Aquatic Microenvironments in Bacterial Ecology and Diversity

by

Dana E. Hunt

B.A. Biochemistry
B.A. Environmental Engineering
Rice University, 2001

Submitted to the department of Civil and Environmental Engineering
in Partial Fulfilling of the Requirements for the Degree of

Doctor of Philosophy
in Civil and Environmental Engineering
at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2008

Signature of Author_____
Department of Civil and Environmental Engineering
October 12, 2007

Certified by_____
Martin F. Polz
Associate Professor
Thesis Supervisor

Accepted by_____
Daniele Veneziano
Chairman, Departmental Committee for Graduate Students

Aquatic Microenvironments in Bacterial Ecology and Diversity

by

Dana E. Hunt

Submitted to the Department of Civil and Environmental Engineering
on October 12, 2007 in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy
in the Field of Environmental Biology

ABSTRACT

Molecular surveys have revealed tremendous bacterial diversity in the world's oceans; yet how do these diverse bacteria with the same essential nutrient requirements co-exist in the same environment? This study examines the role of aquatic microenvironments in generating bacterial diversity: closely related organisms may co-exist in the same environment without competing for resources by a combination of habitat, metabolic, and behavioral differentiation. This hypothesis has been approached from several angles: (i) Within the bacterial family *Vibrionaceae* is there evidence for microenvironmental specialization or functional differentiation? (ii) Is there small scale clustering of bacteria around phytoplankton in the coastal ocean? Microdiverse clusters (< 1% 16S rRNA gene divergence) of *Vibrionaceae* were found to be differentially distributed between zooplankton-enriched, particulate, and planktonic water column microenvironments. However microhabitat preferences may not correspond to metabolic capabilities; chitin metabolism was observed to be a near ubiquitous metabolic characteristic of the *Vibrionaceae*, yet does not appear to be linked to colonization of chitinous zooplankton or particles. Finally, the microscale patchiness of bacterial cells was examined over an annual cycle, revealing seasonal variation and a positive correlation with eukaryotic cell number, suggesting that bacteria may cluster in the nutrient-rich microzones around algae in the environment. This study seeks to answer several fundamental questions about marine bacterial populations: how do closely related species co-exist in the same environment, do bacteria adapt to distinct microscale environments and how important are these microenvironments to bacterial productivity.

Thesis Supervisor: Martin F. Polz

Title: Associate Professor of Civil and Environmental Engineering

ACKNOWLEDGEMENTS:

I would first like to thank my advisor Martin Polz, for his continual support. As my project evolved and shifted Martin allowed me to explore intellectually stimulating ideas, even when they were quite different from what I had originally intended on studying. His critical thinking and mentorship have changed the way I think about science. I would also like to thank my committee members: Ed Delong, Daniele Veneziano, Roman Stocker and Eric Alm, each of whom contributed to my intellectual journey outside of formal committee meetings, sometimes even at the lab bench or computer screen.

My lab mates over the years have made the crazy schedule, frustrations, and joys of my thesis research so much more rewarding. Special thanks to Stefan Bertilsson, Silvia Acinas and Vanja Klepac-Ceraj as my "lab mommies" setting me on a path to scientific competence when I was a clueless first year student. Field work wouldn't have half so enjoyable without the company of Janelle Thompson, Luisa Marcelino and Sarah Preheim. My bioinformatics incompetence was mitigated by valuable collaborations with Dirk Gevers, Lawrence David and Eric Alm. For general lab entertainment the contributions of Ramahi Sarma-Rupavtarm, Chanathip Pharino, Jennifer Benoit, Hong Xue, Ben Kirkup, Jenn Carr, Hans Wildschutte, and Yan Boucher cannot be beat. The undergraduate researchers I supervised, Nisha Vahora and Marion Dumas, were both excellent scientists and kept me on my toes with their insightful questions. The Chisholm, Delong, and Stocker labs were great resources for "borrowing" supplies and equipment or just hashing out ideas.

Further thanks to my friends and family who have put up with my scientific obsession for the last six years even when they didn't understand what I was doing, why I was doing it, or why anyone else would possibly care once I got results. And of course my significant other, Zackary Johnson, contributed both scientific and emotional support. I value his clear thinking and role as a "sounding board" for all of my ideas and late-night discoveries.

TABLE OF CONTENTS:

LIST OF FIGURES:

Chapter 1

Chapter 2

Chapter 3

Chapter 4.

Appendix 1

LIST OF TABLES:

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix 1

Chapter One

Introduction

INTRODUCTION

This thesis investigates the importance of aquatic microenvironments in bacterial productivity and diversity. Aquatic microenvironments are here defined as local resource inhomogeneities on scales within the dispersal range of individuals, implying that organisms can actively seek out these environments. While the importance of mesoscale oceanographic features has been established for metazoans, microenvironmental features may be similarly important for bacteria, as they are at the scale at which bacteria can sense and respond to their surroundings. There are two major types of microscale interactions, those involving (i) colonization of a resource and (ii) motility-driven clustering around sources of dissolved organic matter (DOM). Here, I review these two adaptive strategies using specific examples: bacterial attachment to particles and the chemotaxis of heterotrophic bacteria toward phytoplankton, with emphasis on the roles these microenvironments play in bacterial productivity and diversity.

