimming search patterns (error bars = SEM).**

Due to the use of native coral mucus in the experiments, native coral mucus was examined for microorganisms. The coral mucus collected from live corals, identified between 0 – 3 native motile cells per video with an overall average of 0.08 cells s^{-1} (SEM= 0.07, n = 8) visible. Only straight swimming search patterns were observed as being used by the native motile bacteria in the coral mucus and these bacteria exhibited an average speed of 89 μm s^{-1} (SEM = 17.7, n = 8). No flick or run-reverse search patterns were observed. The native mucus motile cells only remained in the microscope field of view for 1 – 3 frames only, which did not allow enough frames for a potential flick to occur and were not consider 'disguishable'. 10 videos of the native coral mucus were collected and reviewed for motile cells. As *V. coralliilyticus* cells were inoculated in high numbers (1 x 10^6 cells mL^{-1}), this resulted in all motile cells measured in the transect experiments being the inoculated cells. Approximately half of the inoculated cells in coral mucus were motile and exhibited speeds of 59 μm s^{-1} (SEM = 0.4, n = 56).

3.3.2 Native mucus, methocel and mucin viscosity values

Ten viscosity recordings were collected for the native coral mucus using a viscosity cup. Values were as follows, 56, 60.5, 58, 53, 51.5, 57, 55.5, 54, 59.5 and 55 s. These values were averaged and gave a mean value of 56 s, which was incorporated into equation 2 and gave a viscosity value of 29.7 cP (SEM = 0.9, n = 10).

Ten viscosity recordings were collected for the 0.5% methocel control using a viscosity cup. Values were as follows, 56.5, 51.5, 52, 57, 56, 58, 49, 52, 58.5 and 54.5 s. These values were averaged and gave a mean value of 54.5 s, which was incorporated into equation 2 and gave a viscosity value of 28 cP (SEM = 1.0, n = 10).

Ten viscosity recordings were collected for the 1% methocel control using a viscosity cup. Values were as follows, 22.5, 18, 18.5, 22, 20, 23, 23.5, 21, 24.5 and 22 s. These values were averaged and gave a mean value of 21.5 s, which was incorporated into equation 1 and gave a viscosity value of 66 cP (SEM = 0.7, n = 10).

Five viscosity recordings were collected for the 0.1% mucin control using a viscosity cup. Values were as follows, 30.2, 29.6, 32.1, 29 and 30.1 s. These values were averaged and gave a mean value of 30.2 s, which was incorporated into equation 2 and gave a viscosity value of 1.3 cP (SEM = 0.5, n = 5).

Five viscosity recordings were collected for the 0.5% mucin control using a viscosity cup. Values were as follows, 30.5, 32, 29, 31.5 and 29.5 s. These values were averaged and gave a mean value of 30.5 s, which was incorporated into equation 2 and gave a viscosity value of 1.7 cP (SEM = 0.6, n = 5).

Five viscosity recordings were collected for the 1% mucin control using a viscosity cup. Values were as follows, 30.1, 29.5, 29, 31.5 and 30.4 s. These values were averaged and gave a mean value of 30.1 s, which was incorporated into equation 2 and gave a viscosity value of 2.3 cP (SEM = 0.4, n = 5).

Five viscosity recordings were collected for the 2% mucin control using a viscosity cup. Values were as follows, 31.5, 33, 31, 28.5 and 33.5 s. These values were averaged and gave a mean value of 31.5 s, which was incorporated into equation 2 and gave a viscosity value of 2.7 cP (SEM = 0.9, n = 5).

An average of 0.5 s^{-1} 3-step flick search patterns were identified in the 1 cP mucin control, with a mean speed of 65.3µm s^{-1} (SEM = 0.6, n = 120). In contrast, an average of 0.28 s^{-1} run-reverses and 1.2 s^{-1} straight swimming search patterns were

identified at the 1 cP mucin, with a speed of 65.7 µm s^{-1} (SEM = 0.3, n = 120) and 70.7 µm s^{-1} (SEM = 1.4, n = 120) respectively.

Contrastingly, at 66 cP an average of 0.17 s^{-1} flicks was identified with an average speed of 31.0 µm s^{-1} (SEM = 0.7, n = 120) (P = 0.014). Similarly, an average of 0.05 s^{-1} run-reverses and 1.93 s^{-1} straight swimming search patterns was identified with an average speed of 34.6 µm s^{-1} (SEM = 0.7, n =120) and 31.8 µm s^{-1} (SEM = 1.7, n = 120) respectively (Fig. 3.4). An average speed difference of 34 µm s^{-1} was observed between flick search patterns between the low viscosity control of 1 cP and the higher viscosity methocel control of 66 cP. Similarly, an average difference of 31 µm s^{-1} and 39 µm s^{-1} for run-reverse and straight swimming search patterns respectively was observed between the low viscosity of approximately ≤ 2 cP, and high viscosity of approximately ≥ 28 cP controls. Changes in search pattern averages and speed were observed with the increasing viscosity values (P = 0.002) (Fig. 3.4).

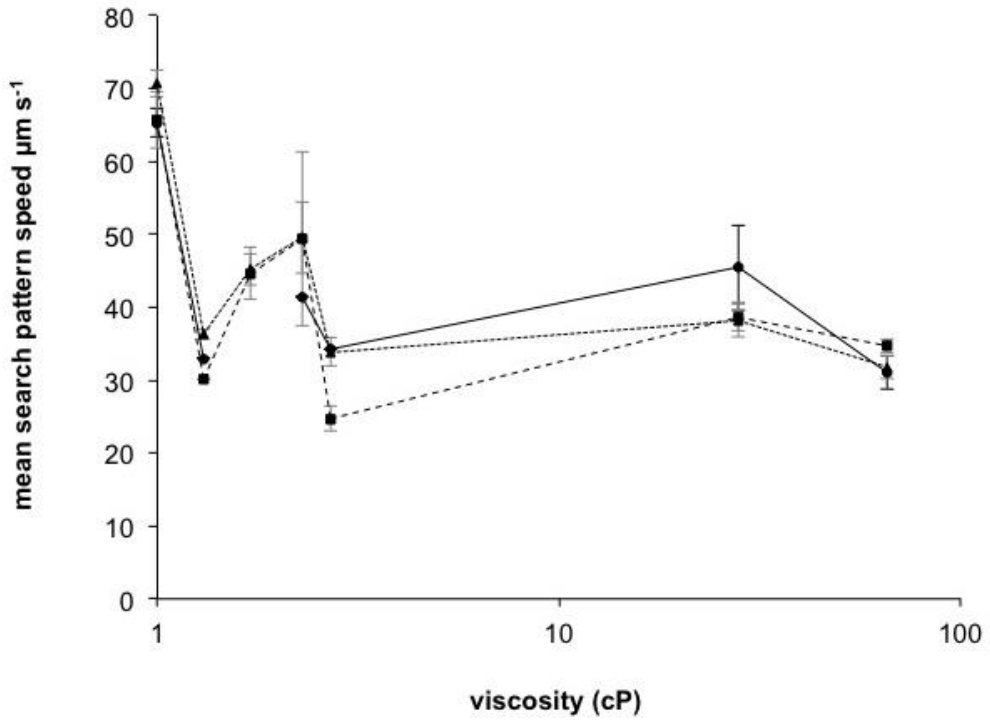


Figure 3.4. Differences in search pattern speed with increasing viscosity. **The mean search pattern speeds identified in solutions of various viscosity (cP). The circles and solid line represents the 3-step flick search patterns, the square and dashed line represents the run-reverse search patterns and the triangle and dotted line represents the straight swimming search patterns (error bars = SEM).**

3.3.3 Motility energy analysis

Examination of the motility energy analysis identified significant differences between the search patterns speeds and the viscosity of the media ($P = < 0.05$) (Fig. 3.5). In lower viscosity media, identified as $\leq$ 2 cP, energy requirements generally decreased with increasing viscosity values. However, at 2.3 cP mucin the energy requirements for all search patterns were 10-fold greater with an mean value of $1.1x10^{-9}$ cm gram seconds (cgs), than that required at the 1.7 cP or 2.7 cP mucin solutions ($P = 0.028$) with values of $6.5x10^{-10}$ cgs and $5.1x10^{-10}$ cgs respectively (Fig. 3.5). A 100-fold difference was observed in the energy requirement between the 1 cP culture media with a mean value of $8.4x10^{-10}$ cgs, and the 28 cP methocel solution with a mean value of $8.4x10^{-9}$ cgs for all search patterns. Search patterns in the 66 cP methocel had an energy requirement of $1.3x10^{-8}$ cgs which was $1.2x10^{-8}$ cgs more flagella energy than the search patterns in 1 cP culture media. Similarly, a 10-fold energy requirement increase was noted between the 1 cP culture media and 28 cP methocel solution ($P = 0.008$). However, there were no significant differences in the flagella energy expenditure between the 3-step flick, run-reverses and straight swimming search patterns ($P = 0.566$) (Fig. 3.5). In addition, the linear regression

analysis using the chow test identified no significant difference between linear regression lines of viscosity and search pattern types (Fig. 3.5).

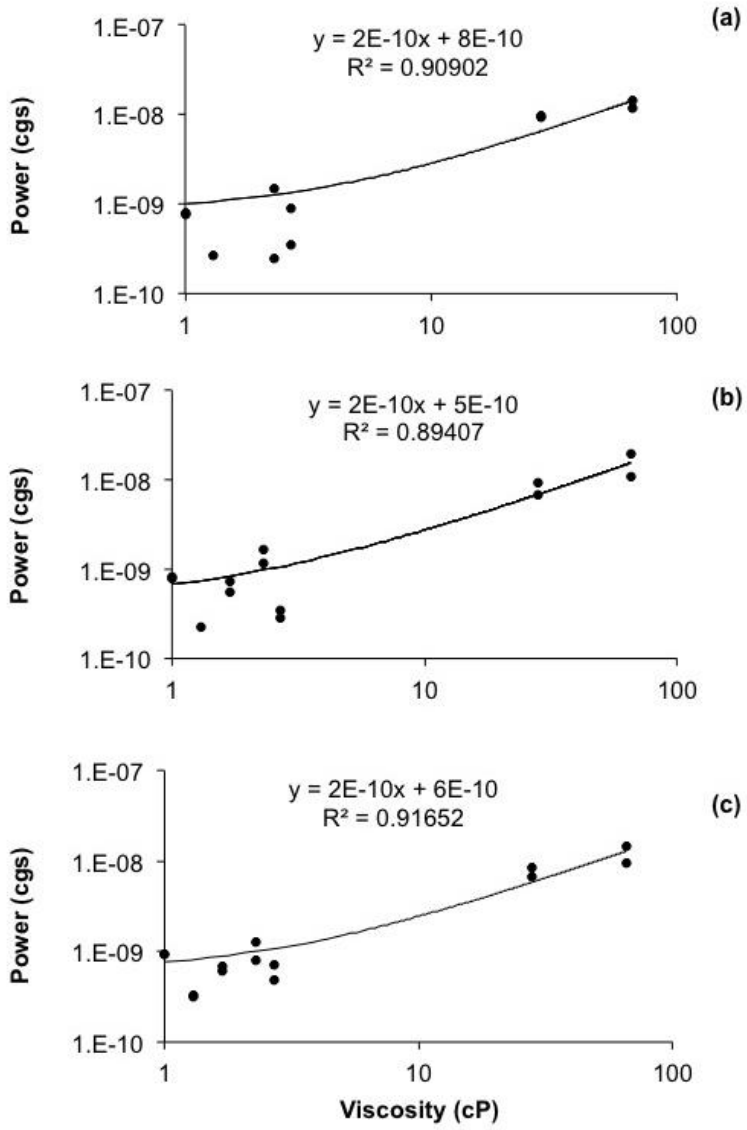


Figure 3.5. Energy requirements for *V. coralliilyticus* at differing viscosities. **The energy requirements for *V. coralliilyticus* in solutions of differing viscosities during (a) 3-step flick search patterns, (b) straight swimming search patterns and (c) run-reverse search patterns.**

3.4 Discussion

Vibrio coralliilyticus is an emerging coral pathogen and has been implicated in causing disease lesions in corals on reefs around the world (Ben-Haim and Rosenberg 2002, Ben-Haim *et al.* 2003, Sussman *et al.* 2008, Pollock *et al.* 2010). Using chemotaxis to find a host is the first step of the initial stage of a bacterial infection, which has been widely studied (O'Toole *et al.* 1996, Otteman and Miller 1997, Josenhans and Seurbaum 2002). The importance of motility and chemotaxis has also been linked to *V. coralliilyticus*, with Meron *et al.* (2009) reporting that *V. coralliilyticus* mutants lacking a flagellum are incapable of causing infection and tissue lesions in corals. We have previously demonstrated the dynamics of *V. coralliilyticus* across oxic and anoxic conditions (Winn *et al.* 2013), which are found spanning the coral tissues. In this study we identify that chemotactic search pattern strategies of *V. coralliilyticus* respond to the changing viscosity parameters in an artificially constructed chamber mimicking the seawater and mucus boundary layer. These results suggest that straight swimming search patterns may be more proficient in mucus layers or viscous liquids than others. In light of previous reports (Garren *et al.* 2013) that have described an increase in velocity of *V. coralliilyticus's* search patterns in mucus regions and chemotactic behavior towards coral mucus, we

assessed whether flick search patterns are prevalent in mucus regions and in higher viscosity media.

3.4.1 Impacts of culture media – mucus interfaces on *V. coralliilyticus* cell motility

V. coralliilyticus alters its search pattern behavior in accordance with day and night oxygen fluctuations, with 3-step flicks occurring more frequently during oxic daylight hours than during anoxic night time hours (Winn *et al.* 2013). In this current study we observed that across a culture media to mucus interface, the search patterns were dependent on the surrounding environment, namely the media or mucus. Three-step flick search patterns decreased to an average of 0.02 s^{-1} (SEM = 0.01; n = 197) search patterns in the mucus region compared to 0.73 s^{-1} (SEM = 0.07; n = 98) flicks in the culture region. This suggests that the mucus may impede or influence the search pattern behavior of *V. coralliilyticus*. This impediment may in part be due to the structural complexities of coral mucus, which has previously been suggested to impede the rotation of the flagella, thus affecting the motility of microbes (Berg 1974, Atsumi *et al.* 1992, Xing *et al*. 2006, Chen *et al.* 2008).

Our results show than whilst chemotactic search patterns differed in the mucus compared to the culture media, *V. coralliilyticus* remains motile and so still has the potential to reach the coral tissue surface. The ability of microorganisms to alter their chemotactic search strategies is primarily due to the capability of the flagellar motor to alter its rotational direction (Berg 2003). This rotation mechanism is fundamental to the forward and reversing movements observed in many search patterns, as well as the 90° directional change caused by the whip like motion of the flagella (Stocker 2011, Xie *et al.* 2011). Changes in viscosity can influence the

flagellar helix and reduce the efficiency of the propulsion of the flagella (Schneider and Doetsch 1974, Shigematsu *et al.* 1998). Some flagellated bacteria have shown an initial increase in search pattern speed when viscosity increased up to 2 cP, however mean search pattern speed values decreased when viscosity increases beyond 2 cP (Shoesmith 1960, Schneider and Doetsch 1974, Shigematsu *et al.* 1998). *V. coralliilyticus* cells utilizing the 3-step flick search pattern strategy exhibited a 14 µm s^{-1} increase in speed in the mucus region than identified in the culture region which is consistent with the observations of Schneider and Doetsch (1974). This indicates that the search pattern speed is not a result of the specific search patterns themselves, rather the regional differences or chemotactic properties associated with the regional changes across the coral mucus boundary layer. In viscous solutions greater than 2 cP, *V. coralliilyticus* displayed a reduction in speed of flick search patterns. A 7.2 µm s^{-1} decrease between the 2.3 and 2.7 cP mucin was also identified which is again consistent with the studies of Schneider and Doetsch (1974), and Shigematsu *et al.* (1998). In these studies, the motility of numerous motile bacteria including *Campylobacter jejuni* was examined in low and high viscosity solutions ranging between 1.7 and 74.7 cP. These studies identified an increase in cell speed corresponding with an increase in viscosity values before reaching a maximum speed threshold at 2.5 cP. Cell speed decreased beyond 2.5 cP. Similarly, both run-reverse and straight swimming search patterns also displayed a reduction in speed of 24.6 µm s^{-1} and 15.7 µm s^{-1} respectively. This decrease may be potentially due to the increased inefficiency of the flagella whilst propelling the cell through mid-range viscous solutions of approximately > 2 cP, despite previous reports that illustrate that only high-range viscous conditions greater than 28 cP increase the amount of drag

and therefore slow down search pattern speeds (Schneider and Doetsch 1974, Shigematsu *et al.* 1998).

Three-step flick search patterns identified in the mucus showed a decrease in speed compared to flick patterns in the culture region. Similarly, run-reverse and straight swimming search patterns also decreased in speed between the 2.3 and 2.7 cP mucin solutions. This is consistent with the report by Schneider and Doetsch (1974). Viscous solutions have been identified to cause dampening, which prevents momentum through the fluid (Lauga and Powers 2009). In addition, Schneider and Doetsch (1974) also reported that polar flagellated bacteria, like *V. coralliilyticus* had a smaller speed increase in viscous solutions than peritrichously flagellated bacteria. This has been explained in Berg (1975) where cells that are pushed by a single flagellum or flagellar bundle appeared to move slower when moving through a viscous medium. This pushing approach by a single flagellum as is the case of *V. coralliilyticus* may explain the decrease in velocity that was identified in the 2.3 – 2.7 cP mucin solution.

The motility energy budget calculated that an additional $5.3x10^{-10}$ cgs of power is required by *V. coralliilyticus* cells to use the 3-step flick search pattern, featuring a 90° reorientation flick, compared to the run-reverse search patterns at 2.3 cP. At 2.7 cP, an additional $3x10^{-10}$ cgs of power is required for a flick search pattern to occur than a run-reverse search pattern and at 28 cP, an additional $1.5x10^{-9}$ cgs of power is required for a flick search pattern, than a run-reverse. For this analysis we separated the non-flicks category into run-reverse and straight swimming to ascertain what power load is required for each of the individual search pattern paths identified. This suggests that since the 3-step flick consists of a run-reverse and a 90° reorientation, it is the 90° reorientation itself that requires the higher power load,

since purely run-reverse search patterns are calculated to require less power than flick search patterns (Stocker 2011, Xie *et al.* 2011). This was identified in the motility energy budget analysis where the equation of the line for run-reverse and flick search patterns presented two different y intercept values of $y = 2 \times 10^{-10}x + 5 \times 10^{-10}$ cgs/cP^{-1} compared to $2 \times 10^{-10}x + 8 \times 10^{-10}$ cgs/cP^{-1} respectively. Figure 5 illustrates that with increasing viscosity the power load for all search patterns increases. With flicks requiring the most power, this may influence their numbers in mucus environments. Also, since 3-step flick search patterns are predominately utilized for reorientation, it may not be necessary to continue to use a reorientation search pattern, such as the 3-step flick when in a high chemotactically beneficial area such as the coral mucus.

Garren *et al.* (2013) found that *V. coralliilyticus* is chemotactic to coral mucus, with *V. coralliilyticus* cell speeds increasing when chemotaxing to the coral mucus. Whilst an increase in speed is necessary for flick search patterns to occur (Son *et al.* 2013), flicks were not identified in greater quantities in the mucus as anticipated. The nutrient uptake movement of the run-reverse and the meandering movement of the straight swimming search patterns compared to the reorientation nature of the 3-step flick may suggest why we saw more non-flick search patterns in the coral mucus. The *V. coralliilyticus* search patterns identified here, showed a mean decrease in search pattern speed when in the mucus region. This speed decrease predominately occurred in the straight swimming and run-reverse search patterns as few flicks were identified in the mucus region. This decrease may be a cellular and motility response to areas of high chemoattractant, as the cells do not need to move any considerable distance to find a suitable habitat for chemotactic support and subsequent colonization and establishment. *C. jenjui* possesses an identical flagellar

arrangement to *V. coralliilyticus* and has been identified to increase its search pattern speed in viscous liquids between 8 and 30 cP before decreasing beyond 30 cP (Ferrero and Lee 1988), this increase and decrease of search pattern speed with viscosity supported the results identified for *V. coralliilyticus* in the high viscous mucin controls > 28 cP.

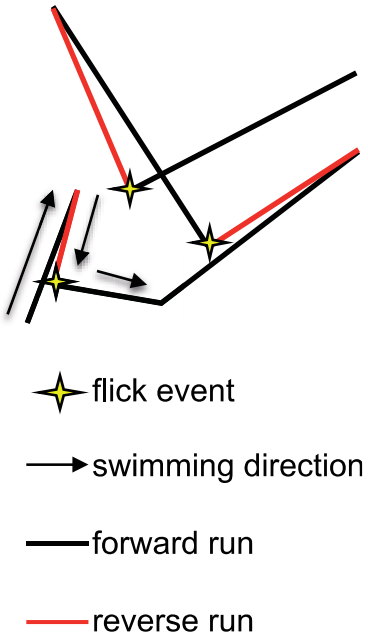
Chemotactic search pattern behavior has been linked to the cell's performance (Larsen *et al.* 2004, Stocker 2011). Specific search patterns, namely the 3-step flick outcompete other cells utilizing other search pattern types for nutrient patches (Larsen *et al.* 2004, Stocker 2011). This indicates that on a basic level, whereby not inferring conscious thought or decision making, chemical gradients or resources may influence the chemotactic search pattern response utilized by the cells (Taktikos *et al.* 2013, Theves *et al.* 2013, Bubendorfer *et al.* 2014). Motile bacteria respond to chemicals in their environment and use search patterns to utilize these chemical patches. Coral mucus contains known attractants including beneficial compounds which have been identified to be a source of chemoattractant for *V. coralliilyticus* (Coles and Strathmann 1973, Benson and Muscatine 1974, Ducklow and Mitchell 1979, Coffroth 1990, Bordas *et al.* 1998, Banin *et al.* 2006, Nithyanand *et al.* 2011). As such, the *V. coralliilyticus* search patterns identified in the coral mucus may be a response to seeking out the chemoattractants, as well as a response to the altered viscosity (Berg 1975, Larsen *et al.* 2004, Taktikos *et al.* 2013).

The results show that *V. coralliilyticus* chemotactic search patterns decrease in speed when in high viscosity solutions. This result confirms the hypothesis by Son *et al*. (2013) that suggests that high viscosity liquids impede and disrupt the flick search pattern utilized by monotrichous bacteria. The unique 90° flick reorientation (Supp. Fig 3.1) is due to a buckling instability of the flagellar hook (2013) which

whilst we only classified the flick based on the angle of the search patterns collected our results suggest flicks occur less frequently in highly viscous media. This may be due to the increasing amount of drag a viscous solution places on a motile cell, which acts to reduce the cell's swimming speed. The swimming speed appears to be an influencing factor on the occurrence of flick search patterns. For example, Son *et al.* (2013) reported that monotrichous bacteria exhibiting speeds of 25 μm s^{-1} or less showed a 70% reduction in flick search patterns than cells swimming at speeds greater than 47 μm s^{-1}. The reduction of flicks is potentially one new way in which mucus may act as a defensive mechanism against possible bacterial invasion. In mucus environments, *V. coralliilyticus* cells can predominately utilize only straight swimming or run-reverse search patterns, which could limit their movement and colonization possibilities.