The ability to physically separate particle-attached and free-living bacteria has revealed differences between these two populations in cell size, abundance, diversity, and activity. The relationship of heterotrophic bacteria with phytoplankton also bears further investigation as photosynthesis is the major source of bacterial carbon in the epipelagic. Yet, the spatial component of phytoplankton- bacterial interactions in the ocean remain unclear. Evidence of ecological specialization among microdiverse (>99% rRNA similarity) bacteria has been observed for both large and small-scale environmental traits and compartments, suggesting that closely related bacteria partition resources in the environment.

Oceanographers historically treated the oceans as homogeneous at scales smaller than kilometers, yet small resource-rich patches could allow more efficient foraging and explain the high productivity of nutrient-depleted waters (McCarthy & Goldman 1979, Azam & Ammerman 1984, Alldredge & Cohen 1987, Blackburn et al. 1998). Recently, mesoscale features, which persist on the scale of kilometers and days, have been incorporated in oceanographic modeling to explain nutrient and energy fluxes (McGillicuddy et al. 1998), patchiness in surface chlorophyll values (Doney et al. 2003) and observed zooplankton abundance (Bochdansky & Herndl 1992, Davis et al. 1992, Folt & Burns 1999). One might ask if bacteria also experience environmental patchiness, potentially on the microscale (micrometers to centimeters) at which they can sense and respond to their environment (Figure 1). Although the extent and importance of these microscale features have been relatively poorly studied, preliminary evidence suggests that adaptation to microenvironments is an important ecological strategy in the marine environment.

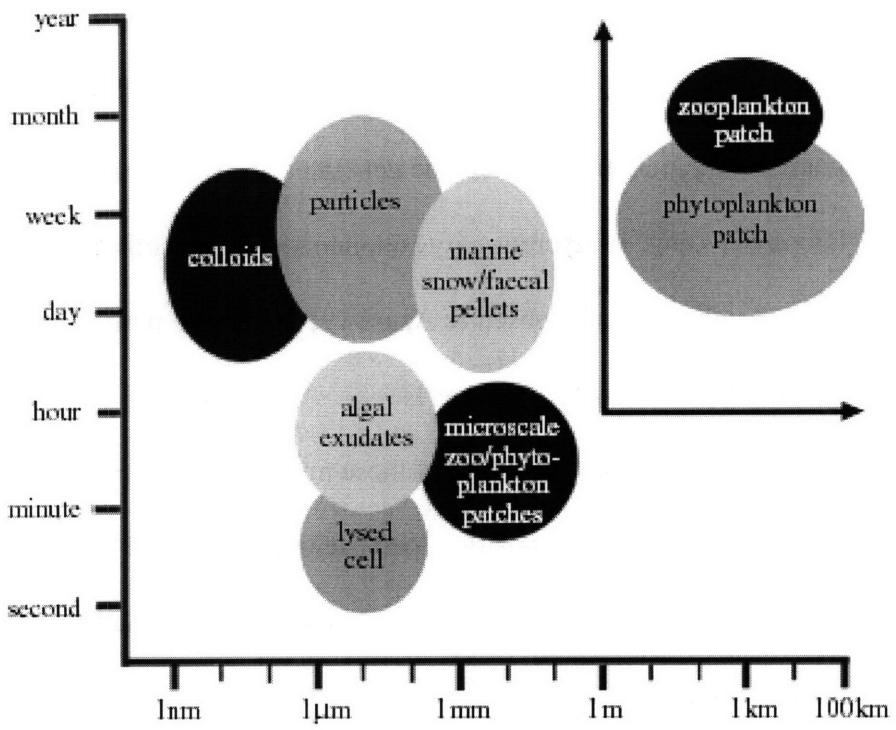


Figure 1 Temporal and spatial extent of micro- and meso-scale features affecting the growth and productivity of marine bacteria. The region to the right and above the arrows indicates features that are captured by standard oceanographic sampling methods (modified from (Dickey 1991, Seymour 2005)). Reprinted from (Polz et al. 2006).

Types and sources of nutrient-rich microenvironments in the ocean

Microscale nutrient patches may be hotspots of bacterial activity allowing bursts of uptake and reproduction that drive much of the total bacterial productivity in a background of low bulk nutrient concentrations (Giovannoni & Stingl 2005). There has been a renewed interest in assigning bacteria roles as either oligotrophs or copiotrophs/ "opportunitrophs" i.e. those which efficiently utilize low levels of background nutrients or those which can exploit nutrient patches (Poindexter 1981, Giovannoni & Stingl 2005,

Polz et al. 2006). Patch-specialized bacteria respond to nutrient pulses with increased uptake and behavioral adaptation, allowing a rapid cycling of resources (Polz et al. 2006). This patchy environmental landscape can be generated by a wide range of sources, including "sloppy feeding" or excretion by zooplankton, lysed cells (Azam & Cho 1987, Fuhrman 1999), fecal pellets (Jacobsen & Azam 1984), detrital particles, eukaryotes, and marine snow (Kiørboe et al. 2002) (Figure 1).