3.5 Acknowledgments

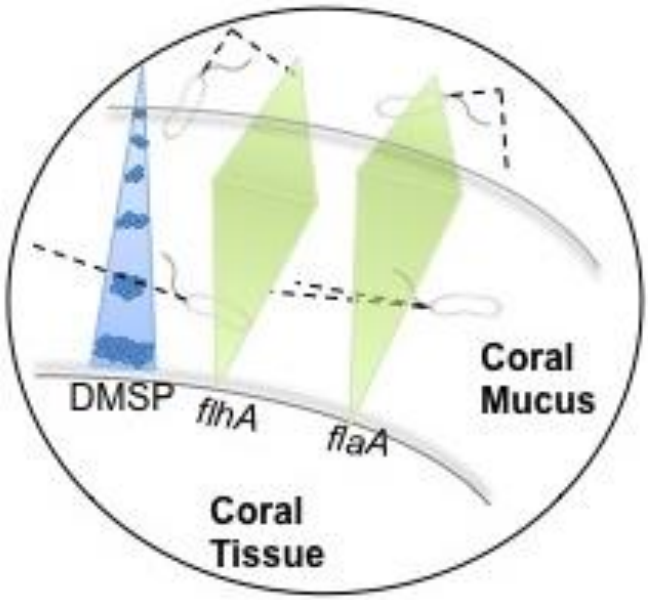
We thank the Australian Research Council and the Centre for Marine Microbiology and Genetics at the Australian Institute of Marine Science for funding support for this research.



Supplementary Figure 3.1. Three-step flick schematic diagram. **A schematic diagram of the 3-step flick, identified by a straight swimming path, a 180° reversal and a 90° reorientation to the side.**

Chapter 4

Coral mucus and DMSP influence flagellar gene copies and chemotaxis of the coral pathogen *Vibrio coralliilyticus*



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Chapter cover figure: A visual summary of the component influencing the search pattern response in chapter 4, gene expression of *flaA* and *flhA* motility genes, which is further discussed in chapter 6.

4.0 Abstract

The flagellin filament outer-sheath protein (*flaA*) and flagellar biosynthesis protein (*flhA*) have previously been linked to the motility and virulence of *Vibrio* species, including *V. alginolyticus* and *V. coralliilyticus* (Wassenaar *et al.* 1991, Josenhans *et al.* 1995, Meron *et al.* 2009). Here, we examined the extent to which gene expression of the *flaA* and *flhA* proteins and chemotactic search patterns were influenced by varying coral mucus and DMSP concentrations to mimic the natural shifts and variance at the coral tissue surface. Various concentrations of DMSP and coral mucus were added to overnight *V. coralliilyticus* cultures and gene expression of *flaA* and *flhA* was analyzed using qPCR. Expression of *flhA* was up-regulated at 7, 13, 20 and 33μM DMSP with peak up-regulation occurring in the presence of 20 μM DMSP. Compared to the control, expression at > 33 μM did not change significantly. In contrast, expression of *flaA* was down-regulated at DMSP concentrations of 7 μM and 67 μM respectively, and up-regulated in the presence of the remaining DMSP concentrations, namely 13, 20, 33 μM DMSP. Peak up-regulation of *flaA* occurred at 13 μM. Three-step flick search patterns reduced by 7.3x when in higher concentrations of DMSP (20 μM) whilst straight swimming search patterns increased 2.2x with increasing concentrations of DMSP. The search pattern and gene expression data suggests that the *flaA* and *flhA* proteins are linked to the differing coral mucus and DMSP concentrations and may fluctuate with changes in nutrient and chemoattractant availability.

4.1 Introduction

Bacteria have the capability to detect and respond to chemical signals within a changing environment (Grebe and Stock 1998). Bacterial cells possess signaling pathways that monitor their external environment and internal physiology (Falke *et al.* 1997). Their capacity to migrate to more suitable environments or a better nutrient supply is critical in providing protection against disadvantageous environments (de Kievit and Iglewski 2000). Despite marine environments being classed as typically nutrient poor (Rothman-Denes and Parsot 2008), coral reefs may provide both cues and resources from its organisms which drive bacterial sensory responses.

Coral reefs contribute significantly to the carbon and nutrient biogeochemical cycles (Froelich *et al.* 1983, Crossland *et al.* 1991, Kleypas *et al.* 1999), which can act as a chemoattractants for bacteria. However, due to the reefs typical confinement to shallow and oligotrophic waters, they are highly dependent on the cycling of nutrients and trace elements by reef-associated bacteria (Raina *et al.* 2010). The coral surface microenvironment is characterized by strong gradients of chemical cues including oxygen, pH, light and photosynthesis in the tissue (Kühl *et al.* 1995). This occurs during the diurnal fluctuations between daylight and nighttime which causes a near depletion of O^2 of <5% air saturation with a pH reduction of >0.5%. These fluctuations occur within minutes of the change in light conditions (Kühl *et al.* 1995). Reef corals are also known major contributors to the production of dimethylsulfoniopropionate (DMSP) and sequentially through the degradation of DMSP by bacteria, dimethylsulfide (DMS) (Yoch 2002, Raina *et al.* 2010), with significant concentrations recorded in scleractinian corals and giant clams (Broadbent *et al.* 2002, Van Alystyne *et al.* 2006, Swan *et al.* 2012).

Whilst dimethylsulfoniopropionate (DMSP) is a major contributing factor of the sulphur cycle (Raina *et al.* 2010), whereby dimethylsulfide (DMS), and its precursor DMSP, has been implicated in the formation of clouds and suggested to exert a major cooling effect on the local and regional climate (Charleson *et al.* 1987, Simó 2001, Yoch 2002), this was not the focus here. Rather, we addressed the signaling response DMSP had on *V. coralliilyticus* and the implication on *V. coralliilyticus's* chemotactic responses on a physical and genetic level. DMSP has been identified as a potent chemotactic cue (Garren *et al.* 2014), which is produced by phytoplankton (Yoch 2002), some dinoflagellates, namely *Prorocentrum sp*. and micro- and macroalgae (Yoch 2002, Stefels *et al.* 2007). DMSP has been identified as a potent chemotactic cue (Garren *et al.* 2014), which has been documented to be produced by marine macro and micro-algae (Stefels *et al.* 2007), and recently by corals (Raina *et al.* 2013). The inclusion and production of DMS and DMSP in corals generates strong chemotactic cues, which may drive bacteria into actively seeking out coral organisms with DMSP also a known chemoattractant to pathogens, including *Vibrio coralliilyticus* (Raina *et al.* 2009, Tout *et al.* 2015).

Bacteria are highly abundant in seawater (Rohwer *et al.* 2001), with some studies suggesting there is a mutually beneficial role between some bacteria and the coral organism (Rohwer *et al.* 2002, Lesser *et al.* 2004). While mutually beneficial bacteria can aid the coral ecosystem in the cycling of carbon and nutrients (Falkowski *et al.* 1993, Bren and Eisenbach 2000, Knowlton and Rohwer 2003, Rhshef *et al.* 2006), the chemoattractants and nutrients present in corals also has the consequence of attracting pathogenic bacteria (Bruno *et al.* 2003, Voss and Richardson 2006, Rypien *et al.* 2010).

In the current study, the expression of two flagellar genes, *flaA* and *flhA* were examined in a strain of V. coralliilyticus (ATCC BAA-450). By introducing differing concentrations of coral mucus and DMSP, we assessed the expressional response of these genes and combined this with visual observations of *V. coralliilyticus* search pattern trajectories. This analysis provided a link between the search pattern repertoire utilized by bacteria and the genes involved in flagella movement.

4.2 Materials and Methods

4.2.1 Bacterial strain and growth conditions

V. coralliilyticus type strain LMG20984 (ATCC BAA-450), isolated from *Pocillopora damicornis* as previously described (Rosenberg and Ben-Haim 2002) was used for all experiments. Pure cultures were stored at -80°C in 15% glycerol stock solutions. Strains were grown on thiosulphate citrate bile salts sucrose agar media (Sigma Aldrich) overnight at 28°C. Liquid cultures were prepared in 30 mL of minimal media solution (Table 4.1), inoculated with one colony and incubated for 16 hours at 28°C with 160 rpm shaking and used during exponential growth phase. Bacteria culture cell density and concentrations were quantified using a spectrophotometer (GeneQuant Pro, GE Healthcare Biosciences).

Table 4.1. The minimal media recipe utilized in this chemotactic study which contained the minimal quantities of nutrients needed for cell growth

Schott bottle Number	Amount	Compound	Full name
1	25g	NaCl	Sodium chloride
dissolved into 700mL*	0.7g	KCl	Potassium chloride
	0.02g	$CaCl_2.2H_2O$	Calcium chloride dihydrate
	0.005g	FeEDTA	Ethylenediaminetetraacetic acid iron (III) sodium salt
	1g	Tris	Trizma base
	5g	$C_4H_4Na_2O_4$	Succinic acid
	1.35g	$C_6H_{12}O_6$	Glucose
2	1g	NH4NO3	Ammonium nitrate
dissolved in 200mL*	0.2g	$MgCl_2.H_2O$	Magnesium chloride
3	0.05g	KH_2PO_4	Potassium phosphate monobasic
dissolved in 100mL*	1g	$Mg.SO_4.7H_2O$	Magnesium sulfate heptahydrate

* distilled water

• add bottles 1, 2 and 3 together and store in the refrigerator for later use

4.2.2 Oligonucleotide construction, DNA extraction and PCR reactions

Nucleotide sequences of the flagellin filament outer-sheath protein (*flaA*) and flagellar biosynthesis protein (*flhA*) genes were retrieved from *Vibrio coralliilyticus* (LMG 20984), using the National Center for Biotechnology Information (NCBI) Entrez Nucleotide Database search tool (http://www.ncbi.nlm.nih.bov/). Sequences were aligned using the CLUSTALW alignment tool (http://www.genome.jp/tools/clustalw) and Geneious v.5.3.6 (created by Biomatters Ltd.; available from http://www.geneious.com) and the most conserved regions within the DNA sequence of the respective genes was selected. Primers for RT-PCR

were designed using Geneious 6.0.5, with the specific gene sequences as input and the following parameters specified: primer Tm – 55-65°C (optimum 60°C); primer length 18-24 bp (optimum 20 bp); primer GC content 45-70%; amplicon length 100-120 bp. Primers were checked for suitability using Geneious and Sigma Aldrich online oligonucleotide design webpage. The primer pairs for *flaA* and *flhA* can be found in Table 4.2.

Table 4.2. Primer pairs for the *flaA* and *flhA* genes

Protein	Forward primer (5' – 3')	Reverse primer (3' – 5')
flaA	TGGCCCAGTATGGATTGGCA	TGAACGAGTTACCATGATTGCGG
flhA	ACGACATCGAGCAGCTTGCG	ATCACCGCCCAATCGACCAC

Extracted *V. coralliilyticus* DNA was used to confirm and test the oligonucleotide design for the *flaA* and *flhA* gene. DNA was extracted from isolated single strain liquid cultures by using the Wizard® Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. All DNA was re-suspended in 100 µl DNA rehydration solution, quantified with a Nanodrop spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored at -20°C until required.

PCR reactions were performed for confirmation of the *flaA* and *flhA* gene and optimization for the annealing temperatures using a gradient PCR. The initial PCR reactions included 10 µl of 5x Reaction Buffer, 1 µl of 20 µM concentration of each primer, 1 µl of Taq DNA polymerase, 5 µl of DNA template and adjusted to a final volume of 50 µl with sterile Milli-Q water. The reaction conditions were as follows:

95°C for 3 min; followed by 30 cycles of 95°C for 30 seconds (s), 60°C for 30 s, 72°C for 30 s; and then a final extension of 72°C for 8 min. The gradient PCR used identical reaction volumes and conditions as mentioned above, except for the annealing temperature which varied between 50-70°C for each of the 12 reaction tubes. Each row of the gradient PCR represented a different temperature to optimize the annealing temperature for future qPCR reactions. Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained, with SYBR® Safe DNA gel stain (Invitrogen™, Carlsbad, CA). The DNA was dried in a vacuum centrifuge (Savant DNA 120) and sequenced (Macrogen, Inc., Seoul, Korea) to confirm the inclusion and purity of the *flaA* and *flhA* gene.

4.2.3 Coral mucus and DMSP supplement experiments

V. coralliiltyicus cultures were grown for 16 hours at 28°C, with 160 rpm shaking, and used during the exponential growth phase. Bacteria culture cell density and concentrations were quantified using a spectrophotometer (GeneQuant Pro, GE Healthcare Biosciences). 1 mL of 16 hr culture was aliquoted into 1.5 mL microfuge tubes with coral mucus and DMSP added at levels between 0.001 dilution ($1x10^{-3}$) and 0.5 dilution ($5x10^{-1}$) for the coral mucus and between 0 and 67 µM of DMSP. Absolute quantities can be seen in Table 4.3. Triplicate samples were prepared for all coral mucus and DMSP concentrations. A no-addition control was also prepared prior to RNA extraction.

Table 4.3. Absolute quantities added to the DMSP and coral mucus experiments

(a)

DMSP	**Tube**						
	1	2	3	4	5	6	7
Concentration (µM)	0	7	13	20	27	33	67
Absolute quantities (µL)	0	20	40	60	80	100	200

(b)

Coral mucus	**Tube**							
	1	2	3	4	5	6	7	8
Dilution factor	0	0.001	0.002	0.005	0.01	0.02	0.1	0.5
Absolute quantities (µL)	0	1	2	5	10	20	50	500

Coral mucus was collected from *Acropora millipora* colonies maintained in outside 300 L aquaria holding tanks at the Australian Institute of Marine Science. Mucus was collected by removal of the coral from the aquarium, removal of residual seawater by gentle shaking and exposing the coral colony to air for two to four minutes. The colonies released substantial quantities of mucus, with 5 mL collected by balancing the coral nubbins over a sterile 50 mL falcon tube. The coral mucus was used either immediately or stored at 4°C and used within 4 hrs of collection. Since coral mucus did not have a specific concentration, we classified the addition of mucus into the experiments in terms of a dilution, ranging between a 0.001 (with 1 µL of mucus to 999 µL of distilled autoclaved water) to 0.5 (with 500 µL of mucus to 500 µL of distilled autoclaved water) dilution, added to a minimal media 16 hr *V. coralliilyticus* culture.

Total RNA was isolated from 1 mL minimal media cultures of *V. coralliilyticus* LMG20984 using the RiboPure™-Bacteria Kit (Ambion® Life Technologies Co., Carlsbad, CA) according to the manufacturer's instructions. An optional DNase 1 treatment at the end of the RNA isolation protocol was used to remove any trace amounts of genomic DNA from the eluted RNA. All RNA was quantified with a Nanodrop spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden), and stored at -80°C until required.

cDNA was synthesized from the total RNA isolated using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen™, Carlsbad, CA) according to the manufacturer's instructions. The reactions included: varying quantities of mRNA to make the concentration up to 50 ng, 2 µL SuperScript III/RNAOUT Enzyme Mix, 10 µL of 2X RT Reaction Mix, 1 µL of *E. coli* RNase H, and adjusted to a volume of 21 µL with DEPC-treated water. The reaction conditions were as follows: 25°C for 10 mins, 50°C for 30 minutes and 85°C for 5 mins to terminate the reactions, which were then chilled on ice. The *E.coli* RNase H was then added and incubated at 37°C for 20 minutes and stored at -20°C until required.

4.2.4 Preparation of quantified standards

16S rRNA, *flhA* and *flaA* genes derived from *V. coralliilyticus* were amplified using designed primers for each gene. 16S rRNA gene was targeted using the universal primers 331f(5'-TCCTACGGGAGGCAGCAGT-3'), 797r

(5'-GGACTACCAGGGTATCTAATCCTGTT-3). The PCR products were cloned into TA-vector, TOPO, using the TOPO-TA cloning kit (Invitrogen). The plasmid DNA was purified using the QIAprep miniprep kit (Qiagen) and the concentration of

purified plasmid was calculated from the absorbance (260 nm) of this preparation. The plasmids were linearized by digestion with *HIND*III (New England Biolabs). Dilutions of linear plasmid DNA for each gene were used as standards for subsequent qPCR analysis.

4.2.5 qRT-PCR

Quantitative real-time PCR analysis was carried out on the cDNA generated from *Vibrio coralliilyticus* strains grown under varying conditions. The SensiMix™ SYBR NO-ROX One Step kit (Bioline Reagents Ltd., NSW, Australia) was used to prepare the cDNA for qPCR. The qPCR reaction tubes each included 12.5 µL of 2x SensiMix SYBR No-Rox reagent, 1 µL of forward primer, 1 µL of reverse primer, 5 µL of template cDNA and were adjusted to a final volume of 25 µL with sterile Milli-Q water. All assays were conducted on a RotorGene 3000 (Corbett Research, Sydney, Australia) real-time analyzer with the following cycling parameters: initial incubation at 95°C for 10 mins (DNA polymerase activation), followed by 40 cycles at 95°C for 15 s (denaturation), 65°C for 15 s (annealing), and 72°C for 15 s (extension). During the extension phase of each thermal cycle, fluorescence was measured in the FAM channel (470-nm excitation and 510-nm detection).

4.2.6 Quantified real-time PCR data analysis

The standard curve method was used for quantification since the efficiencies between the targets and the reference gene were not comparable (Giulietti *et al.* 2001). Standard curves were constructed by plotting the Ct-value against the log template

value of cDNA of each gene. Target concentrations were adjusted by normalizing against the 16S rRNA gene, which served as an endogenous reference, by dividing the average concentration of target DNA by the average concentration of reference RNA.

The number of *V. coralliilyticus* genes/cell was determined with the SYBR-Green PCR procedure using *V. coralliilyticus*-specific primers. DNA from known amounts of *V. coralliilyticus* was added in serial dilutions from 10^1 to 10^8 cells to a series of PCRs. The reactions were carried out in a RotorGene Q thermocycler (Qiagen Hilden, Germany), and the fluorescence was monitored throughout the reaction. The relative gene expression levels were compared by dividing the normalized target concentration by the control sample. Gene expression levels were obtained by quantitative RT-PCR analysis, which were normalized to that of the 16S rRNA gene, since its expression was to found to be unchanged under different temperature and enrichment conditions. The expression levels of 16S rRNA gene was normalized to that of the *flhA* and *flaA* genes. Quantitative values were obtained by using the standard curve method. The C_T value corresponded to the PCR cycle at which the first detectable increase in fluorescence associated with the exponential growth of PCR products occurred. Genes were considered to be up- or down-regulated if their relative expression levels were at least two-fold higher or lower respectively, compared to the control sample (Desroche *et al.* 2005).

4.2.7 Search pattern analysis

Overnight cultures were prepared for search pattern analysis in response to the coral mucus and DMSP additions. Using a slide observation chamber, constructed from

two glass coverslips on a glass slide and covered with a third coverslip (Winn *et al.* 2013), motile cells were examined. All distinguishable cells in each video were tracked and analyzed. Cells were considered distinguishable if they remained in focus for at least 5 frames. All distinguishable cell trajectory paths were classified into one of three search patterns: run-reverse, straight swimming or 3-step flick. Each trajectory path was then measurement and the swimming speed calculated (Winn *et al.* 2013).

4.2.8 Statistical analysis

Statistical analyses were performed using the SPSS v.21.0 software package for Macintosh (SPSS Inc., USA), and independent student t-tests were applied to test for the significance between gene expression values. When the *p*-value was less than 0.05, the result was considered as statistically significant.

4.3 Results

4.3.1 Flagella biosynthesis gene expression patterns

Gene expression of the target flagella biosynthesis genes *flaA* and *flhA*, analyzed using qPCR, demonstrated varied gene expression patterns that were dependent on the addition of DMSP concentrations, a known bacterial chemoattractant in coral mucus (Fig. 4.1a). The expression of the biosynthesis protein, *flhA* was low at the control (no DMSP addition) and 7 μM DMSP concentrations, with values of $7.5x10^3$ (SEM = $1.6x10^2$) and $1.0x10^4$ (SEM = $1.2x10^3$) gene copies/ ng of RNA respectively (Fig. 4.1a). Compared to the control, *flhA* gene expression was significantly greater

at concentrations of 13 (P = 0.032), 20 (P = 0.035), and 27 (P = 0.033) μM, with the highest gene expression at the 20 μM concentration with a value of $1.1x10^5$ (SEM = $1.5x10^4$) gene copies/ ng of RNA. Interestingly, when DMSP concentrations were even higher, at 33 and 67 μM, gene expression of the *flhA* gene was reduced, with values of $8.3x10^3$ (SEM = $3.6x10^2$) and $8.4x10^3$ (SEM = $9.1x10^2$) gene copies/ ng of RNA respectively and did not significantly differ from the control (P = 0.165 and P = 0.430 respectively) or the 7 μM (P = 0.208 and P = 0.261 respectively) DMSP addition experiments.

The expression of the flagellin protein, *flaA* was low at the control (no DMSP addition) and 7 μM DMSP concentrations, with values of $7.7x10^4$ (SEM = $1.4x10^2$) and $1.2x10^4$ (SEM = $2.6x10^3$) gene copies/ ng of RNA respectively (Fig. 4.1a). The highest gene expression of the flagellin protein, *flaA* occurred at 13μM DMSP with a value of $1.3x10^5$ gene copies/ ng RNA (SEM = $2.2x10^4$) which was significantly greater (P = 0.002) than the control of $7.7x10^4$ (SEM = $1.4x10^2$) gene copies/ ng RNA. Similarly, at concentrations of 20, 27 and 33 μM DMSP, gene copy numbers of the *flaA* were significantly higher with values of $1.16x10^5$, $1.1x10^5$, $1.1x10^5$ gene copies/ ng RNA respectively than the control (P = 0.035, P = 0.018, P = 0.002 respectively) (Fig. 4.1a). Gene copies/ ng RNA of the *flaA* gene at 67 μM DMSP was lower with $6.2x10^4$, yet still significantly different to the control (P = 0.042).

The gene expression values of the *flaA* and *flhA* genes, for cells exposed to coral mucus which is a complex mixture of polysaccharides, amino acids and other organic products, were different from the gene expression values observed for DMSP additions (Fig. 4.1b). When the control was directly compared to the 0.001, 0.005 and 0.02 mucus dilution with values of $4.3x10^3$, $4.0x10^3$ and $8.5x10^3$ gene copies/ ng

respectively, there was statistically significant differences observed in the flhA gene expression value (P = 0.018, P = 0.005, P = 0.006 respectively). No significant difference (P = 0.46) in the *flhA* gene expression was observed between the control and the 0.5 mucus dilution, with values of $7.5x10^3$ and $6.9x10^3$ gene copies/ ng respectively. There was also no significant difference between the no mucus control and the 0.002 mucus dilution (P = 0.069) with a value of $1.1x10^4$ (SEM = $1.1x10^3$) gene copies/ ng, the 0.01 mucus dilution (P = 0.784) with a value of $8.0x10^3$ (SEM = $1.4x10^3$) gene copies/ ng, or the 0.1 mucus dilution (P = 0.276) with a value of $8.4x10^3$ (SEM = $7.0x10^2$) gene copies/ ng RNA.