Difficulty in determining the role of these microscale features stems partially from inherent variation in the composition, persistence, and size of these microenvironments (Figure 1). For example, marine snow is defined operationally as particles larger than 0.5 cm; however, these aggregates can contain multiple constituents ranging from algae engaged in oxygenic photosynthesis to anaerobic fecal pellets (Alldredge & Cohen 1987, Turner 2002). Besides providing nutrients to the surface-attached bacteria which degrade complex polymers, marine snow, as well as other particles, may release a plume of dissolved organic matter (DOM) that can be used by free-living bacteria (Kiørboe & Jackson 2001). The influence of these particles is also mediated by their persistence which can be highly variable, where a photosynthesizing algal aggregate can be highly buoyant, fecal pellets fall hundreds of meters per day to the ocean floor, removing their nutrients from the pelagic food web (Turner 2002). The quality of particulate matter may also change over time; with depletion of key nutrients (nitrogen, phosphorous, etc.), microhabitats may become less attractive to bacteria (Jacobsen & Azam 1984, Smith et al. 1992). Colloids, sized between 1 and 1000 μm, are small enough to remain suspended over long periods of time (Wells 1998); although they aggregate into larger particles that are subject to gravity (Chin et al. 1998). However,

prior to aggregation they may be too small to support a bacterial population (Chin et al. 1998).

Bursts of DOM produced by photosynthesizing algae or lysing cells (Figure 1) are then subject to dissipation in the aquatic environment, resulting in ephemeral microenvironments. While these microhabitats are transient, they potentially provide an important stimulus for chemotactic bacteria, which require dissolved organic matter to bind chemoreceptors. Such dissolved monomers could allow bacteria to localize in nutrient-rich patches. Each patch may be chemically distinct; as the materials released from lysed cells, phytoplankton, or zooplankton are likely highly dependent on the species or growth state of the organism. Moreover, the fluid environment limits the lifetime of nutrient patches, with diffusion and turbulence acting to disperse patches of dissolved organic matter (Moeseneder & Herndl 1995).

The two primary means by which bacteria make use of microenvironments in the ocean are colonization/attachment and transient clustering mediated by chemotaxis and motility. The same resource may be used by bacteria employing both methods; marine snow may be both colonized by bacteria and produce a wake of dissolved organic matter used by chemotactic bacteria (Moeseneder & Herndl 1995). Additionally, chemotaxis may be used to locate and colonize particles.

Bacterial attachment to particles

Particles are one of the best studied aquatic microenvironments since attached bacteria can be physically separated from free living cells, and thus particles' roles in biogeochemical cycling and bacterial productivity has been extensively studied. A large

fraction work has focused on marine snow due to its potential for deep-sea carbon export (Alldredge & Silver 1988, Turner 2002). However, smaller particles are orders of magnitude more abundant than marine snow; and such particles are varied in composition including proteins (Long & Azam 1996), transparent exopolymers, colloids (Chin et al. 1998), and other recalcitrant macromolecules.

Evidence suggests that particles represent an important resource for bacteria: a significant fraction are colonized (Long & Azam 1996), bacterial density is higher than in seawater (Caron et al. 1982), and up to half of the total water column community can be particle attached (Crump et al. 1998). Further, attached bacteria can constitute 90% of bacterial biomass production (Crump et al. 1998), suggesting particles provide a significant nutrient resource for bacterial cells (Alldredge 1979, Hebel et al. 1986, Long & Azam 1996). Additionally, attached bacteria have higher per cell levels of hydrolytic enzymes (Karner & Herndl 1992, Smith et al. 1992). These extracellular enzymes convert particulate matter to DOM, not all of which is taken up by attached bacteria, generating a nutrient plume that can be utilized by free-living organisms (Kiørboe & Jackson 2001) and retaining organic matter in the upper ocean.

The majority of studies have observed differences between attached and free-living bacteria (Table 1). However, even in the Flavobacteria, which are thought to be particle specialists, no phylogenetic difference was observed between free-living and attached communities, although they were more abundant in the particle-attached size fraction (Abell & Bowman 2005). While some studies have found certain groups only in particle-attached fractions (Huber et al. 2003), this may be an artifact of limited sample size rather than reflecting a true absence in the free-living fraction. Instead of existing as

a distinct population, particle attached bacteria are likely a subset of the free-living population, as attached bacteria shed offspring which then colonize new particles. Additionally, particle attached bacteria may display distinct physiologies; for example, particle derived isolates are more likely to have antagonistic interactions with other bacteria (Long & Azam 2001a).

Table 1. A review of the literature on the partitioning of bacterial diversity between aquatic microenvironments

Organism	Location	Habitat	How Measured[1]	Related to habitat Yes/No	Reference
Bacteria	Deep-sea vent	Particles	Clone libraries	Yes	(Huber et al. 2003)
Bacteria	Mediterranean	Particles	Clone libraries	Yes	(Acinas et al. 1999)
Bacteria	Columbia river estuary	Particles	Clone libraries	Yes	(Crump et al. 1999)
Bacteria	San Francisco Bay	Particles	DGGE	No	(Hollibaugh et al. 2000)
Bacteria	Freshwater mesocosm	Particles	Functional screens	No	(Worm et al. 2001)
Bacteria	Mediterranean	Particles	T-RFLP	Yes	(Moeseneder et al. 2001)
Bacteria	Freshwater mesocosm	Particles	DGGE	Yes	(Riemann & Winding 2001)
Bacteria	Coastal ocean	Marine snow	Clone libraries	Yes	(DeLong et al. 1993)
Bacteria	Freshwater mesocosm	Diatom aggregates	FISH	Yes	(Knoll et al. 2001)
Bacteria	Salt marsh	Particles	FISH	Yes- □Proteo No -others	(Dang & Lovell 2002)
Bacteria	Estuary	Particles	DGGE	Yes	(Selje & Simon 2003)
Flavobacteria	Southern Ocean	Particles	DGGE	No	(Abell & Bowman 2005)
Photobacterium spp.	Ocean	Fish light organs	MLSA	Yes	(Ast & Dunlap 2005)
Vibrio spp.	Coastal ocean	Sediment/oysters	ERIC-PCR Phage.	No Yes	(Comeau & Suttle 2007)
Vibrio spp.	Chesapeake Bay	Zooplankton	FODC	Yes	(Heidelberg et al. 2002)

[1] DGGE = denaturing gradient gel electrophoresis; T- RFLP= terminal restriction fragment length polymorphism; FISH= fluorescence in situ hybridization; MLSA=

multilocus sequence analysis; ERIC-PCR= Enterobacterial Repetitive-Element Intergenic Consensus Sequence PCR; Phage= phage sensitivity assays; FODC=fluorescent oligonucleotide direct count.

Bacterial clustering around nutrient point sources

Bacterial clustering around small nutrient patches may be responsible for a large fraction of bacterial activity. For example, bacterial productivity decreased by 12-20% when water samples were artificially mixed suggesting that nutrient patches enhance productivity over an even distribution of the same total nutrients (Moeseneder & Herndl 1995). Microbial adaptation to large changes in resource concentrations is also evident in the multiphasic kinetics for the uptake of D-glucose and amino acids (Azam & Hodson 1981, Fuhrman & Ferguson 1986, Ayo et al. 2001), suggesting that either individual bacteria have multiple transport systems or taxa are optimized to different substrate concentrations. In either case, marine assemblages are adapted to order of magnitude variations in nutrient levels and can increase uptake under pulsed nutrient conditions (Azam & Hodson 1981, Fuhrman & Ferguson 1986, Ayo et al. 2001).

Bacterial aggregation has been observed in seawater enrichments where patches of bacteria hundreds of micrometers in diameter developed around microfeatures such as lysed cells (Blackburn et al. 1998); other potential transient sources of dissolved organic matter include excretion events and sloppy feeding by metazoans as well as photosynthate released by phytoplankton. Microscale variability in bacterial cell numbers in environmental samples suggests that patchy resources induce clustering (Table 2); such clustering can also be artificially generated by adding a nutrient source

(Blackburn et al. 1998, Krembs et al. 1998a, Krembs et al. 1998b). These *in situ* observations of aquatic bacterial patchiness at the millimeter to centimeter scale found abundance differences of up to 16-fold (Table 2); however, these bacterial patches are too large to have been formed by chemotactic aggregation which occurs on the scale of hundreds of micrometers (Blackburn et al. 1998). Explanations given for these larger-scale events include differential growth in higher nutrient environments (Duarte & Vaqué 1992, Andreatta et al. 2004), turbulent resuspension of particles/ bacteria (Seymour et al. 2000, Andreatta et al. 2004, Seymour et al. 2005), attachment to particles such as marine snow (Seymour et al. 2004), and differential feeding by predators (Seymour et al. 2000). Using flow cytometery, bacterial populations can be binned by size and DNA content; it appears that large, high-DNA content bacteria, presumably the most active, are the most numerically patchy (Andreatta et al. 2004, Seymour et al. 2004). Although the extent and importance of chemotaxis-driven clustering is not yet known, bacteria appear to be adapted to use of aquatic microenvironments.

The costly energetic investment in motility (Mitchell 2002) implies that bacteria derive a substantial energetic benefit from microenvironments. Most marine isolates display a high-speed "run-reverse" motility, which may allow enhanced response to nutrient pulses that dissipate in tens of seconds (Mitchell et al. 1996, Blackburn et al. 1998), and can reach speed of ~400μm/second (Barbara & Mitchell 2003a). Contradicting the assumption that a low fraction of bacteria are motile, up to 60% percent of cells have been observed to swim (Mitchell et al. 1995, Mitchell 2002). Additionally, marine assemblages have demonstrated chemotaxis towards glucose and amino acids, suggesting that transient nutrient point sources are important in these systems (Fenchel

2001, Barbara & Mitchell 2003b). Older marine chemotaxis models suggesting that bacterial motility is energetically unfavorable should be reconsidered in light of the high speeds, novel search strategies, and sensitive chemotaxic receptors of marine bacteria (Kiørboe & Jackson 2001).

Table 2. Spatial variability in bacterial abundance suggested by ratio of the highest observed concentration of bacteria to the lowest bacterial cell count for each study.