In contrast, the gene expression of the *flaA* gene was statistically significantly (P < 0.05) for all coral mucus additions relative to the control, with a gene expression value of $7.7x10^4$ (SEM = $1.4x10^2$) gene copies/ ng RNA when no mucus was added. All of the other coral mucus additions produced *flaA* gene expression values exceeding $2.0x10^5$ (P = 0.002) gene copies/ ng RNA. Gene expression of the *flaA* gene increased with the increasing additions of coral mucus until the 0.1 coral mucus dilution, which presented a gene expression value of $4.7x10^5$ (SEM = $6.78x10^5$) gene copies/ ng RNA. This presented a 1.8 times (P = 0.014) decrease in the gene expression value when compared to the 0.02 coral mucus dilution with a gene expression value of $8.4x10^5$ (SEM = $5.6x10^4$) gene copies/ ng RNA. The gene expression value of *flaA* decreased from the 0.1 coral mucus dilution onwards. The exception to this trend occurred at the 0.005 mucus dilution which presented a gene expression value of $2.7x10^5$ gene copies/ ng RNA. This value is 2.6x (P = 0.010) lower than the gene expression values at the 0.002, and 2.8x (P = 0.008) lower than the 0.01 coral mucus dilutions with values of $7.0x10^5$ (SEM = $9.04x10^4$) and $7.5x10^5$ (SEM = $9.69x10^4$) gene copies/ ng RNA respectively.

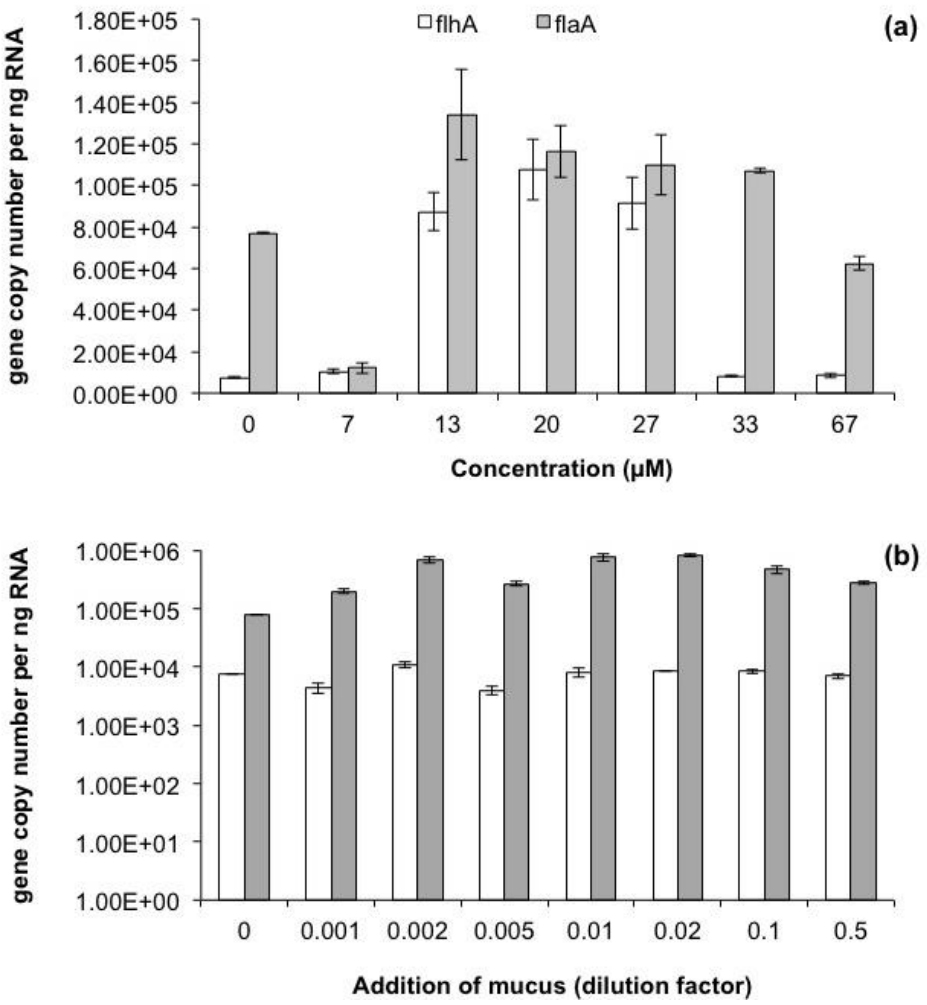

Figure 4.1. Gene expression values in chemoattractant experiments. **The *flaA* and *flhA* gene expression values in chemoattractant experiments, (a) gene copies/ ng RNA across the various DMSP concentrations, (b) gene copies/ ng RNA across the various coral mucus additions (error bars = SEM).**

4.3.2 Chemotactic search patterns of *Vibrio coralliilyticus* in response to DMSP and mucus additions

Search pattern trajectories of motile *V. coralliilyticus* cells in response to increasing DMSP concentrations and mucus additions, showed a reduction in 3-step flick search

patterns in the solutions with a DMSP value greater than 7 μM. The flick decrease occurred with increasing concentrations of DMSP, with the lowest number of flicks observed at 20 μM and 33 μM DMSP concentrations. The average number of flick search patterns was identified to vary between 1.8 flicks/s in 7 μM DMSP (SEM = 0.4), compared to 0.2 flicks/s at 20 μM and 33 μM DMSP. A significant difference was noted in the number of flicks identified between the highest and lowest DMSP concentration (P = 0.04). Straight swimming search patterns illustrated a contrasting trend whereby the majority of straight swimming search patterns were identified in high DMSP solutions. There was a significant increase in the number of straight swimming search patterns identified at 20 μM DMSP compared to 33 μM DMSP, with an average 0.5 (SEM = 0.3) straight swims/s identified compared to 0.9 (SEM = 0.2) straight swims/s respectively (P = 0.001). The average number of run-reverse search patterns remained relatively consistent irrespective of the DMSP concentration, with an average of 0.8 (SEM = 0.5) reverses/s identified at the control compared to 0.9 (SEM = 0.2) reverses/s at the 33 μM DMSP concentration (P = 0.661) (Table 4.4).

The highest speed of flicks and run-reverse search patterns, occurred at the 20 μM DMSP concentration. At this concentration the straight swimming search pattern speed was also high with a value of 93.8 $\mu m/s^{-1}$, the speed was observed to be highest at 7 μM DMSP concentration with a value of 97.4 $\mu m/s^{-1}$ (SEM = 6.0). All search patterns decrease in speed between 20 and 33 μM (P = 0.007) DMSP with both flick and run-reverse speeds near identical (P = 0.356) in 33 μM DMSP conditions. Mean flick speeds fluctuated from 57.2 $\mu m/s^{-1}$ at 0 μM (SEM = 0.9) to

62.8 μm/s^{-1} at 33 μM (SEM = 9.4) (P = 0.05), with a peak speed of 98.1 μm/s^{-1} (SEM = 8.6) (P = 0.001) that occurred at the 20 μM DMSP concentration.

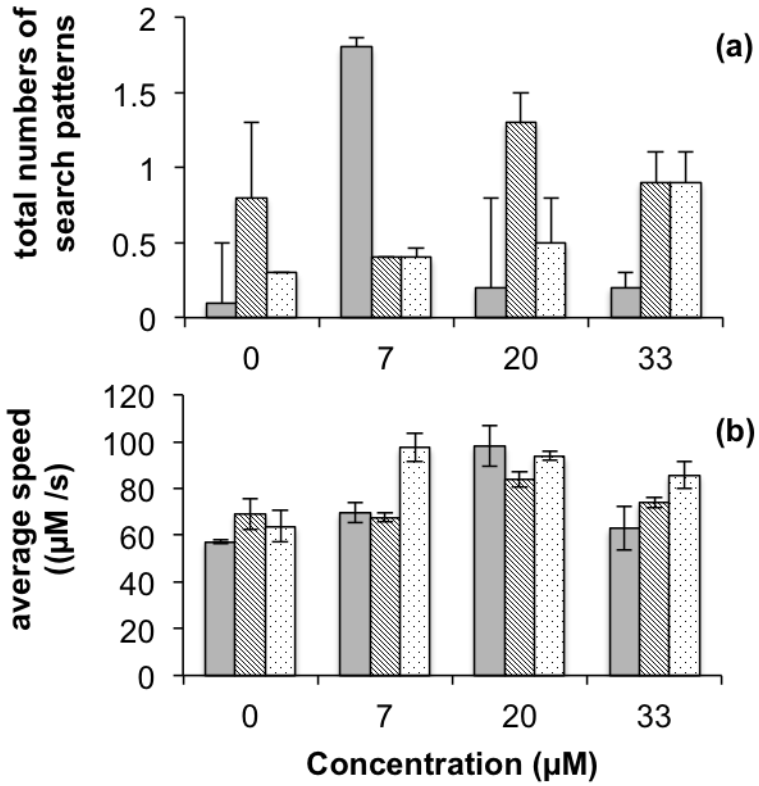

Figure 4.2. Chemotactic search pattern data for the DMSP experiment. **The chemotactic search patterns for the DMSP experiment with, (a) total numbers of all search patterns (SEM), (b) average speed for all search patterns (μm/s) (SEM).**

A reduction in 3-step flick search patterns was identified in solutions of increasing mucus dilutions, with less flicks occurring at higher mucus concentrations compared to the no mucus control. At the 0.5 coral mucus addition, the fewest number of flicks was observed with an average of 0.4 (SEM = 0.1) flicks/s compared

to an average of 1.1 (SEM = 0.07) flicks/s observed in the control (Table 4.5). In contrast, the highest number of straight swimming runs occurred at the 0.5 coral mucus addition, with an average of 1.0 (SEM = 0.3) straight swims/s identified compared to a mean value of 0.3 straight swims/s in the control. Run-reverse search patterns fluctuated between mean values of 0.4 reverses/s in the control, compared to 0.7 reverses/s in the 0.5 coral mucus addition. However, no clear trend was apparent ($P = 0.585$). Run-reverse search pattern numbers were highest within the mid-range concentrations of DMSP (20 μM) and coral mucus (0.005 mucus dilution), with values of 1.33 reverses/s and 1.06 reverses/s respectively. Similarly, the run-reverse and straight swimming speed was found to be the highest at the 20 μM DMSP concentration and at 0.5 coral mucus addition, with values of 84 μm s^{-1} and 77.1 μm s^{-1} respectively for the run-reverses, and 93.8 μm s^{-1} and 83.2 μm s^{-1} respectively for the straight swims. Straight swim numbers were greatest at the highest DMSP concentration (33 μM) and coral mucus additions (0.5), with values of 0.9 straight swims/s and 1.0 straight swims/s respectively. Flick search pattern numbers were similar for both the 0.002 and 0.005 coral mucus additions ($P = 0.898$), and for the 20 μM and 33 μM DMSP concentrations ($P = 0.664$). However, according to the search pattern results, the highest search pattern speeds are identified in the 0.02 coral mucus dilution for reverses and straight swims, and in the 0.5 coral mucus dilution for the flick search patterns. The highest speed of flick search patterns occurred at the 0.5 coral mucus addition with an average of 91.0 μm/s^{-1} (SEM = 11.7), with flick search pattern speeds increasing with increasing mucus concentrations. Run-reverse and straight swimming search patterns did not exhibit the same trend, with the highest speeds for these search patterns recorded at the 1:10

coral mucus addition with 77.1 µm/s^{-1} (SEM = 3.7) and 103.5 µm/s^{-1} (SEM = 3.5) respectively.

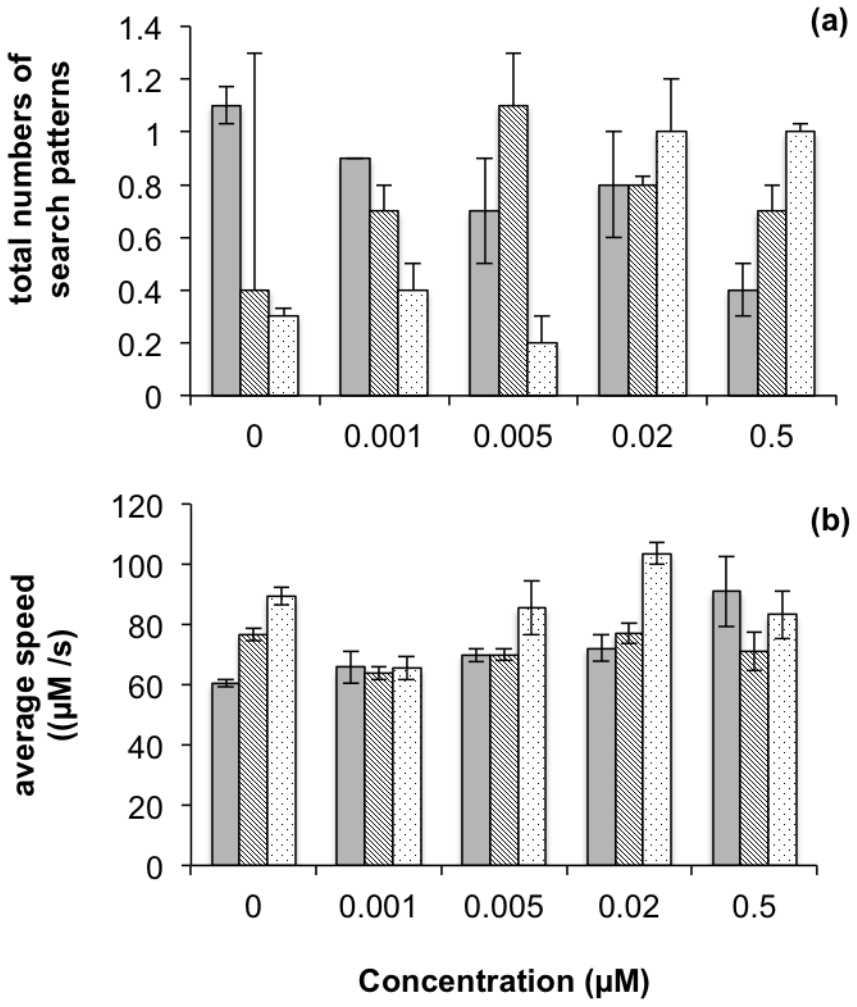

Figure 4.3. Chemotactic search pattern data for the coral mucus additions. **The chemotactic search patterns for the coral mucus addition experiment with, (a) total numbers of all search patterns (SEM); (b) average speed for all search patterns (µm/s) (SEM).**

4.4 Discussion

4.4.1 Chemical cues influence flagella gene expression and search pattern behavior

For many pathogens, flagella are necessary for infection and for the attachment at a host's surface (Toren *et al.* 1998, Banin *et al.* 2001, Josenhans and Seurbaum 2002, Butler and Camilli 2004, Ramos *et al.* 2004). The lack of virulence and pathogenicity by non-motile *flhA* and *flaA Vibrio* strain mutants has been demonstrated by Meron *et al.* (2009) and Milton *et al.* (1996) respectively. This identifies that these two genes are linked to the motility characteristics of *V. coralliilyticus* observed in this study. Whilst non-motile mutants are still able to multiply, the infection capabilities of the bacterium are greatly diminished, with the flagellum suggested to be the best characterized virulence factor (Milton *et al.* 1996). For the coral pathogen *V. coralliilyticus*, the flagellum appears critical to its chemotactic behavior and intrinsically linked to its virulence capability (Meron *et al.* 2009). This is identified in this work where changes in chemotactic search patterns and gene expression occur with fluctuations in DMSP and mucus concentrations. The motility and genetic responses in this study indicate that the flagella and its genetic components show identifiable responses to chemical gradients within the environment. In this study we have determined through gene expression and trajectory analysis, that the chemotactic search patterns of *V. coralliilyticus* are correlated with the expression of the *flhA* and *flaA* genes under changing DMSP and coral mucus concentrations. Although direct comparisons cannot be made, search pattern similarities were identifiable between the coral mucus additions and DMSP concentrations. The linkage of gene expression with chemotactic nutrient sources suggests that the motility and search pattern repertoire of *V. coralliilyticus* may be controlled on a

genetic and protein level. The study provides the first description of the gene expression of key chemotactic genes in the presence of fluctuating concentrations of chemoattractants which may drive the motility responses utilized by chemotactic bacteria in a shifting nutrient environment. This study also provides gene expression data of key motility genes that correlates specifically with the run-reverse search pattern data collected.

The gene expression results indicate that DMSP and coral mucus concentrations used in this study increases the gene copies/ ng RNA for *flaA* and *flhA* until a threshold has been reached. This threshold occurs at 20 µM DMSP for *flhA* and 13 µM DMSP for *flaA* and 0.002 coral mucus addition for *flhA* and 0.02 coral mucus addition for *flaA,* after which gene expression levels decrease by a minimum of 1.2 times. This suggests that these key motility genes may be utilized and expressed in differing quantities on a genetic level in solutions of differing concentrations. Previous reports have identified that mutants lacking these genes are non motile and are unable to chemotax effectively, preventing infection (Meron *et al.* 2009). As these genes regulate the flagellar motor rotation this influences the search patterns utilized (Roman *et al.* 1993).

DMSP is present in coral mucus and the surrounding seawater with concentrations of up to 62 µM measured in the coral mucus (Garren *et al.* 2014). Fluctuations in DMSP and coral mucus concentrations in the environment are the result of mixing with seawater and distance from the coral surface, with much lower concentrations (~ 6nM) documented in the surrounding seawater (Broadbent and Jones 2004, Van Alystne *et al.* 2006). The concentrations and dilutions utilized in this experiment mimicked this mixing to establish an understanding of the genetic and/or physical response to fluctuations in chemoattractants. The 3-step flick and

run-reverse search patterns identified fluctuations in search pattern speed that is consistent with the gene copy trend for the *flhA* gene. This suggests the *flhA* gene maybe influential in the search pattern trajectories utilized by *V. coralliilyticus*. There also appears to be a correlation between the *flaA* gene and the number of flick search patterns, where flick search patterns peaked at 13 µM DMSP with 1.8 flicks/s (SEM = 0.05) before decreasing to 0.2 flicks/s (SEM = 0.1) at the 33 µM DMSP experiment. In addition, straight swimming speeds were also identified to correlate with the *flaA* gene expression values, with straight swim speeds peaking at 13 µM DMSP concentration with a value of 97.4 $\mu m/s^{-1}$ (SEM = 6.0), before reducing to 85.7 $\mu m/s^{-1}$ (SEM = 5.8) in the 33 µM DMSP concentration. The gene copy number results presented here, illustrates that chemoattractants such as DMSP and mucus influence the level of expression of the *flaA* and *flhA* genes. By identifying fluctuations in gene expression associated with the variability in concentrations of chemoattractants indicates these genes have a driving effect on motility. These genes affect the search pattern type and/or speed of the flagellar motor, rather than purely switching the flagellar segment or motor on or off. This provides an understanding into the mechanical response chemoattractants have on the gene expression and motility of *V. coralliilyticus*, and thus the pathogenicity and virulence of this microorganism.

Search pattern speeds for run-reverses and flicks peaked at 20 µM DMSP with a value of 98.1 µm/s (SEM = 8.6) and 93.8 µm/s (SEM = 1.9) respectively, and similarly gene copies were highest at 20 µM for the *flhA* gene with a value of $1.1x10^5$ gene copies/ ng RNA. This elevated search pattern speed is potentially directly related to the flagellar motor switch for which the *flhA* gene is instrumental. In conjunction with this finding, there also appears to be a link between the flick and

run-reverse search pattern numbers with the *flhA* gene copies/ ng RNA whereby when flicks are elevated, run-reverses are reduced and vice versa. This difference in search pattern behaviour may be the result of the cell's niche preference as flicks have been suggested to act as reorientation search paths whilst run-reverses have been suggested to utilize a preferred niche by backtracking (Mueller 1996, Son *et al.* 2013). The *flhA* gene is a component of the flagellar motor which controls the flagellar rotation behavior and consequently cell direction. Since the same trend does not coincide with straight swimming search pattern numbers or speed, it can be suggested that the rotation mechanism which the flagellar motor switch controls may be influencing the directional movement found in both the flick and run-reverse search patterns. The 180° reverse which occurs in both the 3-step flick and run-reverse search pattern relies on the clockwise rotation of the polar flagella in monotrichious bacterium (Terasawa *et al.* 2011, Stocker 2012, Son *et al.* 2013), which is controlled by the flagellar motor switch (Khan and Macnab 1980, Scharf *et al.* 1998, Mcnab 2003). This may account for elevated gene copies/ ng RNA in association with the reverse search pattern path.

Coral mucus, which also contains DMSP, has been previously identified to influence *V. coralliilyticus's* chemotactic search pattern behavior (Garren *et al.* 2014, Winn *et al.* in review). In a previous study, fewer flicks were identified in the coral mucus than in the *V. coralliilyticus* cell culture area (Winn *et al. in review*, chapter 3). In addition, the search pattern speeds were greater in the mucus region than the culture or interface regions for all search patterns, namely the flick ($P = 0.01$), run-reverse ($P = 0.032$) and straight swimming ($P = 0.024$). This supported other studies, which showed that flagellated bacteria show an initial increase in speed up to 2 cP, with coral mucus having a measured cP value of 1.8 (Winn *et al*. in review). This

illustrates that search pattern speed is not dependent on the search patterns themselves, rather the locational differences or chemotactic properties associated with differences in quantities or concentrations of chemoattractants from the mucus. This current study also shows that flicks decrease with increasing amounts of coral mucus from 1.1 flicks/s at the control where no coral mucus was added, to 0.4 flicks/s in the 0.5 coral mucus solution. However, in the current study, there was no consistent trend for the speed of all search patterns. The highest speeds for run-reverse and straight swimming search patterns occurred at the 0.002 mucus dilution, however, the highest speed for flick search patterns occurred at the 0.5 coral mucus solution. The viscosity of the coral mucus solutions could potentially account for the reduction in speed identified at the 0.5 coral mucus solution for the run-reverse and straight swimming search patterns. Due to the variable nature of coral mucus and susceptibility to mixing with seawater, the exact viscosity of coral mucus is inconsistent (Garren *et al.* 2014). This may account for some of the variations observed in search pattern data whereby viscosity has been suggested to impede or influence the search patterns utilized by *V. coralliilyticus* (Winn *et al.* in review). Flicks generally showed a reduction in speed when compared to run-reverse and straight swimming patterns which suggests that the addition of coral mucus and DMSP is having an effect on the flagellar motor rotation causing the speed reduction. This may be potentially due to a threshold being reached which impacts the search pattern behavior. This threshold was reported by Son *et al.* (2013), where it was discovered that monoflagellated bacteria reorient by exploiting a buckling instability of their flagellar hook. This was found to occur 10 ms after the onset of a forward swimming path when the hook is compressed and the associated hydrodynamic load triggers a buckling response (Son *et al.* 2013). Reducing the load on the hook below

the buckling threshold by decreasing the swimming speed results in the suppression of the reorientation path, namely the flick. This has also been identified to occur in other microorganisms when in the presence of viscous solutions (Son *et al.* 2013, Winn *et al.* in review). There appears to be several plausible explanations which could act in combination to account for the search pattern trends identified in this study. Firstly, the viscosity of the liquid media due to the added coral mucus may impede the swimming speed (Atsumi *et al.* 1992, Xing *et al.* 2006). This occurs due to drag inflicted by a viscous liquid (Berg 1974, Chen *et al.* 2011), which is sufficient enough to prevent the speeds required for the buckling instability to occur and therefore, flick events. Secondly, the cells reduce their speed in the presence of coral mucus and elevated DMSP concentrations in order to not undergo the buckling instability that occurs beyond a certain swimming speed in order to maintain their position in a chemotactically desirable habitat. Lastly, the viscous solution aids the cells swimming behavior by assisting with their propulsion through the liquid (Schneider and Doetsch 1974, Ferrero and Lee 1988, Atsumi *et al.* 1996, *et al.* Teran 2010). Whilst, a threshold speed has been identified for *V. alginolyticus* of 51 μm/s (Son *et al.* 2013), this speed may be greater in other bacterial species, as all *V. coralliilyticus* cell speeds exceeded this value. There is no discernable threshold identifiable in these results, however future studies into the threshold comparisons of a variety of bacteria would be informative.

V. coralliilyticus in the presence of corals has access to numerous chemoattractants (Brown and Bythell 2005, Garren *et al*. 2014, Tout *et al.* 2015). Past studies have identified that chemoattractants and viscosity can influence *V. coralliilyticus* search pattern and virulence behavior (Macnab 2003, Meron *et al.* 2009, Garren *et al.* 2014). In this study a significant correlation ($P = 0.006$) was

identified between the search pattern behavior with increasing DMSP concentrations and this correlation was reflected in the expression of the flagella structural and biosynthesis genes, *flaA* and *flhA*. In contrast, when *V. coralliilyticus* cells were mixed with coral mucus there was no clear trend for the expression levels of either the *flaA* and *flhA* gene relative to the mucus concentration. This may explain why the *flaA* and *flhA* appear to be constantly expressed at similar gene copies/ ng RNA despite the variety of dilutions of coral mucus tested. This may be in part due to the complex nature of coral mucus with numerous constituents in addition to DMSP, including polysaccharides, amino acid, sugars, lipids, carbohydrates and chemical elements such as nitrogen and carbon (Ducklow and Mitchell 1979, Tout *et al.* 2015). The fluctuating response is likely to be typical for *V. coralliilyticus* and other motile marine pathogens because rarely is there a single chemoattractant or nutrient present, but rather a complex mix of signals. In a complex nutrient environment such as the ocean (Coffroth 1990, McCook 1999, Bruno *et al.* 2003), bacteria respond to the flux of chemoattractants present, thereby constantly and rapidly changing their gene expression and search pattern responses.

4.5 Acknowledgements

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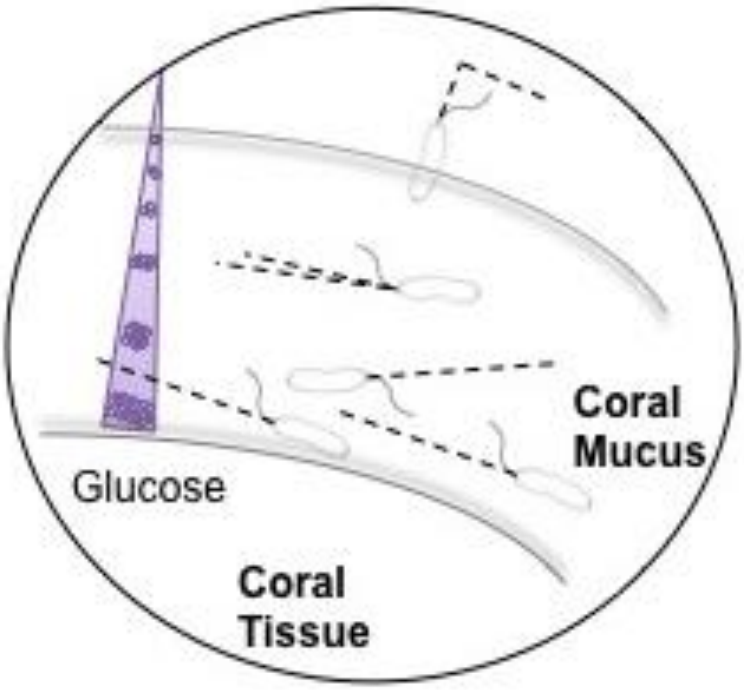
Chapter 5

Cellular energy expenditure modelling for the coral pathogen *Vibrio coralliilyticus*



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Chapter cover figure: A visual summary of the component influencing the search pattern response in chapter 5, the metabolic cost of motility, which is further discussed in chapter 6.

5.0 Abstract

Microorganisms are able to successfully move through the mucus layers of numerous host systems and surfaces allowing for colonization of the host tissue. On corals, the presence of a mucus layer has a higher viscosity than the surrounding seawater. This can impact the cost of motility for bacterial cells, such as *Vibrio coralliilyticus*, implicated as a pathogen in some coral disease outbreaks. Here we introduce a mathematical model for examining the cellular energy expenditure for *V. coralliilyticus's* utilization of different chemotactic search patterns across a culture-to-mucus interface, indicative of the viscosity and nutrient gradients found across the seawater-to-coral mucus interface.

The cellular energy expenditure and metabolic cost, measured in glucose molecules, identified that swimming in a mucus region uses an additional 100 times the amount of glucose molecules than swimming in seawater, irrespective of the chemotactic search patterns utilized. *V. coralliilyticus's* search patterns doubled in speed in the coral mucus region compared to the seawater region with the energetic cost increasing by 1.5 orders of magnitude. This presents an interesting result where despite a significant increase in energetic cost to the cell, motility in the mucus region continues to occur. Furthermore, also identified is the cells' ability to alter its' diffusivity of a chemoattractant, based on its motility. Diffusivity only varied by a factor of 2 across the seawater to mucus region, compared to a 4 orders of magnitude increase in diffusivity values when cells utilized long run lengths compared to shorter ones. These results imply that the benefit of reaching the mucus region seems to far outweigh the cost involved in relocating to this area. This result coupled with the diffusivity findings suggests that *V. coralliilyticus* cells' are much more in

behavioral control than previously given credit for. This suggests that the motility search patterns identified in cells are not necessarily random or purely the result of the external conditions, rather the cells have developed ways to make favorable use of their environment wherever possible.

5.1 Introduction

Bacterial cells must regulate and allocate their resources successfully to survive under different environmental conditions (Rothman-Denes and Parsot 2008). The partitioning and allocation of these finite resources must be finely tuned to allow for enough energy for critical cellular processes including reproduction, growth, nutrient sensing, motility and seeking out resources (Rothman-Denes and Parsot 2008). Resource allocation may vary across different bacterial species, with individual species using different allocation techniques due to differences in cell appearance including cell size, flagellar arrangement and number (Rothman-Denes and Parsot 2008). The physical characteristics of bacteria including size and external projections such as flagella can influence the energetic cost of motility. The body size of bacteria has been identified to influence the energetic cost of movement with size differences of just 0.1 μm resulting in 100,000-fold change in energetic cost of chemotaxis (Mitchell 2002). Furthermore, in motile bacteria the possession and use of flagella, including the ion pumps and receptors results in the consumption of significant cell resources (Macnab 1987, Eisenbach 1996, Mitchel and Barbara 1999). In particular, *Escherichia coli* has a high genetic and biosynthetic expense that is linked to the production and maintenance of more than 60 proteins required for chemotaxis (Macnab 1987). This expense is thought to be greater for marine bacterium, particularly *Vibrio* species which possesses two propulsion systems driven both

proton and sodium ions (Alldredge *et al.* 1986). In addition *Vibrios* have been suggested as having two chemosensory systems, with one each for starving and growing cells (Malmcrona-Friberg *et al.* 1990), with either the dual propulsion system or the dual-growth state suggesting that the sensory and motility costs are high in marine bacteria (Mitchell *et al*. 1995).

Comparably, the energetic expense of chemotactic motility in *E. coli* is a small fraction of the total energy available (Purcell 1977), whereby cells must produce enough power to swim at 30 μm s^{-1} to compensate for the brownian motion and diffusion of the nutrients or attractants present within their environment (Purcell 1977, Berg 1993). With energy costs inferred to be higher in marine bacteria and with marine bacteria identified to swim at greater speeds this energy expense may be significantly greater due to the lower ambient nutrient and attractant concentration and higher swimming speeds, which have been identified as having to be in excess of 100 μm s^{-1} to compensate. In many cases individual organisms deal with different biological cues such as the presence or absence of nutrients differently (Rothman-Denes and Parsot 2008). This suggests that motility patterns may not be uniquely used for specific environments such as a low nutrient environment, rather these patterns are dependent on individual organism preference.

The partitioning and allocation of resources must be regulated by bacteria, in particular their membrane lipid composition in order to survive under different environmental conditions and to avoid unnecessary energy expenditure (Rothman-Denes and Parsot 2008). In addition, it has been identified that a limited supply of energy during some cell growth may create greater resistance to the breakdown of the permeability barrier and the production of more light-harvesting or energy-converting membranes (Sojka *et al.* 1969). Furthermore, the metabolism of bacteria

has been identified as being an open system that is characterized by a continuous input and output of matter and energy (Thauer *et al.* 1977). This means that each cell possesses a system that is capable of transforming both the chemical and physical energy taken up into biologically useful energy which can be utilized at a later time to perform work (Thauer *et al.* 1977). This energy transformation and utilization involves the ATP (adenosine 5'-triphosphate) system (Thauer *et al.* 1977).

The allocation of resources is also influenced by external factors such as nutrient availability, whereby the energetic cost of motility was lower in culture media as nutrient availability is unlimited compared (Purcell 1977) to oceanic environments where nutrient concentration is low and the bacterial swimming speeds are high (Purcell 1977). Physical attributes such as cell size can limit several cellular attributes including locomotory methods, maximum attainable speed and motion duration which determine the best strategies for searching for food, prey defense and predator attack (Mitchell 2002). Microorganisms can adjust the expression of specific sets of genes and the activity of proteins in reaction to external stimuli (Rothman-Denes and Parsot 2008). For the same stimuli cue such as the presence or absence of nutrients, different strategies are adopted by different bacterial species (Rothman-Denes and Parsot 2008).

The ability to seek out resources requires cellular ability to be motile, however bacteria have the capability to optimize the suitable use of energy they take from nutrients, and in some cases ceasing motility for a length of time may be energetically beneficial (Mitchell *et al.* 1995). Mitchell (1991) found that in marine environments motility is energetically expensive due to the low nutrient concentration and small cell sizes, which makes is advantageous for motility to be able to be switched on or off as required (Mitchell *et al.* 1995). In Kjelleberg (1993)

and Dusenbery (1997) the relationship between bacterial cell size and the energetic cost of movement is established, which correlates with the universal allometric equation for size and movement in animals (Mitchell 2002). In some cases photosynthetic bacteria that grow with a limited supply of energy show an elevated bacteriochlorophyll content and increased resistance to breakdown of the 'permeability barrier' by these agents (Sojka *et al.* 1969).

The ATP (adenosine 5'-triphosphate) system is the universal molecular carrier for biological energy whereby the energy taken up by the cells is used to drive the endergonic synthesis of ATP from ADP (adenosine 5'-diphosphate) and inorganic orthophosphate (Thauer *et al.* 1977). In many anaerobic bacteria the amount of ATP formed per mole of energy substrate fermented can be regulated (Iannotti *et al.* 1973) which allows the organism to optimize the thermodynamic efficiency of energy transformation (Thauer *et al.* 1977). The amount of free energy required to synthesize 1 mol of ATP is variable and highly dependent on intracellular concentrations with none of these parameters known with certainty and remains controversial (Banks and Vernon 1970, Thauer *et al.* 1977) with values of -6.79 kcal/mol (-28.4 kJ/mol) (Rosing and Slater 1972), -7.6 kcal/mol (-31.80 kJ/mol) (Guynn and Veech 1973) and -8.74 kcal/mol (-36.57 kJ/mol) (Alberty 1969, Phillips *et al.* 1969, Shikama 1971) all obtained. This suggests that some variability is present which may possibly be linked to the individual cell type or external environmental factors. In the case of coral bacteria this energy cost may be influenced by external factors including viscosity, nutrient diffusivity and the thickness of the coral mucus layer. Using a modelling approach, this study examines the energetic cost of motility for V. coralliilyticus invasion, by taking into account the viscosity variance and

fluctuations of know chemoattractant, DMSP that are present within the coral organism and surrounding seawater.

5.2 Materials and Methods

5.2.1 Run length and duration measurements

The search patterns collected from Winn *et al.* (in review) were examined to assess the length of runs between each consecutive turn. Between each microscope video frame the run length of the search patterns collected were examined and collated, and classified according to the search pattern type. The run length was calculated as the distance between any directional changes. Individual search pattern run durations for all search patterns utilized by *V. coralliilyticus* were identified using the collated data from Winn *et al.* (in review).

5.2.2 Cell speed measurements

Cell speed values at distances across a cell culture to mucus interface were collated from Winn *et al*. (in review, chapter 3). The cell speed data taken from Winn *et al.* (in review, chapter 3) was averaged, and the means stated and utilized in this study.

5.2.3 Bacterial diffusion

The diffusion coefficient is a constant between the molar flux due to molecular diffusion and the gradient in the concentration of the nutrient and was calculated using Berg's diffusion coefficient equation (Berg 1993),

$$D = \frac{v^2 \tau}{3(1-\alpha)} \quad (1)$$

where α is the mean of the cosine of the angle between successive runs, τ is the straight runs of mean duration, *v* is the average cell speed.

5.2.4 Power energy budget

Viscous drag can influence the torque and thrust generated by the rotation of the flagellar filament. Numerical estimates of the required power expenditure to overcome the drag in differing viscosities were calculated by:

$$P = 6\pi\eta a v^2 \quad (2)$$

where P is power, η is dynamic viscosity, a is cell radius of a sphere and v is cell velocity. All calculations were done in cgs units (Mitchell 1991).

The efficiency of the flagella-motor complex and of nutrient uptake are fixed at the commonly reported 1% (Berg and Purcell 1977, Purcell 1977, Berg and Turner 1979, Lowe *et al.* 1987), with mechanical limitations determining this efficiency with approximately 100 glucose molecules required to produce 1.8×10^{-11} ergs (Werner 1983, Mitchell 1991). So that a direct comparison between the mechanical energy output and the chemical energy potential could be made, the values calculated in ergs s^{-1} were converted to molecules of glucose consumed sec^{-1}.

5.2.5 Flux calculations

The diffusion flux of nutrients into the cell depends on active transport and was calculated using:

$$J = 4\pi \mathrm{CaD_c} \quad (3)$$

Where J is the flux, C is the concentration and *a* is a constant value, D_c is the diffusivity coefficient (Berg 1993).

Due to our trajectory line being only 2400 μm in length and our focus on the coral surface mucus boundary layer, the DMSP concentrations used in our simulations focused on the DMSP concentrations known to immediately surround the coral tissue itself. Since DMSP can be found at concentrations up to 60 μM in the coral mucus and decrease to as low as ~ 6 nM in the seawater surrounding the coral organism (Broadbent and Jones 2004, Van Alstyne *et al.* 2006, Swan *et al.* 2012, Garren *et al.* 2013), for the simulations of flux of *V. coralliilyticus* presented here, the DMSP concentrations utilized increased systematically from 0 – 60 μM across the seawater-mucus transect.

5.2.6 Species comparison

Search pattern motility characteristics including run lengths and swimming speeds and in some cases, diffusivity measurements were collated from previous studies to use for comparison to the diffusivity and flux calculations, calculated in this study for *Vibrio coralliilyticus* (Berg and Brown 1972, Poole and Armitage 1988, Packer and Armitage 1994, Karim *et al.* 1998, Larsen *et al.* 2004, Duffy *et al.* 1995, Xie *et al.* 2011 Qian *et al.* 2013). This allowed for comparison amongst multiple species to observe whether diffusivity, glucose equivalence and flux are similar across bacterial species or whether there is a variance perhaps due to the differences in the number of flagella, flagella arrangement and the motility search patterns that are utilized (Table 5.1).

Comparative diffusivity values were collated from this study and others (Kiørboe *et al.* 2002, DeLoney-Marino *et al.* 2013, Graff *et al.* 2013) for the species comparison. The diffusivity values were calculated for all the chemotactic search patterns utilized by motile bacterial cells, including 3-step flick, run and tumble, run-reverse, random walk and the straight swim. These values were calculated by varying the cosine of the angle value between runs. As not all bacteria utilize identical search patterns such as the random walk, only commonly utilized search patterns were compared. Whilst motile cells have been identified to diffuse approximately 1000 times faster than non-motile cells, it is unclear which chemotactic search patterns presents the greatest influence on the diffusivity variation (Kim 1996). Here, we assess the diffusivity variation across multiple bacterial species and the influence of the search pattern on those diffusivity values.

5.3 Results

The mean search pattern speeds of *V. coralliilyticus* were plotted against the seawater-mucus interface (Fig. 5.1). This interface mimics the natural variance in viscosity and chemoattractants, as previously identified due to diffusion between the coral epidermis and surrounding seawater (Swan *et al.* 2012, Garren *et al.* 2013). For cells identified in the mucus, cell search pattern speeds were on average 2 fold higher than comparable search pattern speeds in the culture. Mean flick search pattern speeds were identified as 35.4 μm s^{-1} (SEM = 1.6, n = 3) in the culture compared to 68.6 μm s^{-1} (SEM = 2.6, n = 3) in the mucus. Mean run-reverse search pattern speeds were identified as 32.3 μm s^{-1} (SEM = 1.4, n = 3) in the culture compared to 72.3 μm s^{-1} (SEM = 1.0, n = 3) in the mucus. Mean straight swim search pattern speeds were

identified as 35.7 μm s^{-1} (SEM = 1.3, n = 3) in the culture compared to 69.3 μm s^{-1} (SEM = 1.9, n = 3) in the mucus. The shift in speed that is identified here for all search patterns is predicted to be a result of the slight increase in viscosity due to the increasing quantity of coral mucus with distance and therefore proximity to a simulated coral epidermis layer (Fig. 5.1).

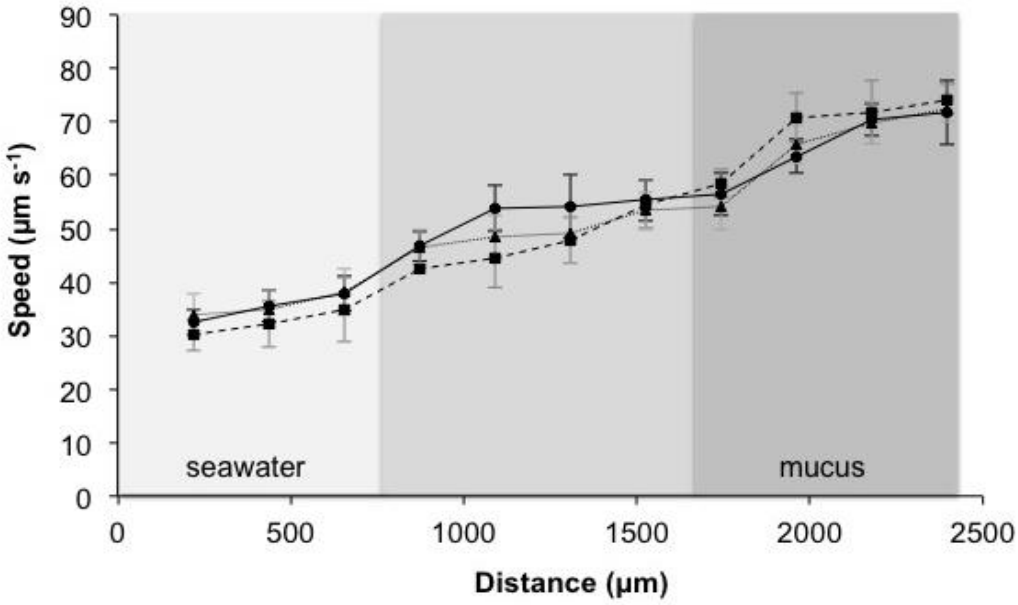

Figure 5.1. Chemotaxis to coral mucus. Experimental data showing changes in chemotactic speed from 1 cP in seawater to 2.3 cP in mucus. Error bars = SEM. Circles and a solid line represents flicks, squares and a dashed line represents run-reverses and triangles and a dotted line represent straight swims.

Individual search pattern run durations ranged between 0.2 and 2.4 seconds (Fig. 5.2). This range occurred due to the straight swim search patterns, which showed no changes in direction and gave the greatest run durations. This is compared to the shorter run durations between each directional change for the run-reverse and flick search patterns. We identified that flick and run-reverse search patterns had the

shortest run duration of 0.2 s with 83% and 65% respectively. In contrast, straight swimming search patterns had longer run durations with only 30% of the runs consisting of 0.2 s durations. Purely straight swimming search patterns had to have a run duration of at least 1.0 s due to the requirements that each cell was viewed for a minimum of 5 frames. However, straight swims also occurred in the run-reverse and flick search patterns which accounted for the 30% of straight swims that were identified of having a run duration of 0.2 s. The straight swimming run durations also showed a bi-modal trend, which is identified by two peaks at the 0.2 s and 0.8 s run duration, with 30% of bacterial runs and 31% of bacterial runs occurring respectively, for the 0.2 s and 0.8 s run durations (Fig. 5.2).

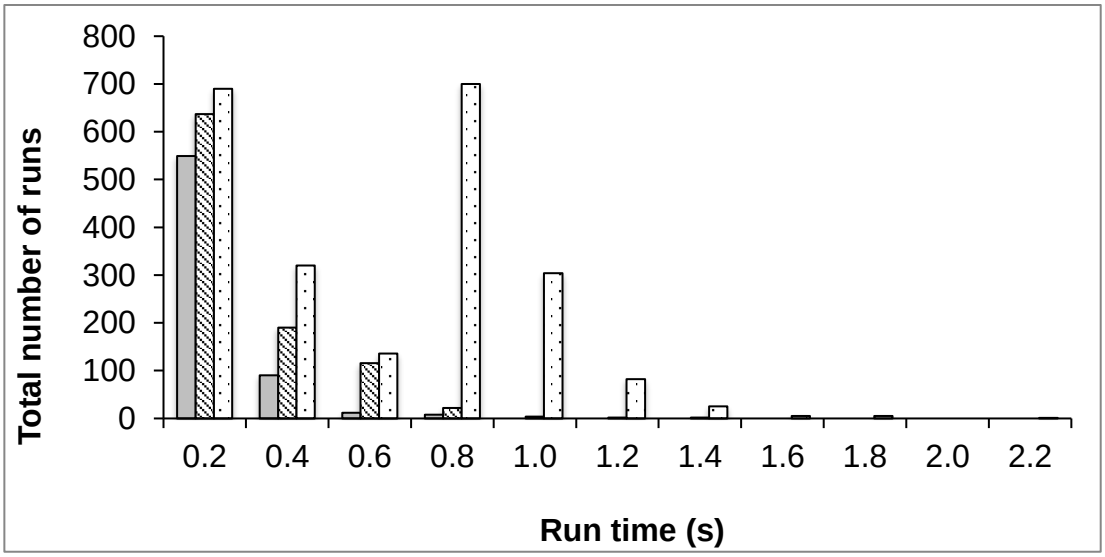

Figure 5.2. The total number of the cells' run times (in seconds) of chemotactic search patterns. Cells were tracked for a minimum of 5 microscope video frames. The distance of the cell's actual path between each frame was recorded and measured to determine the run time. Note. Only the actual search pattern path was measured, when search patterns tracked back on themselves, such as in reverses the gaps between the run paths were not measured. Circles and a solid line represent flicks, squares and a

dashed line represent run-reverses and triangles and a dotted line represent straight swims.