Volume of sample	*Ratio of highest to lowest concentration observed*	*Reference*
1 ml	7	(Daubin et al. 2003)
50 μl	16	(Seymour et al. 2000)
100 nl	<5	(Müller-Niklas et al. 1996)

Patchy resources may be preferentially exploited by certain groups of bacteria with capabilities for motility and chemotaxis (Jackson 1987, Mitchell 2002, Barbara & Mitchell 2003b, Polz et al. 2006). As with studies of particle-attached and free-living populations (Table 1), there is some controversy about whether bacterial types vary over small spatial scales. Differences in bacterial diversity between microscale seawater samples may reflect the influence of various microhabitats including clustering around point sources, attachment to particles, sampling error, or mixing of water masses with distinct origins. Denaturing gradient gel electrophoresis (DGGE) is used to obtain

fingerprints of ribotype diversity and found variable patterns of diversity in 1 μl (Long & Azam 2001b), but not in 25 μl samples (Kirchman et al. 2001), suggesting that the bacterial phylogenetic patchiness scale occurs between these two volumes. Bacteria exhibited enhanced diversity in the presence of particles (Long & Azam 2001b), but interpretation of these banding patterns as indications of distinct populations clustering remains tenuous. As DGGE captures only the most abundant ribotypes (Long & Azam 2001b) and PCR exhibits stochastic variability, the significance of variations in banding patterns are difficult to assess (Kirchman et al. 2001). In order to gain an accurate picture of community structure in small scale samples, experiments should measure both bacterial diversity and the relative abundance of these sequence types.

Coupling of phytoplankton and prokaryotes

The region surrounding photosynthesizing algae has been proposed as a high productivity microenvironment for bacteria, yet there have been few studies that link prokaryotes and eukaryotes in a spatially explicit manner. Bulk coupling of photosynthesis and bacterial production is well established; primary production is thought to be the main driver of bacterial metabolism in the photic zone of the pelagic, and bacterial abundance is positively correlated with chlorophyll levels (Gasol & Duarte 2000, Li et al. 2006). Although heterotrophs depend on carbon fixed by phytoplankton, they concurrently compete with them for macronutrients such as nitrogen and phosphorous. In nutrient-limited culture, bacteria out-compete algae for phosphorous (Rhee 1972), resulting in nutrient-starved algae that release more DOM (Guerrini et al. 1998, Mindl et al. 2005). However, over time, a feedback mechanism comes into play

and heterotrophs become co-limited by phosphorous and carbon, thus limiting their abundance relative to the algae (Mindl et al. 2005). Relationships with bacteria can also be beneficial for algae, vitamin B_{12}-requiring phytoplankton can obtain this compound through a symbiotic relationship with bacteria (Croft et al. 2005); and although the function bacteria serve is unknown, it is difficult to culture phytoplankton axenically. Moreover, algae may act on bacterial physiology through the release of cAMP, a metabolic regulator in bacteria that increases the production of catabolic enzymes, potentially making additional nutrients available to the algal cells (Azam & Ammerman 1984). While large scale coupling of bacteria and phytoplankton is driven by carbon fixation, finer scale interactions may include elements of mutualism, commensalism, and parasitism.

In order to gain better access to released photosynthate, bacteria may cluster around phytoplankton; Bell and Mitchell (1972) first suggested the importance of the "phycosphere", the region surrounding a photosynthesizing alga analogous to the rhizosphere of plants. Up to 60% of photoassimilated carbon can be leaked or released by algae (Hellebrust 1974), and this material is thought to be a major source of organic matter used by bacterioplankton (Lancelot 1979, Azam & Cho 1987). The extent to which bacteria cluster around algae remains controversial with models suggesting outcomes ranging from no clustering (Jackson 1987) and clustering only on specialized low-turbulence regions (Mitchell et al. 1985) to estimates that at any given time up to 20% of chemotactic bacteria reside in the phycosphere (Bowen et al. 1993).

Yet clustering of bacterioplankton around algae has not been observed *in situ*. In order for chemotaxis-driven bacterial patchiness to occur, several conditions have to be

met: a fraction of the population has to be actively motile, chemoeffectors have to be spatial localized, and samples at the appropriate spatial scale must be examined. In previous assays looking at clustering, the sample sizes have been either too large (Table 2) or did not find an association. For example, there was no bacterial clustering observed in either natural or algae amended (to 1000 cells/ml of *Chaetoceros muelleri*) seawater samples (Müller-Niklas et al. 1996). Possible explanations for this finding are that the majority of the bacterial cells were non-motile or the algae were not a good chemoattractant. A lag in induction of bacterial motility may have initially limited clustering in another study, where bacterial patchiness was observed at the 100 μm scale only several hours after amendment with lysed diatoms (Krembs et al. 1998a). Further, this patchiness was not associated with the distribution of algal cells; a potential explanation for this observation is that algal nutrients indirectly stimulated patchiness by up-regulating motility or shifting the bacterial population to more motile phylotypes (Krembs et al. 1998a). These results suggest that while bacteria may cluster around phytoplankton in aquatic systems, this association may not be a general phenomenon perhaps occurring only when specific conditions are met.

Additionally, phytoplankton exert a control on the phylogenetic composition of the bacterial population, presumably through the quality and quantity of organic matter produced (Fandino et al. 2001, Schäfer et al. 2002, Pinhassi et al. 2004, Grossart et al. 2005, Grossart et al. 2006, Kent et al. 2007). In the lab, phytoplankton strains appear to maintain specific bacterial populations in co-culture (Grossart et al. 2006). Certain groups such as the *Roseobacter* appear to be adapted to a phytoplankton-associated lifestyle; as they are enhanced in the presence of algae (Grossart et al. 2005), exhibit

chemotaxis towards algal products (Miller et al. 2004) and degrade the algal osmolite dimethylsulfide (DMSP) (Moran et al. 2003, Moran et al. 2004) or photosynthetic byproduct glycolate (Lau & Armbrust 2006).