The number of glucose molecules increased with increasing viscosity. However, the number of glucose molecules did not vary irrespective of search pattern type (Fig. 5.3). The glucose molecules sec^{-1} on averaged increased from 13 (SEM = 1.0, n = 3) to 118 (SEM = 5.9, n = 3) from the low viscosity seawater region to the higher viscosity mucus region (Fig. 5.3a). The cost of motility did not vary between the three search patterns examined. The cost in glucose molecules sec^{-1} increased from 13 (SEM = 1.2, n = 3) to 114 (SEM = 8.3, n = 3), 11 (SEM = 0.9, n = 3) to 130 (SEM = 3.5, n = 3) and 13 (SEM = 1.0, n = 3) to 120 (SEM = 6.2, n = 3) from the seawater to the mucus region for flicks, run-reverses and straight swims respectively (Fig. 5.3b). On average, this indicated a 12 times increase in cost, in glucose molecules sec^{-1}, as a function of distance from the seawater to mucus region. An increase in speed was identified with elevated cost, in terms of glucose molecules s^{-1}, with no difference in cost noted between search pattern types. On average, the speed of the search patterns increased 2.5 times across the seawater to mucus transect, from 34.5μm s^{-1} (SEM = 1.5, n = 3) to 70.1μm s^{-1} (SEM = 3.1, n = 3). This increase in search pattern speed coincided with an increase in cost of one order of magnitude from 13 (SEM = 0.6, n = 9) to 118 (SEM = 3.6, n = 9) glucose molecules s^{-1} (Fig. 5.4).

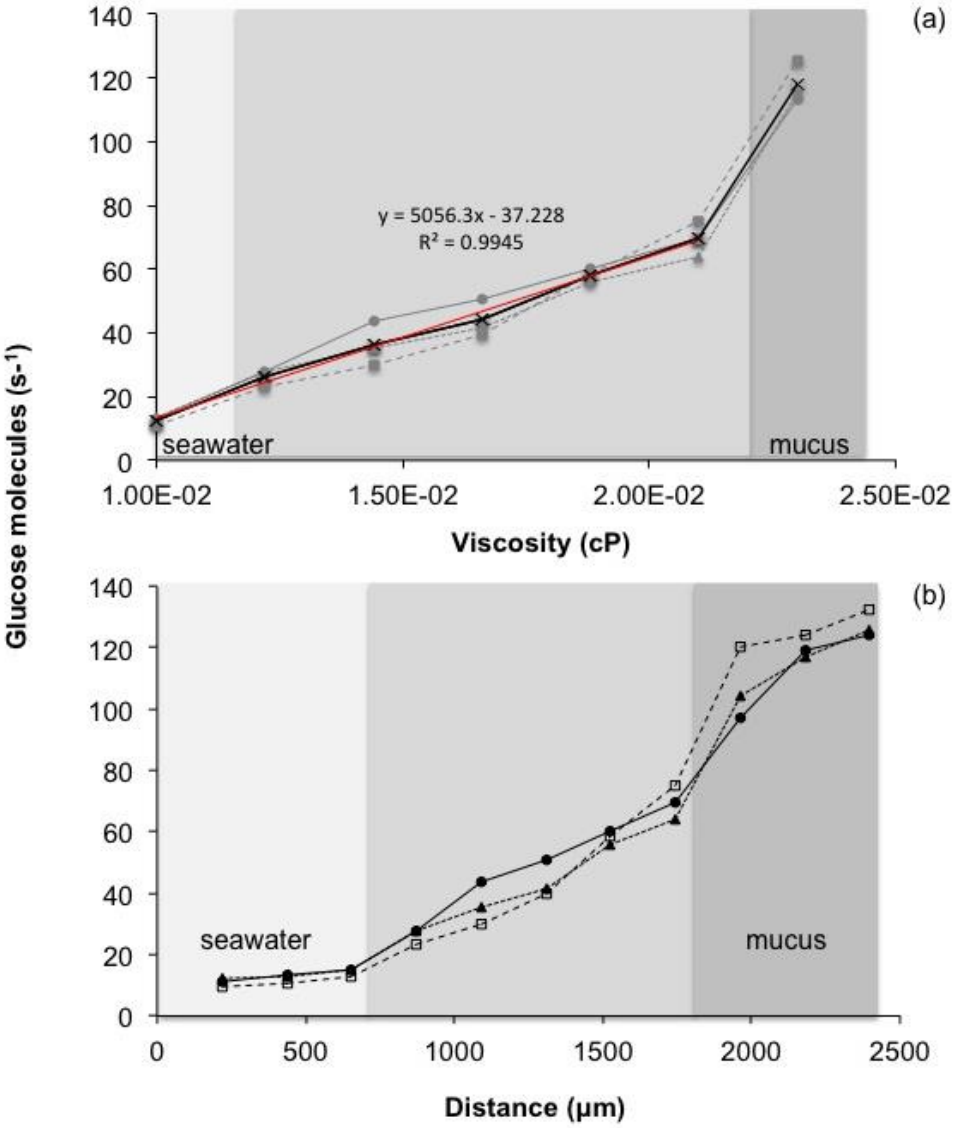

Figure 5.3. The influence of viscosity on the metabolic cost of movement. (a) The metabolic cost, in glucose molecules s^{-1}, experienced by *V. coralliilyticus* in utilizing the three common chemotactic search patterns across a seawater to coral mucus interface identifying how the cost of motility increases with increasing viscosity experienced by cells when moving into the mucus region surrounding the coral tissue surface. A trend line (identified by the red line and x data points) is plotted through the majority of the data with a R^2 value of 0.9945. The final data point is not included in the trend line as it presents as an

outlier which may be an effect from the mucus region; (b) The metabolic cost, in glucose molecules s^{-1}, experienced by *V. coralliilyticus* in utilizing the three common chemotactic search patterns across a seawater to coral mucus interface identifying how the cost of motility increases when cells move into the mucus region surrounding the coral tissue surface. In both figures, circles and a solid line present flicks, squares and a dashed line represents run-reverses and triangles and a dotted line represent straight swims.

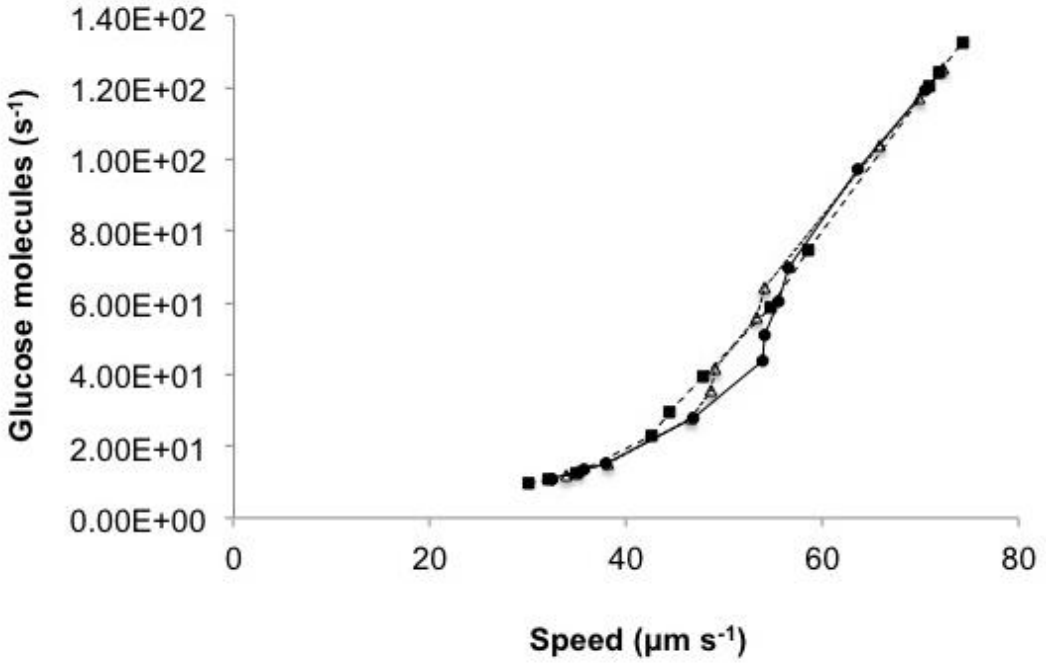

Figure 5.4. The influence of speed on the metabolic cost of movement. The cost, in glucose molecules s^{-1} associated with the speed of chemotactic search patterns utilized by *V. coralliilyticus*. Circles and a solid line represents flicks, squares and a dashed line represents run-reverses, triangles and a dotted line represents straight swims.

Choosing to swim straight by utilizing run lengths of 1.4 s, increased *V. coralliilyticus's* diffusivity by a factor of 2 compared to flick search patterns (Fig. 5.5a). The diffusivity values for *V. coralliilyticus's* straight swims increased from $1.7x10^{-5}$ μm^2 s^{-1} to $2.7x10^{-5}$ μm^2 s^{-1} from the seawater to mucus region, compared to the diffusivity values for flick search patterns which increased from $8.8x10^{-6}$ μm^2 s^{-1} to $1.4x10^{-5}$ μm^2 s^{-1} from the seawater to mucus regions. Midway between the seawater boundary and mucus, the diffusivity values were $3.6x10^{-6}$ μm^2 s^{-1} for straight swims, and $1.8x10^{-6}$ μm^2 s^{-1} for flick search patterns, identifying that whilst the diffusivity trend presented peaks and troughs there was a 1.5 times increase in diffusivity values for straight swim and flick search patterns towards the mucus region (Fig. 5.5a). Diffusivity values decreased with increasing cost, with little differences observed between search pattern paths (Fig. 5.5b). As such, the decrease in cost was identified during the interface region which plateaued once reaching the mucus region with a diffusivity value of $4.1x10^{-8}$. This exponential decrease in cost, of 1.5 orders of magnitude coincided with a ½ order of magnitude decrease in diffusivity.

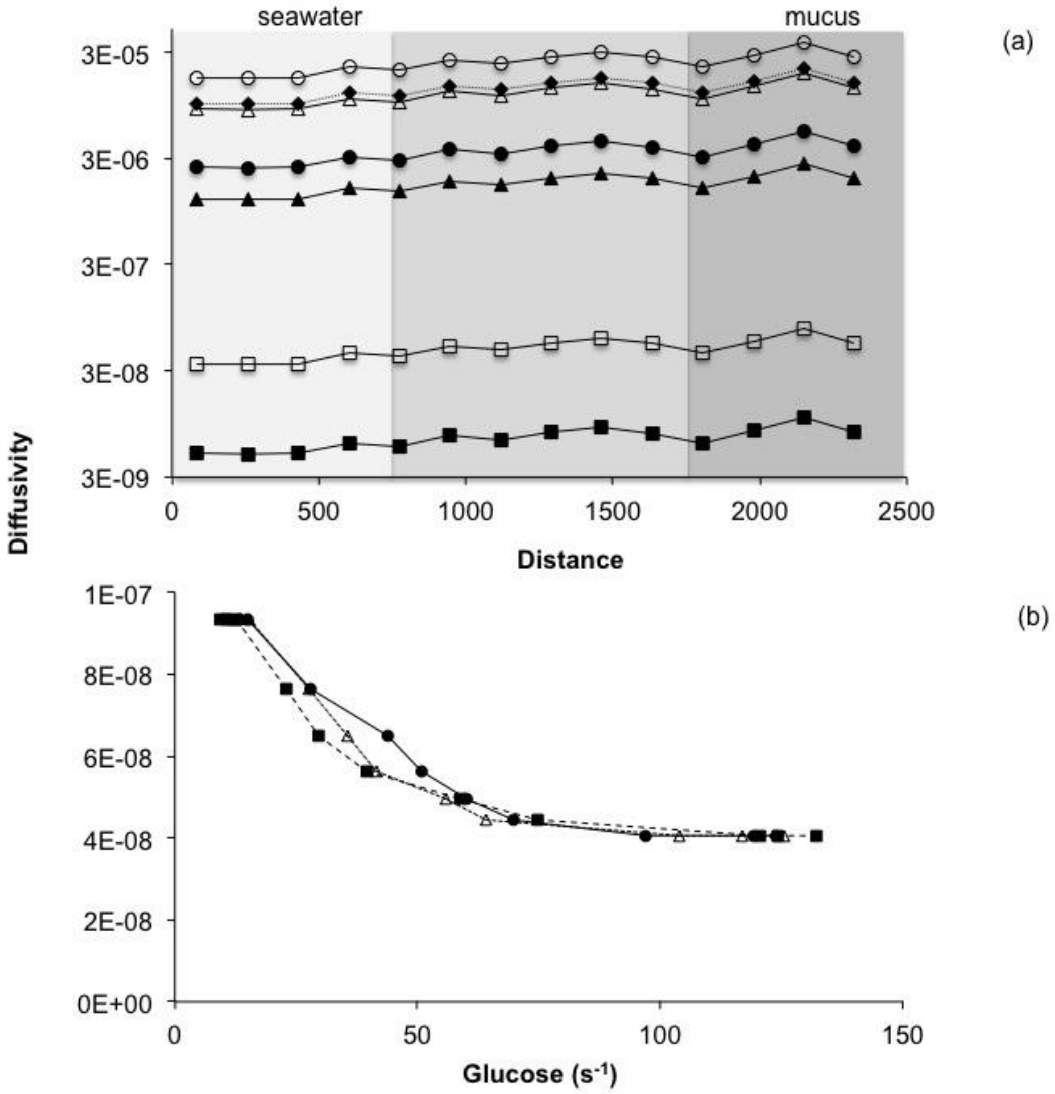

Figure 5.5. Calculated diffusivity values. (a) The diffusivity values of utilized chemotactic search patterns of varied run times (in seconds) across a seawater to coral mucus interface which increases in viscosity from l-r. Squares represent run-reverses, triangles represent flicks, circles represent straight swims. The solid shapes represent run times of 0.2 s, and the open shaped represents run times of 1.4 s. The diamond shape represent 0.8 s straight swim run times; (b) The DMSP diffusivity values calculated with *V. coralliilyticus's* three prominent chemotactic search patterns compared to the metabolic cost involved (in glucose

molecules) of maintaining the diffusivity values. Circles represent flicks, squares represent run-reverses and triangles represent straight swims.

Flux values of DMSP molecules increased across the transect line with a value of 367 $mol.m^{-2}.s^{-1}$ calculated in the seawater region surrounding the coral surface mucus layer at 86 µm along the analyzed transect line where a concentration of approximately 4 µM DMSP is expected. This is compared to the calculated flux values of DMSP molecules in the mucus region with a value of 1760 $mol.m^{-2}.s^{-1}$ near the coral tissue surface within the mucus region at 2300 µm along the analyzed transect line where a concentration of approximately 56 µM DMSP is expected (Fig. 5.6a). A 14 times increase in DMSP flux values was identified between the culture and mucus region. This increase in flux plateaued in the interface region between the seawater and mucus regions, with an increase in flux of only 200 $mol.m^{-2}.s^{-1}$ across an 872 µm distance along the transect line. Flux was identified to increase with increasing cost, in glucose molecules s^{-1} (Fig. 5.6b). Once again, in the interface region the flux values leveled off, increasing slightly from 1100 to 1440 $mol.m^{-2}.s^{-1}$.

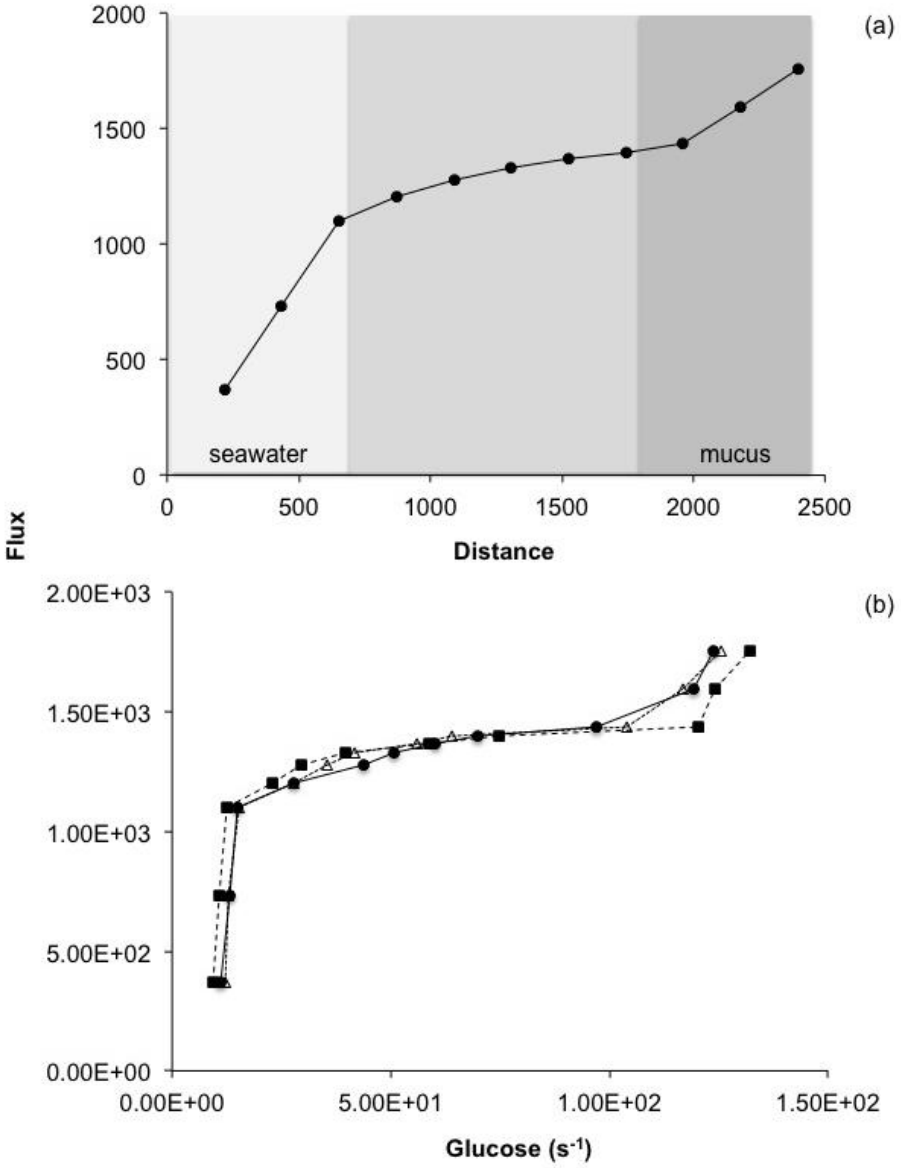

Figure 5.6. Calculated DMSP flux values. (a) The flux of DMSP variance across a seawater to coral mucus interface; (b) The flux of DMSP concentration values associated with the cost, in glucose molecules s^{-1} required. Circles and the solid line represents flicks, squares and a dashed line represents run-reverses and triangles and a dotted line represents straight swims.

For the species comparison, the diffusivity values was dependent on the search patterns utilized, which are identified in Table 5.1. For *Escherichia coli*, a diffusivity value of $2.7x10^{-7}$ μm^2 s^{-1} was identified for straight swimming search patterns, compared to a diffusivity value of $7.4x10^{-8}$ μm^2 s^{-1} for the random walk search pattern. In contrast, *Pseudomonas putida* had a diffusivity value of $2.6x10^{-6}$ μm^2 s^{-1} for straight swimming search patterns, but since *P. putida* has not been identified to exhibit a random walk pattern only the straight swimming diffusivity values were used for comparison with other bacteria. Flagella number did not appear to influence diffusivity (Fig. 5.7) with monoflagellated bacteria showing diffusivity values that varied between high and low values. Monoflagellated bacteria also identified as having some of the highest diffusivity values calculated.

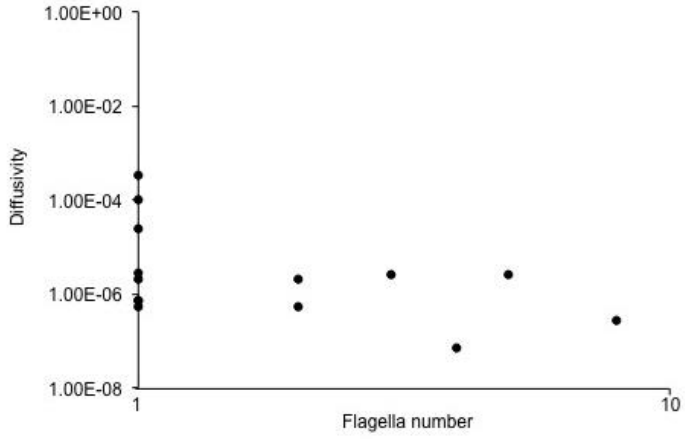

Figure. 5.7 Calculated diffusivity values depending on the flagella number found across numerous bacterial species ranging from monotrichous flagella to peritrichous bacteria.

Diffusivity values compared to multiple bacterial species did vary somewhat although clear groupings of diffusivity values according to individual search pattern

use was identified (Fig. 5.8). Run reverses had the lowest diffusivity value for all bacterial species compared, with straight swims having the highest. This identifies that irrespective of flagella number, orientation or species type diffusivity values are influenced by the angle within the search pattern run.

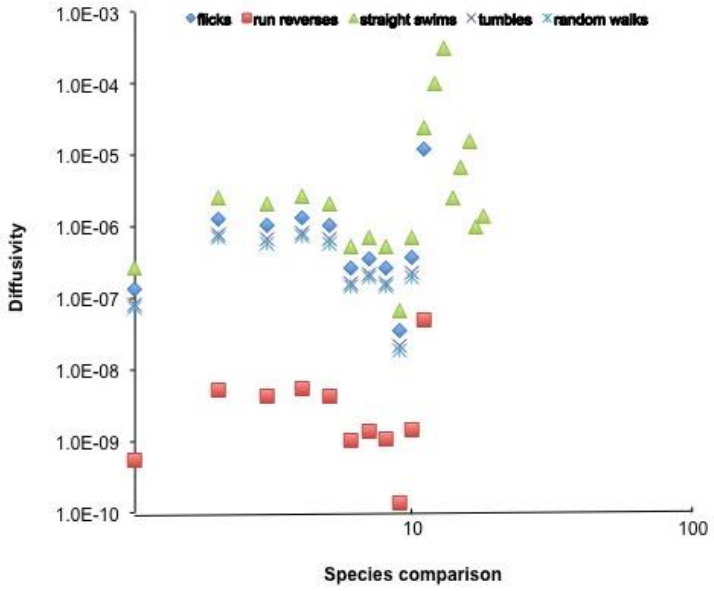

Figure 5.8 Diffusivity values calculated for each type of search pattern across several bacterial species ranging from monotrichous flagella to peritrichous bacteria.

The diffusivity calculations performed and the diffusivity values collected from other research papers, identified an approximate 3 times difference between the diffusivity values of the same bacteria utilizing different search patterns (Berg and Brown 1972, Poole and Armitage 1988, Packer and Armitage 1994, Duffy *et al.* 1995, Karim *et al.* 1998, Millikan and Ruby 2002, Gillad *et al.* 2003, Larsen *et al.* 2004, Xie *et al.* 2011, Stocker 2011, Qian *et al.* 2013). These calculations of diffusivity values of other bacterial species reiterates the current findings whereby *V.*

coralliilyticus's search patterns and run lengths can influence its' diffusivity values, as seen in the other bacterial species.

5.4 Discussion

Vibrio coralliilyticus has been identified to require, on average, more energy using the flick search pattern than any other search pattern in its repertoire. In addition, greater energy expenditure is required to swim in regions of higher viscosity, such as the coral surface mucus layer which can be linked to the increase in drag associated with viscous liquids (Schneider and Doetsch 1974, Cone 2009). These results show that search pattern usage including type and run length, in seconds, has an implication on the cells energy expenditure. Past studies have identified that a reduction in swimming speed does not reduce cell energy expenditure (Mitchell 1991). However, with the unlikelihood cells can actively decide to control their motility (Balázsi *et al.* 2011, Norman *et al.* 2013, Steinert 2014, Lyon 2015) and with evidence of changes in run length and search pattern differences noted in bacterial motility studies (Kiørboe *et al.* 2004, Taktikos *et al.* 2013), this suggests that some form of control with the motility mechanisms for motile bacterial cells is at play (Erban and Othmer 2004, Galajda *et al.* 2007, Hu and Tu 2014).

V. coralliilyticus run-length distribution of run-reverses and flicks presented an exponential decline with a shift towards longer runs (Fig. 5.2). These longer runs may be beneficial to motile bacteria by increasing the diffusivity of surrounding nutrients and chemoattractants into the cell, especially for the straight swim search patterns (Taktikos *et al.* 2013). However, the longer runs of all search patterns result in an increase in diffusivity values. This gives some speculation to the mechanism of

individual chemotactic search patterns, whereby potential chemoreceptors and subsequent flagellar motor responses create uniformed and differentiated search patterns utilized for movement (Taktikos *et al.* 2013). This chemoreceptor and subsequent motility response coincides with the use of flicks as a reorientation mechanism (Stocker 2011, Son *et al.* 2013, Norris *et al.* 2014), because if nutrient diffusivity was high it would be counter productive to not use a search pattern that offers the greatest diffusivity values, such as the straight swim. Of the three search patterns identified in *V. coralliilyticus*, flicks have the shortest run time, suggesting that this pattern is not conducive to long term use whilst swimming and is only used sporadically when required (Taktikos *et al.* 2013, Wu and Tuba 2013).

With bacterial cell search patterns identified to hinder the glucose molecule expenditure differently, other physical properties of a liquid may also impose an effect on the bacterial cellular energy requirements. As identified in this study, flicks and straight swims use more glucose molecules in low viscous solutions with a value of 13 glucose molecules s^{-1} compared to the reverses which use a value of 11 glucose molecules s^{-1} (Fig. 5.3a). Once cells move into the seawater-mucus interface and mucus regions the glucose molecules used are lower for the flick search patterns than for the run-reverses or straight swims (Fig. 5.3b). Cells that adopt a straight swim pattern are often described as meandering through the solutions, with the distances travelled between frames on average considerably shorter than the distances between frames associated with flicks and reverses (Taktikos *et al.* 2013). This may suggest that this meandering pattern means a larger total swimming length or time is required to reach the coral surface which corresponds with an increase in the energy required for movement.

Interestingly, flicks utilize less energy reserves (in glucose molecules per second) in the mucus region than compared to the other search patterns. This result may actually be a representation of the limited number of flicks identified in the mucus region due to unfavorable conditions associated with an increase in drag created by the viscosity of the solution. Son *et al.* (2013) suggested that the higher drag, associated with viscous solutions may actually impede the flick search pattern, as the critical speed required for the buckling instability that results in a flick event is unable to be reached.

From the seawater culture to mucus, diffusivity varies by a factor of 2. However, between search patterns types diffusivity varies by 4 orders of magnitude (Fig. 5.5a). This indicates that viscosity does not influence the diffusivity as much as search pattern choice and run times. If a bacteria uses long run lengths then the diffusivity is 4 orders of magnitude higher than if a bacteria uses short run lengths. Search pattern preference is also shown to influence diffusivity, meaning that this initiates a behavioral control rather than a viscosity control. This means that the bacteria can choose the level of diffusivity based on the search pattern run time and strategy. The choice of search patterns alters the rate of diffusivity whereby the backtracking nature of some search patterns reduces these values (Taylor and Koshland 1974, Thar and Kühl 2003). Cells that backtrack over previous paths may have already exhausted the nutrient supply and hence diffusivity values are lower (Thar and Kühl 2003). Surprisingly, greater diffusivity occurred with a decrease in cost, in glucose molecules sec^{-1} (Fig. 5.5b), which indicates that the greatest diffusivity is not necessary linked to higher energy consumption search patterns.

DMSP flux values increased from the seawater to mucus regions near the coral tissue surface, with values leveling off in the interface region. With greater flux

values identified in the mucus region and lower values in the surrounding seawater, this change in flux is potentially linked to the DMSP concentrations present within the coral environment. As DMSP concentrations reduced in the surrounding seawater (Swan *et al.* 2012, Garren *et al.* 2013), we infer that this reduction resulted from the mixing with seawater thereby reducing its concentration and creating what may account for a DMSP gradient. This mixing has been identified to occur for other solutions associated with the coral organism including the surface mucus layer which becomes less viscous as it mixes with the surrounding water (Garren *et al.* 2013). The elevated flux values associated with elevated cost, in glucose molecules sec^{-1}, are potentially linked to the high cost of movement in viscous conditions. In the mucus, the higher flux of DMSP molecules was associated with greater energetic cost, in glucose molecules sec^{-1}. This result coincides with higher viscosity values in the mucus which therefore demands a higher energy consumption or requirement, in glucose molecules, to combat against the challenges a higher viscous media creates.

The comparative analysis of multiple bacterial species (Supplementary Table 5.1) identified that diffusivity values from cells utilizing identical search patterns did not vary. The bacteria examined here, were either peritrichous or monoflagellated, and this did not appear to influence the diffusivity values (Fig. 5.7, Fig. 5.8) (Taylor and Koshland 1974, Koshland Jnr 1977). Nor did the bacteria's pathogenicity appear to influence the diffusivity values, as these values appeared independent of whether the bacterium was pathogenic or non-pathogenic. Run-reverse search patterns presented diffusivity values that were 2 to 3 orders of magnitude lower than straight swimming search patterns with the straight swimming search patterns having the greatest diffusivity values for all bacteria.

Our results infer that contrary to previous studies (Norman *et al.* 2013, Steinert 2014, Lyon 2015) cells possess the capability to fine-tune their movements to make use of their environmental surroundings and nutrient availability. Controlling the rate of the diffusion of the chemoattractant, DMSP through altered run times (in seconds) identifies that some level of behavioral control or cognitive control occurs. Although this control may be in the form of mechanical changes within the flagellar motor in response to chemosensory cues (Blair 1995, Hu and Tu 2014), this nonetheless signifies a highly adaptable and responsive organism.

5.5 Acknowledgements

We'd like to thank the Australian Research Council and the Centre for Marine Microbiology and Genetics at the Australian Institute of Marine Science for funding support for this research.

Supplementary Table 5.1. Species comparison of search pattern diffusivity values ($\mu m^2 s^{-1}$)

Species	Diffusivity values					Reference
	Straight swimming	Flick	Run-reverse	Tumble	Random walk	
Escherichia coli	$2.66x^{-07}$*	$1.34x10^{-7}$*	$5.38x10^{-10}$*	$8.33x10^{-8}$*	$7.39x10^{-8}$*	Berg and Brown 1972
Pseudomonas putida	$2.56x^{-06}$*	$1.29x10^{-6}$*	$5.16x10^{-9}$*	$8.00x10^{-7}$*	$7.10x10^{-7}$*	Duffy *et al.* 1995
Pseudomonas aeruginosa	$2.11x^{-06}$*	$1.07x10^{-6}$*	$4.27x10^{-9}$*	$6.61x10^{-7}$*	$5.87x10^{-7}$*	Qian *et al.* 2013
Spiroplasma melliferum	$2.11x^{-06}$*	$1.07x10^{-6}$*	$4.27x10^{-9}$*	$6.61x10^{-7}$*	$5.87x10^{-7}$*	Gillad *et al.* 2003
Rhodobacter sphaeroides	$5.23x^{-07}$*	$2.64x10^{-7}$*	$1.06x10^{-9}$*	$1.64x10^{-7}$*	$1.45x10^{-7}$*	Packer and Armitage 1994
Rhodobacter sphaeroides	$6.98x^{-07}$*	$2.53x10^{-7}$*	$1.41x10^{-9}$*	$2.19x10^{-7}$*	$1.94x10^{-7}$*	Poole and Armitage 1988
Vibrio anguillarum	$5.28x^{-07}$*	$2.67x10^{-7}$*	$1.07x10^{-9}$*	$1.65x10^{-7}$*	$1.47x10^{-7}$*	Larsen *et al.* 2004
Vibrio alginolyticus	$2.67x^{-06}$*	$1.35x10^{-6}$*	$5.4x10^{-9}$*	$8.37x10^{-7}$*	$7.42x10^{-7}$*	Xie *et al.* 2011, Stocker 2011
Helicobacter pylori	$6.84x^{-08}$*	$3.46x10^{-8}$*	$1.38x10^{-10}$*	$2.14x10^{-8}$*	$1.90x10^{-8}$*	Karim *et al.* 1998
Camplyobacter jejuni	$7.23x^{-07}$*	$3.65x10^{-7}$*	$1.46x10^{-9}$*	$2.27x10^{-7}$*	$2.27x10^{-7}$*	Karim *et al.* 1998
Vibrio fischeri	$5.93x^{-06}$*					Millikan and Ruby 2002
Vibrio cholerae	$3.65x^{-06}$*					Graff *et al.* 2013
Curacaobacter baltica	$2.60x^{-06}$**					Kiørboe *et al.* 2002
Frigobacterium sp.	$6.80x^{-06}$**					Kiørboe *et al.* 2002
Microscilla fulvescens	$1.60x^{-05}$**					Kiørboe *et al.* 2002
Rhizobium sp.	$1.00x^{-06}$**					Kiørboe *et al.* 2002
α-Proteobacterium	$1.40x^{-06}$**					Kiørboe *et al.* 2002

* calculated diffusivity values in this manuscript through collated run lengths and swimming speeds obtained from research papers

** diffusivity values taken from research papers to use for comparison

Chapter 6

Motility responses to natural fluctuations in corals and their protective boundaries: A general discussion

6.1 Overview

Reef microbial communities are integral players that contribute to the functioning of the coral reef ecosystem (Rosenberg *et al.* 2007). The resilience and adaptability of reef microbes, specifically their motility responses in the presence of structural and nutritional fluctuations is fundamental to their establishment and growth within a coral host (Voss and Richardson 2006, Harvell *et al.* 2007). Despite their prevalence and role in an array of coral tissue diseases, their infection pathway is often overlooked and remains speculative, particularly regarding the initial stages of infection (Lesser *et al.* 2007). The research presented in this thesis was stimulated by the need to gain a greater understanding of how *Vibrio coralliilyticus* initially responds to the coral mucus and its chemical constituents. Specifically, the focus is on their ability to penetrate the protective surface layer in an often turbulent and nutrient-depleted environment. The reoccurring theme throughout this thesis has been that of the motility response of the *V. coralliilyticus* flagella, following the nutrient and viscosity fluctuations found within a coral organism. This final chapter will discuss the major findings of the thesis and address the results from each of the experimental chapters within the context of the specific thesis aims outlined in Chapter 1. The final section of this chapter synthesizes the results and shows how the chapters combined provide a broad understanding of a key step in bacterial pathogenicity, namely locating and finding a susceptible host.

6.1.1 *Vibrio coralliilyticus* search patterns across an oxygen gradient

The data presented in Chapter 2 addressed the extent to which the natural oxygen variances present on corals during night-time hours affected the chemotactic search

patterns of *Vibrio coralliilyticus*.). We acknowledge that the frame rate for the video microscopy is low and this may be a disadvantage for examining the flick mechanism, however this was not the aim here. Rather, this low frame rate was an advantage as we were able to examine motility and search pattern numbers as an average value, rather than individual search pattern dynamics.

Large natural variations in oxygen occur within coral niche habitats, spanning hyperoxic conditions during the day to anoxic conditions during the night at the tissue/mucus interface (Nilsson *et al.* 2004, Thurber *et al.* 2009, Morrow *et al.* 2011, Wijgerde *et al.* 2012). Coral associated bacterial communities must be able to tolerate extremes in oxygen concentrations and other stimuli and associated nutrients to survive and potentially flourish. Chapter 2 identifies that *V. coralliilyticus* remains motile irrespective of the oxygen concentrations present within a coral organism (Winn *et al.* 2013).

6.1.2 Increased drag stops chemotactic flicks

In Chapter 3, I addressed the influence the coral surface mucus layer and variations in viscosity had on the chemotactic search patterns of *Vibrio coralliilyticus*. A fundamental shift in *V. coralliilyticus* chemotactic search patterns was identified as cells crossed from a culture media to mucus interface mimicking the coral mucus to seawater interface (Fig. 3.2). In higher viscous regions, such as the coral mucus, 3-step flick search pattern numbers decreased. This reduction in flicks may be a mechanical response to the limitations viscosity places on cell speed, whereby speed is reduced to such an extent that the buckling mechanism that instigates the 90° flick event, is prevented (Xie *et al.* 2011).

The comparative analysis of viscous controls that mimic the native mucus solutions exhibited a reduction in speed in solutions with viscosities beyond 2.5 cP. This result coincides with the idea that the viscosity of the solutions limits the speed acceleration (Magariyama *et al.* 1995, Atsumi *et al.* 1996) required for flick events to occur (Xie *et al.* 2011, Stocker 2012) and in conjunction, limiting the number of flicks identified in viscous solutions which coincides with the search pattern results in chapter 3 (Fig. 3.4). Remaining motile in viscous solutions also presents a confronting issue for bacterial cells, whereby the cells must be prepared to input additional energy required for motility in viscous regions before benefiting from the nutrient uptake or chemoattraction.

6.1.3 Coral mucus and DMSP influence flagellar gene copies and chemotaxis of the coral pathogen *Vibrio coralliilyticus*

The data presented in Chapter 4 addressed to what extent concentration gradients of coral mucus and DMSP limit the gene expression values of the *flaA* and *flhA* flagellar genes and chemotactic search patterns of *Vibrio coralliilyticus*. With flick search patterns identified as a reorientation mechanism (Stocker 2011), the results from this chapter suggest that *V. coralliilyticus* cells were utilizing the flick to reorientate into a more beneficial chemotactic environment. At 20 µM DMSP, the most utilized search pattern identified was the run-reverse, which correlates with the notion that reverses allow bacteria to effectively utilize and backtrack over favorable nutrient patches (Johansen *et al.* 2002). As DMSP is used as an infochemical for host detection and not metabolized by *V. coralliilyticus* (Garren *et al.* 2014), the concentration gradient present between the mucus and surrounding seawater may

account for the search pattern results and may indicate that DMSP acts as a beacon to identify the proximity to the coral tissue surface (Swan *et al.* 2012, Garren *et al.* 2014). As the gene expression of *flhA* and *flaA* was identified to differ with a variance in DMSP and coral mucus concentrations, search pattern speed appeared to correspond with these given concentrations. This could suggest that the flagellar motor rotation is indicative of search pattern behavior.

6.1.4 Cellular energy expenditure modelling for the coral pathogen *Vibrio coralliilyticus*

The data presented in Chapter 5 modelled and analyzed the metabolic cost, in glucose molecule equivalence, required by *V. coralliilyticus* cells in utilizing chemotactic search patterns. From the seawater to the mucus, the metabolic cost of motility, measured in glucose molecules, was identified to increase in the mucus region (Fig. 5.3). The identified exponential increase of metabolic cost could be an indicator of the increased energy required for the functioning of the flagellar motor under regions of increasing drag (Wall and Kaiser 1999). Along with viscosity, an increase in speed was also linked to elevated metabolic cost (Fig. 5.4). This may further support the finding that increased cell motility is identified in nutrient depleted or starved cells (Mueller 1996) which coincides with the earlier results discussed in 6.1.1 to 6.1.3.

Diffusivity has been identified to be dependent on the size and motility ability of bacterial cells with flagellated cells noted in having higher diffusivity rates than their non-flagellated counterparts (Mueller 1996, Mitchell 2002). Furthermore, elevated diffusivity has been identified under starvation conditions, whereby

presumably desperate cells absorb as much nutrient as possible compared to regions of high nutrients where cells only absorb nutrients as required (Mueller 1996). This may also account for the variance in diffusivity values identified between the species compared in chapter five (Table 5.1), as the nutrient content used in the studies could have influenced their behavior and nutrient uptake.

6.2 Thesis synthesis: Demonstration of invasive indicators for penetrating the coral surface mucus boundary layer

Bacterial search pattern behavior is the physical response to fluctuations in nutrients in the surrounding environment that can occur due to the flagella. This structural component allows microbes to move towards and utilize nutrient patches (Silverman and Simon 1974, Macnab 1999, Harshey 2003). Although bacterial motility has been recognized for approximately 300 years (Mitchell and Kogure 2006), new findings in search pattern behavior and mechanisms are continuing to occur (Altindal *et al.* 2011, Stocker 2011, Son *et al.* 2013, Tout *et al.* 2015). Even though past research has provided thorough and well-established model organisms of some bacterial species, namely *Escherichia coli* (Berg and Brown 1972, Budrene and Berg 1991, Blackburn *et al.* 1998), recent work is only beginning to uncover the roles and mechanisms of the motility structures and characteristics found in other bacterial species. This provides new bacterial models for species with different flagellar arrangement and number (McCarter 2001, Terashima *et al.* 2008). This thesis aimed to generate chemotactic motility signatures for the mono-flagellated coral pathogen *Vibrio coralliilyticus* under conditions mimicking those identified in the native coral organism.

Previous chemotaxis and motility studies have shown that pathogenic bacteria respond positively to nutrients and chemoattractants by swarming nutrient patches (Blackburn *et al.* 1998, Blackburn and Fenchel 1999, Stocker 2012), however the specifics involved in the changes to the motility patterns in the presence of nutrient and viscosity gradients and the potential link with flagellar motor gene expression has not been explored in depth (Kroos *et al.* 1988). Results from this thesis demonstrated that coral mucus regions led to a shift in the chemotactic search patterns utilized and gene expression of key components of the flagellar motor. Thus, the overall behavioral responses of *Vibrio coralliilyticus* associated with fluxes of nutrients, chemoattractants and oxygen gradients was likely driven by the nutritional requirements of the cell. Alternatively, gene expression of the *flhA* biosynthesis protein under fluctuating DMSP concentrations mimicking the DMSP gradient created by the mixing of seawater and concentration decline with greater distance from the coral surface was shown to peak at a midrange concentration of 20µM. This coincided with the highest quantities of run-reverse search pattern strategies, which have been suggested to be the strategy utilized in a region that is most favorable (Johansen *et al.* 2002). Overall, this thesis demonstrated that *V. coralliilyticus* remains motile irrespective of the oxygen decline or viscosity increase, although search patterns exhibited markedly different trends based on nutrient concentration and viscosity level.

Additionally, this thesis showed that chemotactic search patterns present different energetic costs and associated diffusivity values. Previous studies have identified variations in diffusivity values with different bacterial species (Duffy *et al.* 1995, Karim *et al.* 1998, Kiørboe *et al.* 2002, Larsen *et al.* 2004, Graff *et al.* 2013). However, the majority of these studies tend to focus on individual search patterns

(Packer and Armitage 1994, Kiørboe *et al.* 2002, Millikan and Ruby 2002, Gillad *et al.* 2003, Larsen *et al.* 2004). This thesis, however, has demonstrated *V. coralliilyticus's* ability to alter the their diffusivity values by favoring specific chemotactic search patterns. Furthermore, while diffusivity values also differ with run length, this indicates that *V. coralliilyticus* and potentially other bacteria may have a level of behavioral control over their motility which can aid their ability to take up nutrients.

The process of bacterial infiltration through the coral mucus microenvironment and the variations present in coral mucus and DMSP concentrations through this area with the associated response on chemotactic search patterns utilized by *V. coralliilyticus* is outlined in figure 6.1. This identifies that the viscosity, coral mucus and DMSP gradients identified across the mucus layer influence the motility characteristics including speed and search pattern type utilized by *V. coralliilyticus*. Figure 6.2 presents a closer look at each of these factors in turn, and the associated response on *V. coralliilyticus's* motility. Whilst each of the processes in the circles in figure 6.2 can occur interchangeably and simultaneously throughout the invasion process, this visual summary provides a break down of the key factors identified in this thesis that influence the search pattern motility behavior of *V. coralliilyticus*.