Ecologically coherent role of sequence clusters?

Aquatic microenvironments may be important habitats for aquatic bacteria, but at what level of sequence divergence does ecological differentiation among microdiverse sequence clusters emerge? A recent study found that the majority of bacterial diversity is found at less than 1% 16S rRNA gene sequence divergence (Acinas et al. 2004); and 16S microdiversity has been observed in vibrio isolates, despite multiple rRNA operons (Thompson et al. 2005). Yet we have little understanding of the metabolic or ecological diversity that may underlie even small changes in rRNA sequence; although data suggests that similar ribotypes mask extensive genomic diversity (Welch et al. 2002, Rocap et al. 2003, Jaspers & Overmann 2004, Thompson et al. 2005). However, at some level ribotype-based clusters may function as ecological units; as members of these clusters appear to co-vary on seasonal cycles and along environmental gradients (Thompson et al. 2004, Johnson et al. 2006).

There has been a renewed focus on establishing natural taxonomic units for bacterial populations based on the distribution of bacterial types in relation to physical, chemical and biological parameters in the environment rather than divisions dependent on arbitrary sequence distances (Polz et al. 2006). Analysis of metagenomic datasets reveals strong environmental preferences (e.g. soil, ocean) along phylogenetic lineages, with a distance-dependent decay (von Mering et al. 2007). This long-timescale affinity to

macroenvironments must be reconciled with observed specialization of bacteria populations even in qualitatively similar environments, such as the leaves of different trees species (Lambais et al. 2006), varieties of coral (Rohwer et al. 2002) or diatom cultures (Grossart et al. 2005). Further, microdiverse bacterial strains display biogeographic differentiation (Vogel et al. 2003, Whitaker et al. 2003, Vos & Velicer 2006, Ramette & Tiedje 2007). Yet, current and historical environmental differences are difficult to deconvolute from neutral drift due to geographic isolation. Researchers are now working to link the distributions of closely related microbial taxa with physical characteristics of the environment: depth distribution (Field et al. 1997, Lopez-Lopez et al. 2005), light levels (Rocap et al. 2002, Ferris et al. 2003), temperature (Selje et al. 2004, Thompson et al. 2004, Johnson et al. 2006, Sikorski & Nevo 2007), attachment to particles (Casamayor et al. 2002), chemical concentrations (Johnson et al. 2006, Ramette & Tiedje 2007) and association with eukaryotes (Gordon & Cowling 2003, Ward et al. 2004, Ast & Dunlap 2005, Buchan et al. 2005, Nightingale et al. 2006, Smith et al. 2006). The relationship between microdiverse clusters and environmental parameters suggests that environmental and even microhabitat specialization occurs among closely related bacteria. Thus it may be possible to identify ecologically-based clusters by examining bacterial diversity and environmental heterogeneity at the appropriate resolution.

The relative rates at which genomes evolve via gene transfer, selection, point mutation, etc. will determine the relationship between marker gene sequences and preferred environmental niche. The distribution of marine bacteria has largely relied upon highly conserved markers such as the 16S rRNA gene which may evolve too slowly to detect ecological adaptations of closely related bacteria. More variable markers such

as housekeeping or virulence genes may be more appropriate means to delineate ecological populations (Ward et al. 2004, Hanage et al. 2006). Homologous recombination and lateral gene transfer may serve to obscure ecologically cohesive groupings. Conversely, if frequent acquisition of ecological-adaptive genes through horizontal gene transfer determines an organism's niche, then standard phylogenetic methods may not be related to ecologically-meaningful sequence groupings.

To assess the importance of microenvironments and avoid convoluting factors such as endemism and macroecological changes, the well-mixed coastal water column is a good location to investigate microhabitat differentiation; as in soils and sediments the microscale features develop at extremely fine scales and lakes are subject to greater biogeographic effects. Although aquatic environments are considered unstructured, ecological specialization develops rapidly in liquid laboratory culture (Rainey & Travisano 1998, Maharjan et al. 2006), suggesting that sympatric speciation can occur in the absence of physical barriers. Investigation of marine microscale features has focused largely on particles and examined diversity at the phylum level, with little information about resource partitioning between closely related organisms (Table 1). However, specialization of microdiverse bacteria on different habitats has been suggested for soil bacteria (Mummey & Stahl 2004, Ramette & Tiedje 2007), pathogens (Nightingale et al. 2006), and aquatic bacteria (Buchan et al. 2005), indicating that utilization of microenvironmental habitats is a common feature among bacteria of diverse lifestyles and population structures (Vos & Velicer 2006). Microenvironmental specialization may lead to differential population structure in organisms adapted to different microhabitats

through changes in rates of reproduction, genetic exchange, or predation- leading to differences in effective population size etc.

SUMMARY

Microbiologists have recently begun to grasp the staggering diversity of bacteria in the world's oceans and are only now investigating how this diversity is maintained. However, the extent to which bacteria interact with microscale environmental compartments has not been determined. At the bacterial scale, the ocean is rich with microscale patches, such as particles, photosynthesizing or lysing cells, and zooplankton. Each of these may provide a unique chemical environment for bacterial adaptation and differentiation. By investigating spatial, temporal and metabolic partitioning in marine bacterioplankton we hope to address the roles microscale features play in bacterial diversity.

GOALS OF THIS THESIS

This thesis asks two specific questions related to microenvironments in the oceans: (i) do closely related bacteria develop microhabitat specialization and functional differentiation in aquatic environments, and (ii) is chemotactic clustering around algae an important lifestyle in the coastal ocean? These questions were addressed by combining field sampling of the *in situ* distribution of bacteria in a temperature temperate coastal estuary (Plum Island Sound, Ipswitch, MA), with physiological characterization of bacterial isolates, and modeling the interactions underlying these observed associations.

This research focuses on bacteria of the family *Vibrionaceae* as ubiquitous, heterotrophic bacterioplankton that metabolize a broad range of substrates (Thompson & Polz 2006) and are known to attach to the chitinous exoskeletons of zooplankton (Heidelberg et al. 2002). In chapter 2, microdiverse clusters of *Vibrionaceae* were observed to be differentially distributed in size-fractionated seawater corresponding to zooplankton-enriched, particulate, and planktonic water column microenvironments in spring and fall samples. This uneven distribution between seasons and seawater fractions suggests that these microdiverse clades specialize on distinct water column microhabitats. Although clusters corresponding to named bacterial species generally have distinct environmental preferences, preferred habitats switches can occur between clades differing by only a single base pair in the *hsp60* gene, suggesting that habitat switches occur on short timescales as well. Moreover, metabolic differentiation was investigated in the *Vibrionaceae*; chitinoclastic ability was near ubiquitously distributed among vibrio isolates (Chapter 3), even among isolates that were found as largely free-living (as observed in Chapter 2). This finding suggests either these clades occasionally use particulate chitin resources or degrade chitin oligomers, or alternately that unused traits such as chitinoclastic ability are maintained in the genome. The persistence of unused metabolic capabilities may allow rapid adaptation to new niches. Moreover, these results suggest strong competition among the vibrios for resources such as chitin, as does the rapid differentiation and wide range of microhabitats utilized (as observed in Chapter 2)

Finally, clustering of bacteria around phytoplankton was investigated in the marine environment (Chapter 4). Field observations found correlations between the numbers of prokaryotic and eukaryotic cells in microscale samples over a seasonal cycle.

This relationship was most pronounced under high eukaryotic cell concentrations, presumably phytoplankton blooms; which squared with a conceptual model suggesting chemotaxis toward and clustering around phytoplankton is only energetically efficient for high algal concentrations. This prediction was confirmed experimentally by observing a *Roseobacter* strains clustering around and attaching to dead diatoms (*Thalassiosira weissflogii*). Further an isogenic bacterial motility mutant did not colonize dead diatoms, suggesting that motility is necessary to utilize this important resource. This study seeks to answer fundamental questions about marine bacterial populations: how do closely related species co-exist in the same environment, are metabolic characteristics tightly linked to an organism's preferred habitat and how important are microenvironments to bacterial productivity.

WORKS CITED:

Abell GCJ, Bowman JP (2005) Ecological and biogeographic relationships of class Flavobacteria in the Southern Ocean. FEMS Microbiology Ecology 51:265-277

Acinas SG, Anton J, Rodriguez-Valera F (1999) Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Applied and Environmental Microbiology 65:514-522

Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL, Polz MF (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430:551-554

Alldredge AL (1979) The chemical composition of macroscopic aggregates in two neretic seas. Limnology and Oceanography 24:855-866

Alldredge AL, Cohen Y (1987) Can microscale chemical patches persist in the sea - microelectrode studies of marine snow, fecal pellets. Science 235:689-691

Alldredge AL, Silver MW (1988) Characteristics, dynamics and significance of marine snow. Progress in Oceanography 20:41-82

Andreatta S, Wallinger MM, Piera J, Catalan J, Psenner R, Hofer JS, Sommaruga R (2004) Tools for discrimination and analysis of lake bacterioplankton subgroups measured by flow cytometry in a high-resolution depth profile. Aquatic Microbial Ecology 36:107-115

Ast JC, Dunlap PV (2005) Phylogenetic resolution and habitat specificity of members of the *Photobacterium phosphoreum* species group. Environmental Microbiology 7:1641-1654

Ayo B, Unanue M, Azua I, Gorsky G, Turley C, Iriberri J (2001) Kinetics of glucose and amino acid uptake by attached and free-living marine bacteria in oligotrophic waters. Marine Biology 138:1071-1076

Azam F, Ammerman JW (1984) Cycling of organic matter by bacterioplankton in pelagic marine ecosystems: microenvironmental considerations. In: Fasham MJR (ed) Flows of energy and materials in marine ecosystems. Plenham Press, New York, p 345-360

Azam F, Cho BC (eds) (1987) Bacterial utilization of organic matter in the sea, Vol. Cambridge University Press, New York, NY

Azam F, Hodson RE (1981) Multiphasic kinetics for D-glucose uptake by assemblages of natural marine-bacteria. Marine Ecology-Progress Series 6:213-222

Barbara GM, Mitchell JG (2003a) Bacterial tracking of motile algae. FEMS Microbiology Ecology 44:79-87