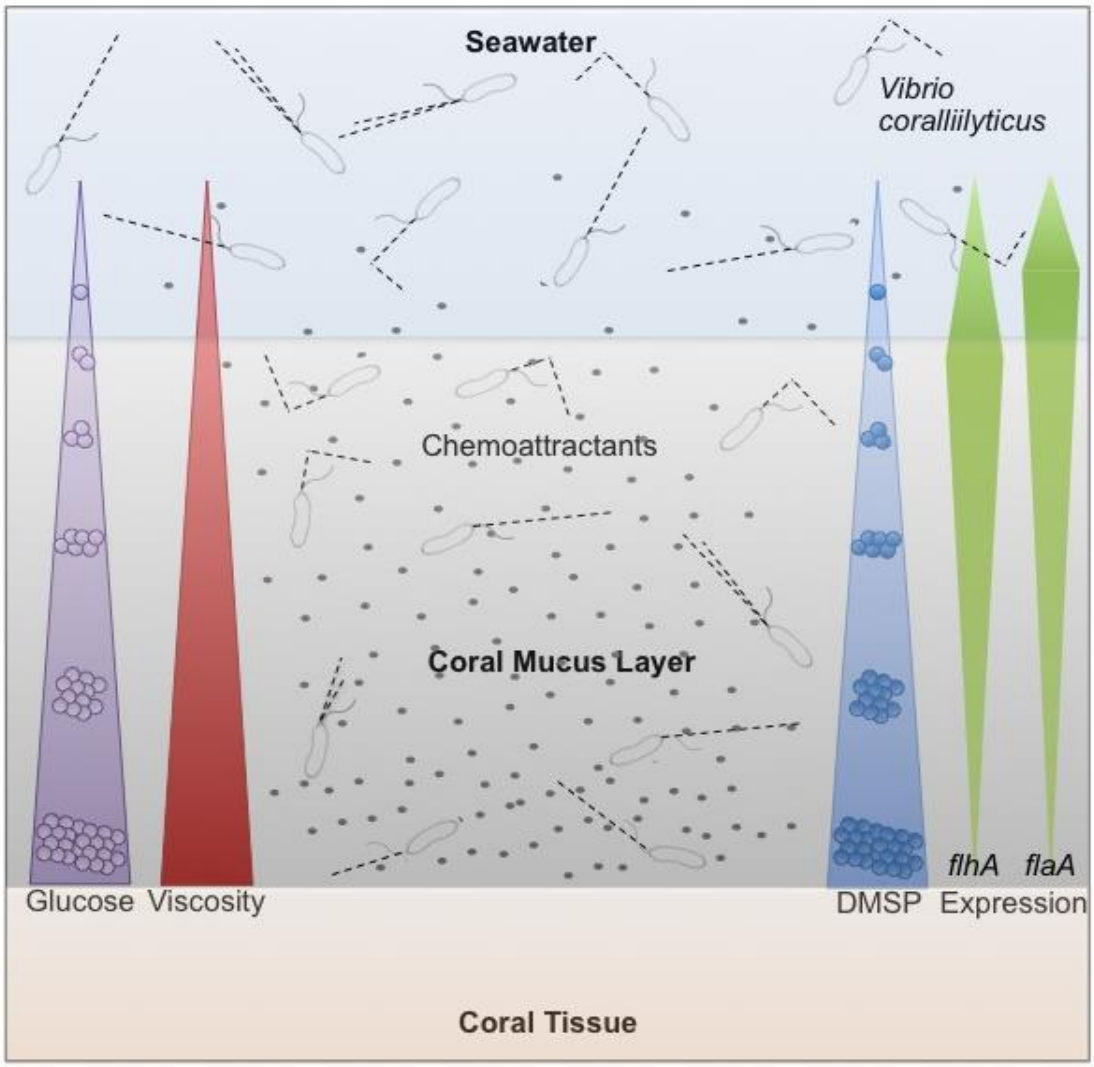

Figure 6.1. The coral surface mucus layer microenvironment. The coral surface mucus layer is full of gradients of chemoattractants, such as DMSP (blue triangle and molecules). These chemoattractants (grey dots) increase towards the coral tissue surface which may form a chemosensory beacon enticing *V. coralliilyticus*. The increase in viscosity associated with the coral mucus (red triangle) alters the chemotactic patterns of *V. coralliilyticus*. Increased viscosity increases with metabolic cost, calculated here in glucose molecules (purple triangle and circles). The gene expression values (green diamond) of key motility genes, namely *flaA* and *flhA* is highest at midrange concentrations of DMSP and coral mucus and is indicated by the diamond width.

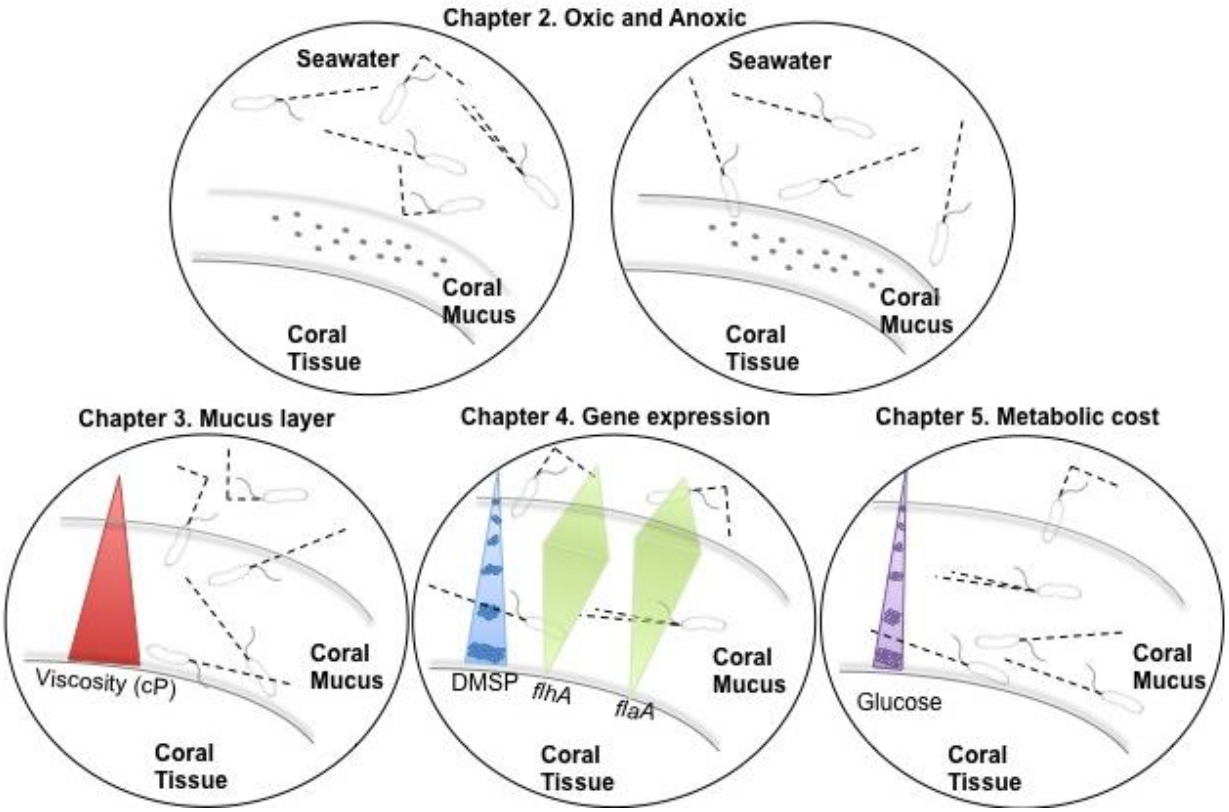

Figure 6.2. A visual summary of the components influencing the chemotactic patterns in *V. coralliilyticus*. Each circle and its' corresponding chapter represents a component that was examined in this thesis with it providing a visual summary of the major results identified in each chapter. *Vibrio coralliilyticus* search pattern responses in: top row (l – r): oxic conditions, anoxic conditions. Bottom row (l – r): through the coral mucus layer, gene expression of *flaA* and *flhA* motility genes, and the metabolic cost of motility.

This thesis provides a novel insight into how natural fluctuations found within the coral organism affects the chemotactic search pattern behavior and gene expression of *V. coralliilyticus*, and for the first time, demonstrates how the motility patterns can differ spatially within regions that mimic the natural fluctuations identified in the coral organism. This thesis also assessed the extent pathogenic organisms, namely *V. coralliilyticus*, can manipulate and counteract seemingly protective host mechanisms. This study highlights the complexity and flexibility of bacterial pathogens inhabiting compromised coral organisms, whilst increasing our understanding of the roles these microbes play in the health of the reef ecosystem and provides another model organism for the understanding of bacterial chemotactic motility.

6.3 Future work; the next steps stemming from this research and addressing the current gaps in research

The next stage of research for this study is to examine the overall progression of infection of *V. coralliilyticus* through the mucus and tissue layers of the coral. Targeted infection experiments could form one part of the analysis where coral nubbins are infected, and analysed periodically through the infection stage. A multi-pronged approach to the analysis would be required to allow for the collection of *V. coralliilyticus cells* for motility and genetic analysis throughout the infection stages. This future study would examine how *V. coralliilyticus* infiltrates the boundary mucus layer and various layers of the coral tissue and in order to gain establishment and growth. This would also allow for the examination of *V. coralliltycus's* chemotactic response throughout the infection process.

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Appendix 1

Manuscript the author contributed to during her candidature

The following publication is a manuscript the author contributed to during her candidature in which she developed techniques and concepts that contributed to her thesis.

Pollock FJ., Krediet CJ., Garren M., Stocker R., Winn K., Wilson B., Huete-Stauffer C., Willis BL and Bourne DG (2015). Visualization of coral host–pathogen interactions using a stable GFP-labeled *Vibrio coralliilyticus* strain. Coral Reefs. 34 (2), 655 – 662.

Appendix 2

Published Manuscript Arising From and Related to this Thesis

OPEN ACCESS Freely available online

PLOS ONE

Vibrio coralliilyticus Search Patterns across an Oxygen Gradient

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Abstract

The coral pathogen, *Vibrio coralliilyticus* shows specific chemotactic search pattern preference for oxic and anoxic conditions, with the newly identified 3-step flick search pattern dominating the patterns used in oxic conditions. We analyzed motile *V. coralliilyticus* cells for behavioral changes with varying oxygen concentrations to mimic the natural coral environment exhibited during light and dark conditions. Results showed that 3-step flicks were 1.4× (P = 0.006) more likely to occur in oxic conditions than anoxic conditions with mean values of 18 flicks (95% CI = 0.4, n = 53) identified in oxic regions compared to 13 (95% CI = 0.5, n = 38) at anoxic areas. In contrast, run and reverse search patterns were more frequent in anoxic regions with a mean value of 15 (95% CI = 0.7, n = 46), compared to a mean value of 10 (95% CI = 0.8, n = 29) at oxic regions. Straight swimming search patterns remained similar across oxic and anoxic regions with a mean value of 13 (95% CI = 0.7, n = oxic: 13, anoxic: 14). *V. coralliilyticus* remained motile in oxic and anoxic conditions, however, the 3-step flick search pattern occurred in oxic conditions. This result provides an approach to further investigate the 3-step flick.

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Introduction

Bacteria use chemotaxis and motility search patterns to position themselves in chemical gradients, locate nutrient sources and initiate pathogenesis [1,2]. These search patterns include run and reverse, run and tumble, straight swimming and the recently described, the 3-step run, reverse and flick [2,3,4,5]. The majority of evidence suggests that only one search strategy is adopted by a single bacterial species [6,7], with marine bacteria suggested to adopt a 'back and forth' swimming behavior pattern, known as a run and reverse, utilizing turbulence-induced shear in the ocean [8]. While the interactions of the run and reverse pattern with its environment is clear, how the 3-step flick pattern, henceforth 'the flick', which is a cyclic motion: forward, reverse, flick and repeat [5,9] interacts with the physical environment is less clear. What is known so far is that by introducing a consistent directional change, the flick allows species such as *V. alginolyticus* to access nutrient patches quicker than bacteria using the run and tumble strategy such as *Escherichia coli* [5]. The key trait of the flick search strategy seems to be a combination of the run and reverse strategy, where the 180° backtracking gives bacteria traveling down a gradient an opportunity to re-exploit nutrient patches found moments earlier, with the run and tumble strategy uses frequent reorientation to find new nutrient sources [3].

Pathogenic and symbiotic *Vibrio* species, including the coral pathogen *V. coralliilyticus*, are reliant on chemotaxis to invade and colonize host species. Abundant in the coral mucus [10,11] and the microbial community of diseased corals [12,13], *Vibrios* alter the microbial metabolism in corals [14]. *V. coralliilyticus* causes the bleaching and tissue lysis of corals at temperatures greater than 25°C, and is further implicated in the disease of other marine organisms including bivalves, during winter months when temperatures are lower [15]. Meron *et al.* [16] demonstrated that the flagellum in *V. coralliilyticus* is critical for infection, including the adhesion to the corals and chemotaxis towards coral mucus.

During coral infection, *V. coralliilyticus* must move through the surrounding seawater, the coral mucus surface layer and into the coral tissue cells for establishment and growth. This infection route, indicative of most coral pathogens, may illustrate unique search pattern strategies to assist in moving through numerous environments of differing viscosity and nutrient complexity. Local marine environments and conditions are constantly changing due to turbulence [9]. Changes in conditions and nutrient concentrations occur diurnally and nocturnally [17]. Oxygen saturation levels are reduced in coral tissue after being exposed to the darkness, with records showing that coral tissues have been shown to have <2% oxygen saturation after 5 minutes in the dark [17]. In contrast, during light conditions coral tissues exhibit up to 250% oxygen saturation [17]. Furthermore, work by Kühl *et al.* [17] showed that oxygen levels remained steady in the surrounding water in both light and low-light conditions, however these levels have shown to differ during light and low-light conditions in the 0.1–1 mm layer on the coral tissue surface as well as inside the coral tissue [17]. This study examines the chemotactic search pattern changes of *V. coralliilyticus* in response to fluctuating oxygen levels. This will provide insight into how *V. coralliilyticus* behaves in

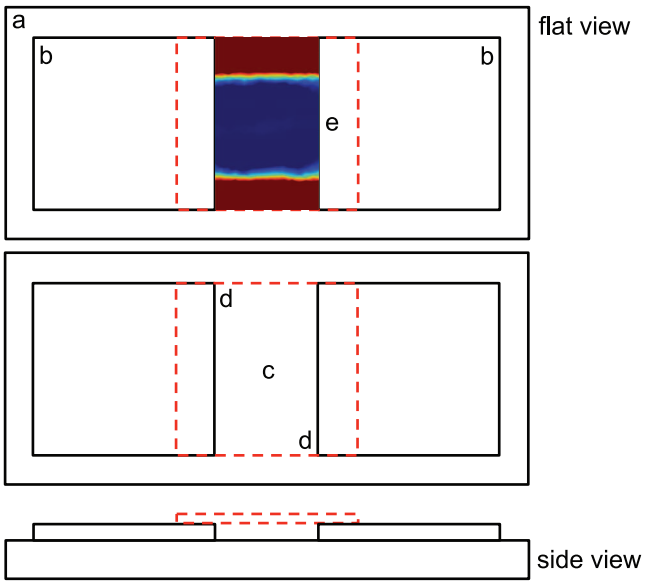

Figure 1. The measurement locations within the observation chamber. (a) Microscope slide, (b) cover slips, (c) center recordings, (d) edge recordings. A 3D numerical model depicts how oxygen diffuses from the top and the bottom of the coverslip labelled (e). Red depicts high oxygen concentrations with a vertical length of 5250 μm, whilst the blue depicts low oxygen concentrations. The chamber has a vertical length of 26,250 μm. The red dashed line illustrates the chamber cover, which sits on top of two cover slips.
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fluctuating oxygen levels from the surrounding seawater, surface mucus layer or boundary layer and the coral tissue.

Materials and Methods

Bacterial Culture and Growth

The single, polar flagellated *V. coralliilyticus* type strain, LMG20984 was used for all experiments. Pure cultures were stored at −80°C in 15% glycerol stock solutions. Strains grew on Thiosulphate citrate bile salts sucrose agar media (Sigma Aldrich) overnight at 28°C. Liquid cultures were prepared in 30 mL of Luria-Bertani broth (Sigma Aldrich), incubated 16 hrs overnight at 28°C with 160 rpm shaking and harvested during exponential growth, diluted with LB to 1/10 to adjust cell density to OD 0.1±0.01, and visualized within 1 minute.

Microscope Observation Chamber and Microscope Video Analysis

A volume of 50 μL of cell suspension was added to an observation chamber constructed from two glass coverslips on a glass slide and covered with a third coverslip (Fig. 1). The distance between the glass slide and coverslip was 0.17 mm, with a channel width of 15 mm. Swimming cells were observed at the edge and center of the coverslip, midway between the coverslip and the microscope slide. The oxygen distribution was approximated by modeling, which illustrated that oxygen levels began to deplete within millimeters from the edge of the observation chamber. Additionally, cells were also observed at the coverslip surface. Within the microscope observation chamber, measurements were made mid chamber within 2 mm of the coverslip edge and center (Fig. 1). Swimming *V. coralliilyticus* cells were observed under dark-field, 200× magnification video-microscopy (AxioCamMrc5 camera, Carl Zeiss AxioSkop microscope) with the field of view at mid-depth in the microscope-slide chamber. The video image was recorded continuously for 10 seconds at 2.4 frames s^{-1} using a time-lapse module (Carl Zeiss AxioVision 4). Search strategies of *V. coralliilyticus* were recorded and plotted across spatial gradients. Cell swimming paths of *V. coralliilyticus* were measured frame-by-frame and traced off the computer screen using overhead transparencies. Cell paths were defined as in Barbara and Mitchell [18], with speed and turn angles calculated according to Barbara and Mitchell [19]. To ensure that all patterns could potentially include a complete flick, only cells visible for at least 5 frames were used (Fig. S1).

Oxic to Anoxic Condition Transect Line Experiments

A volume of 50 μL of 1/10 dilution of overnight culture was added to the created observation chamber. Triplicate transects were collected ranging from the oxic to the anoxic center of the observation chamber. Each transect consisted of 40 square eyepiece quadrats of 172 by 172 μm placed next to each other. The transect line spanned from the oxic to anoxic regions across 6,880 μm. At each quadrat a 10 second, 49-frame video was recorded. All distinguishable cells in each eyepiece quadrant video were tracked and analyzed. Cells were considered distinguishable if they remained in focus for at least 5 frames. All distinguishable cell trajectories were classified into one of three search patterns: run and reverse, straight swimming or 3-step flick (Table S1). Each trajectory was then measured and the swimming speed was calculated.

Aerobic and Anaerobic Culture Control Samples

Cell cultures and the headspaces were bubbled with either pure air or nitrogen for 10 minutes, and grown in conditions as mentioned above. Methylene blue and resazurin indicators (Sigma-Aldrich) were used to confirm whether a solution was saturated throughout the experiment [6,20,21,22]. Methylene blue produced a blue/green color in oxygen-saturated cultures, where oxygen levels were above 0.5%, whereas resazurin produced a bright pink color in nitrogen-saturated cultures, where oxygen levels were below 0.1%. At values between 0.1 and 0.5%, a blue/violet color was produced [6]. For gas experiments, the slide preparation including the construction of observation chambers, the opening of culture bottles and the transfer of liquid culture onto the slides were all prepared within gas filled plastic bags to minimize any changes to saturated cultures. In addition, cell-free media broth used for dilutions was also bubbled with air or nitrogen. Slides were viewed immediately and video recordings were taken within 1–2 minutes after the removal from the gas-filled bag. Previous testing showed that cultures remained colored, either green-blue or pink for aerobic or anaerobic tests respectively, for an average time of 180 seconds. This was calculated by measuring how long the color held when viewing the cultures under the microscope. Ten replicates were measured with color holding values ranging from 160 to 220 seconds. To remain certain that *V. coralliilyticus* was being viewed in oxic or anoxic conditions all videos were recorded well before the lower value in this time window.

Vibrio coralliilyticus Search Pattern Changes Over Time

Microscope videos in oxic and anoxic conditions were collected at 5 minute intervals between 0 and 25 mins to measure the behavioral changes of *V. coralliilyticus* over time. Slides were prepared as stated above. New observation chambers were prepared for each oxygen condition of the timed experiment, which was run in triplicate (Table S2).

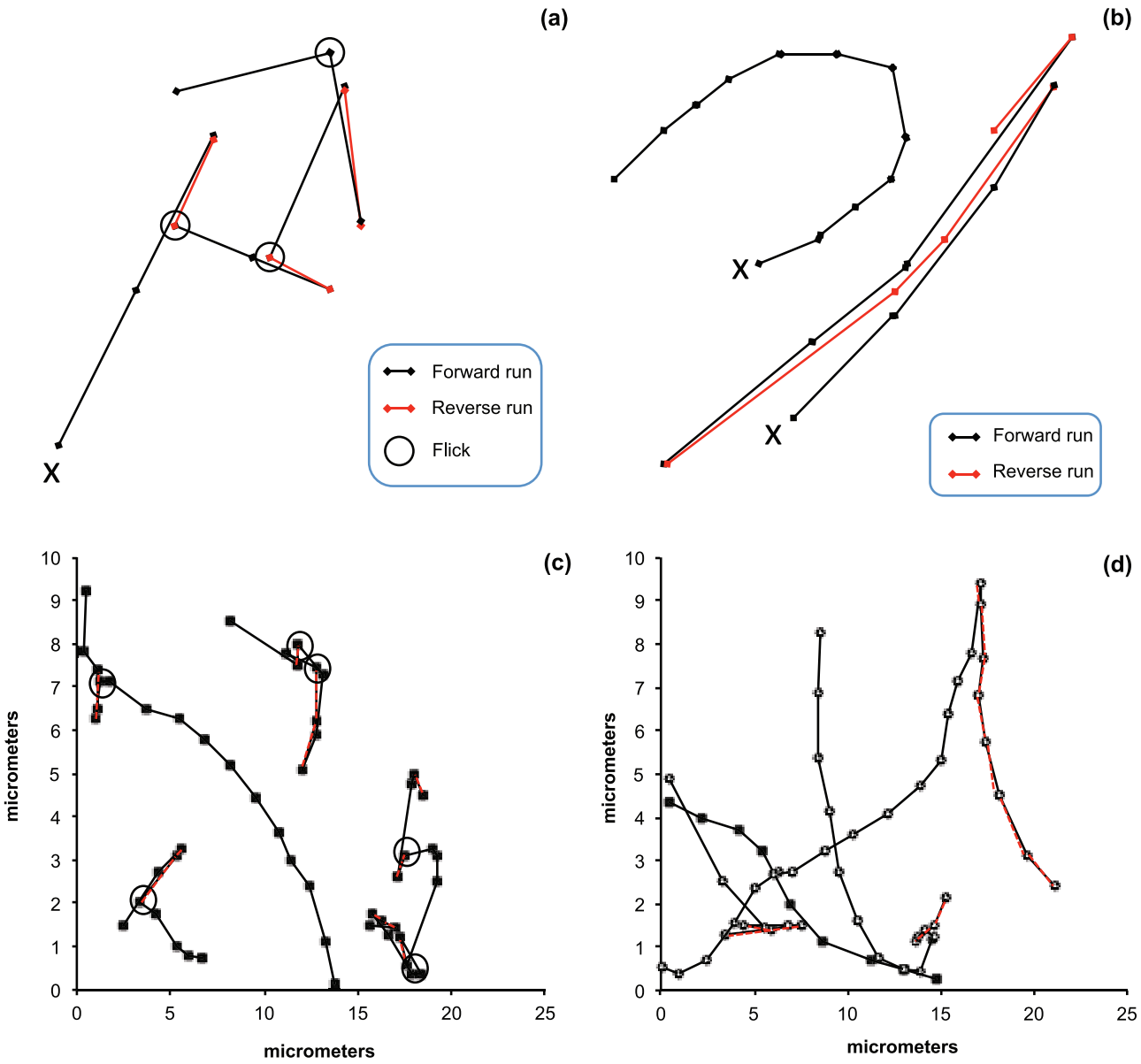

Figure 2. Schematic and observed chemotactic search patterns of *V. coralliilyticus*. The search pattern path starts at X; (a) cyclic 3-step flick search pattern, shown as a forward run, a reverse; seen as a 180° reorientation, and a 90° flick of the flagellum and repeat; (b) run and reverse and swimming search pattern. Run and reverse search patterns are characteristic of a 180° reorientation and reversal. Straight swimming search patterns shows no reversals; (c) 3-step flick search pattern trajectories (closed squares) collected from *V. coralliilyticus* overnight cultures collected from the oxic region of the observation chamber. For clarity, the open circles mark the 90° flicking events and the red-dashed lines indicate the 180° reversals; (d) search pattern trajectories collected from *V. coralliilyticus* overnight cultures collected from the anoxic region of the observation chamber, the search patterns exhibited here are run and reverses (open circles) and straight swimming (closed squares).
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Mathematical Modeling

Numerical simulations of oxygen diffusion in the presence of bacteria were produced using the MatLab-derived COMSOL Multiphysics with the shown geometries using transport of dilute species with convection and diffusion models (Fig. 2). Previous studies have illustrated multiple ways in which this model has been used [23,24,25]. Dilute species transport was represented by.

$$\partial c/\partial t + \mathbf{u}\cdot\nabla c = \nabla\cdot(D\nabla c) + R \qquad (1)$$

where c is concentration (mol/m^3), D is the diffusion coefficient (m^2/s), R is the reaction rate for the species (mol/(m^{3}s)) and in this case is used to signify flux, $\mathbf{u}$ is the vector velocity (m/s). Low Reynolds number, incompressible flows are characterized by

$$\nabla\cdot\mathbf{u} = 0 \qquad (2)$$

However, a modeled chamber depth of 0.2 mm and isothermal conditions damped convective flow and left only molecular diffusion. Equations (1) and (2) were coupled in the MatLab-derived COMSOL Multiphysics 4.0a as a finite element 3 dimensional, free tetrahedron mesh model. The minimum mesh size was 10^{-7} m.

Statistical Analysis

All cell trajectories were classified into search patterns and the results were analyzed using SPSS Statistics (version 19.0).

Table 1. Maximum, and mean search pattern speeds (μm s^{-1}) and total search pattern numbers (n) identified in oxic and anoxic regions of the observation viewing chamber.

Search pattern	Observation chamber region				Aerobically grown cultures	Anaerobically grown cultures
	Edge (Oxic)		Center (Anoxic)			
	Chamber	Surface	Chamber	Surface		
Straight swimming	56*, 21 (n = 13)	18*, 14 (n = 7)	46*, 18 (n = 14)	21*, 14 (n = 22)	47*, 32 (n = 9)	65*, 46 (n = 40)
Run-reverse	47*, 21 (n = 21)	35*, 22 (n = 12)	49*, 20 (n = 46)	29*, 15 (n = 9)	76*, 45 (n = 13)	59*, 48 (n = 6)
Combined non-flicks	57*, 25 (n = 25)	35*, 19 (n = 19)	49*, 19 (n = 60)	29*, 14 (n = 31)	76*, 40 (n = 22)	61*, 46 (n = 46)
3-step flicks	37*, 17 (n = 17)	41*, 25 (n = 21)	43*, 20 (n = 38)	25*, 17 (n = 10)	79*, 45 (n = 38)	56*, 46 (n = 5)

* = maximum search pattern speed.
n = number of recordings.
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Independent sample t-tests were utilized to calculate P values to establish whether flick or non-flick search patterns differ in total numbers or speed across the two observation chamber locations. T-tests were also used to determine any statistical differences in search pattern behavior during the time series.

Results

Vibrio coralliilyticus Search Pattern Preferences in Oxic and Anoxic Conditions

V. coralliilyticus search paths were identified and classified into three search strategies, straight swimming, run and reverse and the 3-step flick (Fig. 2). In oxic areas of the observation chamber, 53 flicks, 29 run and reverses and 13 straight swimming search patterns with mean speeds of 17 μm s^{-1} (95% Confidence Interval (CI) = 2.0, n = 53), 21 μm s^{-1} (95% CI = 4.1, n = 29) and 21 μm s^{-1} (95% CI = 8.4, n = 13) respectively, were observed (Table 1). In anoxic areas of the observation chamber, 38 flicks, 46 run and reverses and 14 straight swimming search patterns with mean speeds of 20 μm s^{-1} (95% CI = 2.6, n = 38), 20 μm s^{-1} (95% CI = 2.5, n = 46) and 18 μm s^{-1} (95% CI = 5.5, n = 14) respectively, were observed (Table 1). Flick search patterns in oxic areas of the observation viewing chamber, namely the edge, were 1.4 times more frequent in number than at anoxic, or center areas of the viewing chamber (P = 0.006) (95% CI = 1.5, n = oxic: 53; anoxic: 38) and were significantly slower than the non-flick search patterns (P = 0.016) (95% CI = 2.9, n = flicks: 53; non-flicks: 42) seen at oxic areas of the observation chamber. In anoxic conditions, run and reverse search patterns were 1.6 times more frequent than in oxic conditions (P = 0.040) (95% CI = 2.3, n = oxic: 29; anoxic: 46). In oxic or anoxic conditions there were no differences in swimming search patterns (P = 0.778) (95% CI = 1.4, n = oxic: 13; anoxic: 14).

Vibrio coralliilyticus Search Patterns Change from Oxic to Anoxic Environments

The chemotactic search pattern behavior of *V. coralliilyticus* changed over the oxic-anoxic interface, identified over a microscope transects line. Three-step flick search pattern numbers decreased 8-fold (P = 0.048), from a mean value of 8 flicks (95% CI = 0.4; n = 24) in oxic environments to a mean value of 0.5 flicks (95% CI = 0.6; n = 3) in anoxic environments. Run and reverse search patterns decreased 5-fold (P = 0.048), from a mean value of 5 run and reverse search patterns (95% CI = 0.51; n = 15) in oxic conditions through to 1 run and reverse (95% CI = 1.13; n = 3) in anoxic conditions. Straight swimming search pattern numbers increased from a mean value of 5.7 straight swimming search patterns (95% CI = 1.2; n = 17) in oxic conditions to a mean value of 9 straight swimming search patterns (95% CI = 0.8; n = 27) in anoxic conditions (Fig. 3a).

Search pattern velocities were shown to decrease 2-fold from oxic to anoxic environments (P = 0.002), from 63.2 μm s^{-1} (95% CI = 2.0, n = 58) to 21.9 μm s^{-1} (95% CI = 3.4, n = 32) respectively, although a large variation in search pattern velocity was identified (Fig. 3). Search pattern velocities remained steady at 23.2 μm s^{-1} from the 2000 μm distance location onwards (P = 0.578, 95% CI = 0.6, n = 400). Velocities did not differ significantly between the three types of search patterns identified, namely the flick, run and reverse and straight swimming (Fig. 3b).

Aerobic and Anaerobic Controls

Motile cells of *V. coralliilyticus* were observed in cultures grown under both aerobic and anaerobic conditions. Immediate visualization of these cell cultures showed that in aerobically grown cultures, 38 flicks, 13 run and reverses and 9 straight swimming patterns with mean speeds of 45 μm s^{-1} (95% CI = 5.6, n = 38), 45 μm s^{-1} (95% CI = 8.4, n = 13) and 32 μm s^{-1} (95% CI = 0.7, n = 9) respectively, were observed (Table 1). Cultures grown under anaerobic conditions demonstrated 5 flicks, 6 run and reverses and 40 straight swimming search patterns with mean speeds of 46 μm s^{-1} (95% CI = 7.0, n = 5), 48 μm s^{-1} (95% CI = 6.9, n = 6) and 46 μm s^{-1} (95% CI = 3.5, n = 40) respectively (Table 1). In aerobic conditions, flick search patterns were 7.6 times greater than in anaerobic cultures (P = 0.024). In addition, a 2.2 fold increase in run and reverse search patterns (P = 0.010, 95% CI = 8.4, n = 13) and a 4.4 fold decrease (P = 0.003, 95% CI = 6.6, n = 9) in straight swimming search patterns were seen in aerobic conditions compared to anaerobic conditions. Search patterns speeds did not differ across aerobic or anaerobically grown cultures (P = ≥0.05, 95% CI = 2.6, n = 111) (Table 1).

Vibrio coralliilyticus Search Pattern Changes Over Time

During a 25-minute time series experiment, the search pattern numbers in anoxic conditions leveled off by the 5-minute mark, whereas in oxic conditions, search pattern numbers began to level off from 10 minutes onwards. In anoxic conditions, flick search patterns decreased by 14 times (P = 0.022, 95% CI = 8.0, n = 38), whilst non-flick patterns increased by 8 (95% CI = 3.4, n = 26) over the 25-minute experiment, though this result was not significant (P = 0.230) (Fig. 4a). Straight swimming search patterns remained constant (P = 0.333, 95% CI = 3.1, n = 203). Similarly, in oxic conditions, flick search patterns decreased 1.8 times (P = 0.014,

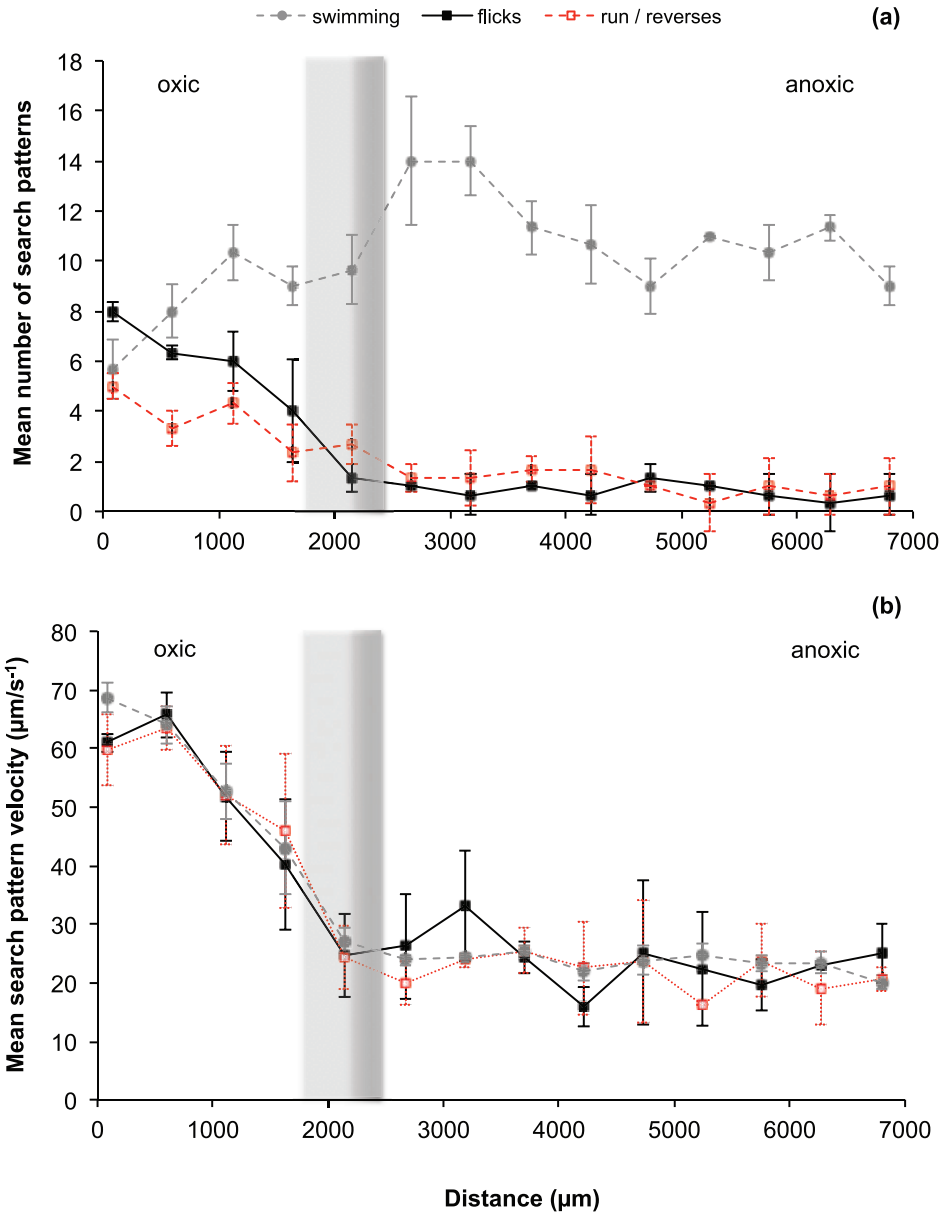

Figure 3. *Vibrio coralliilyticus* search patterns along an oxygen gradient. (a) The mean total number of search patterns seen along the transect line consisting of 40 quadrat boxes (error bars = 95% CI); (b) Mean velocity (µm s^{-1}) of search patterns across the transect line (error bars = 95% CI). The location of the grey gradient lines represents the location where the oxygen transition occurs in Fig. 1 (orange and green area).
doi:10.1371/journal.pone.0067975.g003

95% CI = 2.5, n = 120), whilst non-flick search patterns were variable and demonstrated no significant trends (P = ≥0.05, 95% CI = 3.4, n = 122). All search pattern speeds except the straight swimming patterns remained relatively constant at oxic and anoxic conditions over time (Fig. 4b).

Discussion

Oxygen concentrations can differ between the surrounding water layer and the various microbiomes present in coral organisms, namely the surface mucus layer and coral tissue [17]. During darkness the coral can experience complete hypoxia [26,27,28]. As such, coral microorganisms must be able to survive in changing oxygen conditions [14]. We provide evidence that the coral pathogen, *V. coralliilyticus* is capable of utilizing chemotactic search patterns in oxic and anoxic conditions whilst maintaining search pattern velocities across oxic to anoxic conditions. This indicates that regardless of oxygen concentrations *V. coralliilyticus* are capable of remaining motile.

Our results indicate that *V. coralliilyticus* uses the newly discovered cyclic flick search pattern, first found in *V. alginolyticus*, which is morphologically distinct from the run and reverse or run and tumble patterns adopted by *E. coli* [5,8,14]. The search pattern behavior identified in oxic and anoxic conditions in *V. coralliilyticus* may be the result of saturated oxic conditions, similar to that seen in *Salmonella typhimurium* and *Escherichia coli* [29], whereby in high concentrations, dissolved oxygen is a repellent and in low conditions (0.25 mM), an attractant [29]. Whilst it is known that the 3-step flick allows for a 90° directional change to relocate to a more suitable nutrient environment [5,14], it is

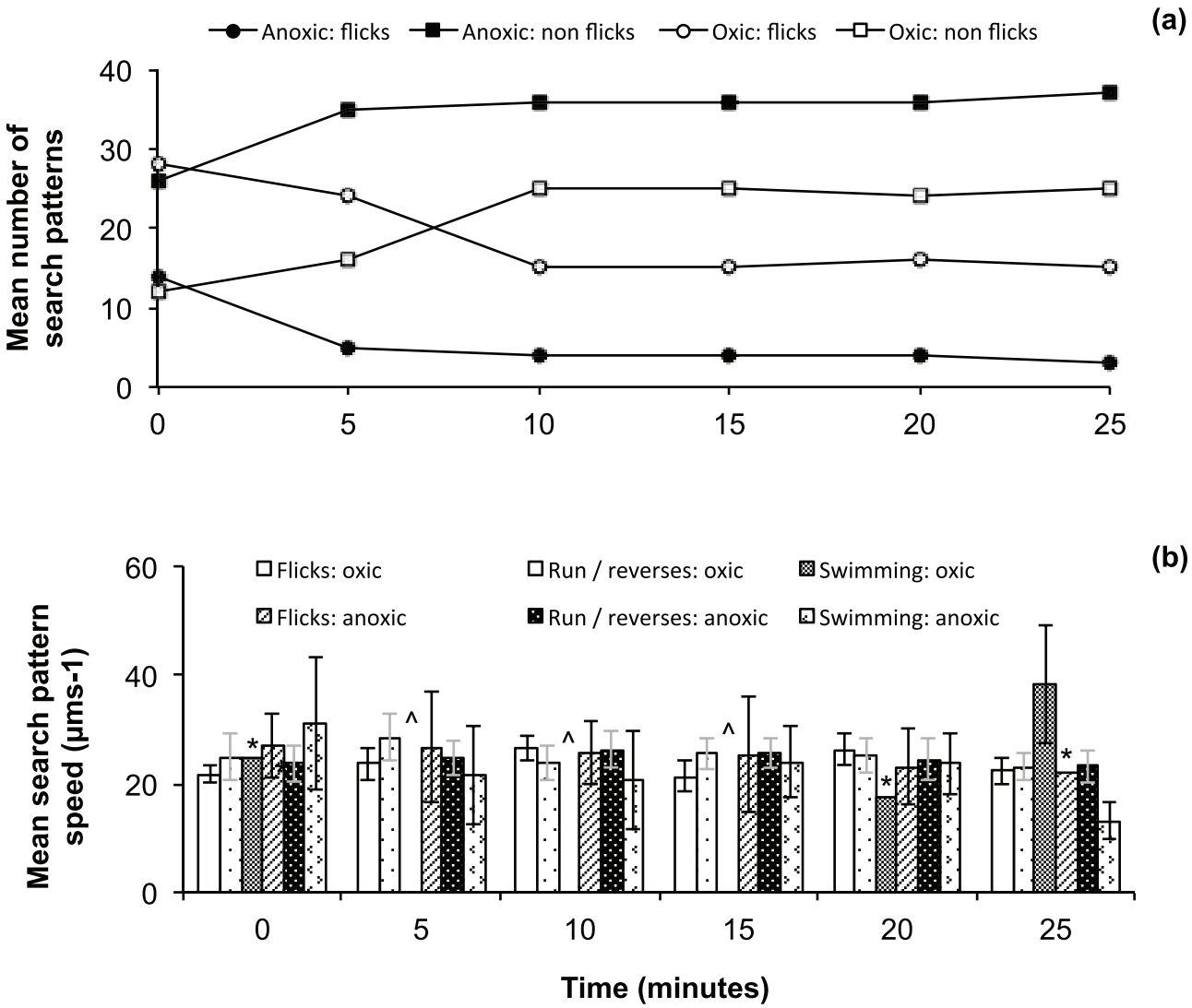

Figure 4. *Vibrio coralliilyticus* search patterns over a 25 minute time series. (a) The mean total number of search patterns seen at the edge of the cover slip and center of the cover slip over a 25 minute time series. The solid squares indicate the number of non-flick search patterns at an anoxic area of the chamber, open squares indicate the number of non-flick search patterns at an oxic area of the chamber, closed circles indicate the number of flicks search patterns at an anoxic area of the chamber, open circles indicate the number of flick search patterns at an oxic area of the chamber; (b) The mean search pattern velocities seen at oxic and anoxic regions of the cover slip chamber over a 25 minute time experiment. * = only 1 search pattern observed so no 95% CI value could be calculated;^ = missing data as no search patterns were identified.
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unknown whether bacteria may use the 90° flick in this search pattern to quickly move away from undesired nutrient patches as often the initial flick is ≥5 µm in length and continues for a distance of ~ 15 µm [5]. Our work adds to this by showing that the 3-step flick is present in oxygenated environments and not in deoxygenated environments. Determining the critical oxygen concentration for turning off the flick, and why oxygen might be needed provides pathways for further investigating the 3-step flick.

Changes in search pattern behavior of *V. coralliilyticus* in oxic and anoxic conditions may not be a response to the oxygen concentrations directly, or lack thereof. Instead, as suggested by Armitage [30] bacteria can monitor changes in the electron transfer rate using redox sensors, which are signaled through sensory pathways. This sensory system converts environmental signals into a rotational change of the flagellar motor [31] allowing for cell relocation or revisiting older nutrient patches [32]. All cells possess an ability to sense oxygen and activate these adaptive sensory processes, which are fundamental to survival when oxygen becomes limited [32]. The observation chamber, which is open on two sides, creates an oxygen gradient from the interplay of microbial oxygen consumption and diffusion from the edges (Fig. 5). This simulates the diurnal and nocturnal changes that occur in the surface mucus layer and coral epithelial tissue highlighted in Kühl *et al.* [17]. The behavioral responses arising from this oxygen gradient, illustrates the behavioral complexities of a *V. coralliilyticus* infection, where *V. coralliilyticus* cells remain motile in both oxic and anoxic conditions. With *V. coralliilyticus's* motility fundamentally linked to infection and establishment in corals [20], knowledge of its behavioral characteristics could provide greater insight into infection rates and mechanisms.

Supporting Information

Figure S1 A sample video recording of *Vibrio coralliilyticus* cells. The cells tracked can be identified by the open circles, and the

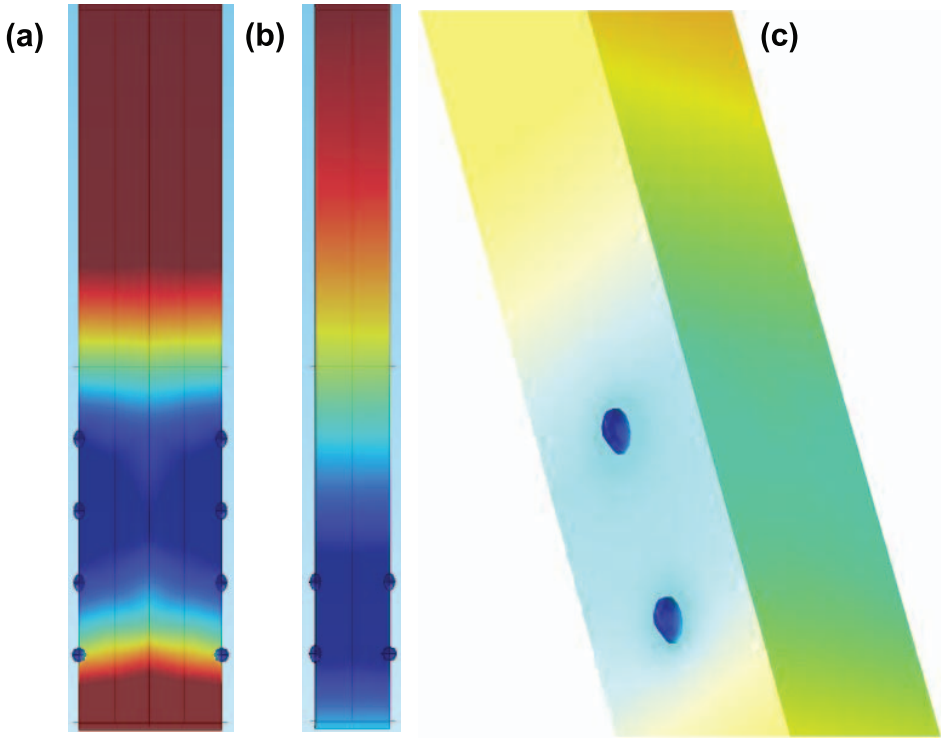

Figure 5. Three-dimensional numerical models of oxygen diffusion microscope slide chamber where oxygen diffuses from the top and the bottom. Blue dots represent bacteria that absorb oxygen as it diffuses past. The columns are 100 μm high (a and b). The relative concentration difference between blue and red is 30 mM. Orthogonalized spacings represent average cell distributions seen in the experiments with (a) for low cell density regions and (b) for cell cluster regions. Real distributions will be heterogenously rather than uniformly distributed. The diagonal in (c) is the rotated volume showing the z axis and the position of the bacteria in that direction.
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specific search pattern trajectories used have been overlaid. The red and yellow lines are flicks, the orange and white lines are straight swimming, and the blue line is a run and reverse. Examples of the 90° flicking events are marked by an X.
(MOV)

Table S1 Experimental number of recordings (n) for *V. coralliilyticus* search pattern preference from oxic to anoxic conditions in Figure 3.
(DOCX)

Table S2 Experimental number of recordings (n) for *V. coralliilyticus* search pattern changes over time in Figure 4.
(DOCX)

Author Contributions

Conceived and designed the experiments: JGM DGB KMW. Performed the experiments: KMW. Analyzed the data: JGM DGB KMW. Contributed reagents/materials/analysis tools: JGM DGB KMW. Wrote the paper: JGM DGB KMW.

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