Barbara GM, Mitchell JG (2003b) Marine bacterial organisation around point-like sources of amino acids. FEMS Microbiology Ecology 43:99-109

Blackburn N, Fenchel T, Mitchell J (1998) Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science 282:2254-2256

Bochdansky AB, Herndl GJ (1992) Ecology of amorphous aggregations (marine snow) in the Northern Adriatic Sea .3. Zooplankton interactions with marine snow. Marine Ecology-Progress Series 87:135-146

Bowen JD, Stolzenbach KD, Chisholm SW (1993) Simulating bacterial clustering around phytoplankton cells in a turbulent ocean. Limnology and Oceanography 38:36-51

Buchan A, Gonzalez JM, Moran MA (2005) Overview of the marine *Roseobacter* lineage. Applied and Environmental Microbiology 71:5665-5677

Caron DA, Davis PG, Madin LP, Sieburth JM (1982) Heterotrophic bacteria and bacterivorous protozoa in oceanic macro-aggregates. Science 218:795-797

Casamayor EO, Pedros-Alio C, Muyzer G, Amann R (2002) Microheterogeneity in 16S ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Applied and Environmental Microbiology 68:1706-1714

Chin WC, Orellana MV, Verdugo P (1998) Spontaneous assembly of marine dissolved organic matter into polymer gels. Nature 391:568-572

Comeau AM, Suttle CA (2007) Distribution, genetic richness and phage sensitivity of *Vibrio* spp. from coastal British Columbia. Environmental Microbiology 9:1790-1800

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature 438:90-93

Crump BC, Armbrust EV, Baross JA (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. Applied and Environmental Microbiology 65:3192-3204

Crump BC, Baross JA, Simenstad CA (1998) Dominance of particle-attached bacteria in the Columbia River estuary, USA. Aquatic Microbial Ecology 14:7-18

Dang HY, Lovell CR (2002) Seasonal dynamics of particle-associated and free-living marine *Proteobacteria* in a salt marsh tidal creek as determined using fluorescence in situ hybridization. Environmental Microbiology 4:287-295

Daubin V, Moran NA, Ochman H (2003) Phylogenetics and the cohesion of bacterial genomes. Science 301:829-832

Davis CS, Gallager SM, Solow AR (1992) Microaggregations of oceanic plankton observed by towed video microscopy. Science 257:230-232

DeLong EF, Franks DG, Alldredge AL (1993) Phylogenetic diversity of aggregate-attached vs free-living marine bacterial assemblages. Limnology and Oceanography 38:924-934

Dickey TD (1991) The emergence of concurrent high resolution physical and biooptical measurements in the upper ocean and their applications. Reviews of Geophysics 29:383-413

Doney SC, Glover DM, McCue SJ, Fuentes M (2003) Mesoscale variability of Sea-viewing Wide Field-of-view Sensor(SeaWiFS) satellite ocean color: Global patterns and spatial scales. Journal of Geophysical Research-Oceans 108

Duarte CM, Vaqué D (1992) Scale dependence of bacterioplankton patchiness. Marine Ecology-Progress Series 84:95-100

Fandino LB, Riemann L, Steward GF, Long RA, Azam F (2001) Variations in bacterial community structure during a dinoflagellate bloom, analyzed by DGGE and 16S rDNA sequencing. Aquatic Microbial Ecology 23:119-130

Fenchel T (2001) Eppur si muove: many water column bacteria are motile. Aquatic Microbial Ecology 24:197-201

Ferris MJ, Kuhl M, Wieland A, Ward DM (2003) Cyanobacterial ecotypes in different optical microenvironments of a 68 degrees C hot sring mat community revealed by 16S-23S rRNA internal transcribed spacer region variation. Applied and Environmental Microbiology 69:2893-2898

Field KG, Gordon D, Wright T, Rappé M, Urbach E, Vergin K, Giovannoni SJ (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. Applied and Environmental Microbiology 63:63-70

Folt CL, Burns CW (1999) Biological drivers of zooplankton patchiness. Trends in Ecology & Evolution 14:300-305

Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. Nature 399:541-548

Fuhrman JA, Ferguson RL (1986) Nanomolar concentrations and rapid turnover of dissolved free amino-acids in seawater - agreement between chemical and microbiological measurements. Marine Ecology-Progress Series 33:237-242

Gasol JM, Duarte CM (2000) Comparative analyses in aquatic microbial ecology: how far do they go? FEMS Microbiology Ecology 31:99-106

Giovannoni SJ, Stingl U (2005) Molecular diversity and ecology of microbial plankton. Nature 437:343-348

Gordon DM, Cowling A (2003) The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology-SGM 149:3575-3586

Grossart HP, Kiorboe T, Tang KW, Allgaier M, Yam EM, Ploug H (2006) Interactions between marine snow and heterotrophic bacteria: aggregate formation and microbial dynamics. Aquatic Microbial Ecology 42:19-26
Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. Environmental Microbiology 7:860-873
Guerrini F, Mazzotti A, Boni L, Pistocchi R (1998) Bacterial-algal interactions in polysaccharide production. Aquatic Microbial Ecology 15:247-253
Hanage WP, Fraser C, Spratt BG (2006) Sequences, sequence clusters and bacterial species. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1917-1927
Hebel D, Knauer GA, Martin JH (1986) Trace metals in large agglomerates (marine snow). Journal of Plankton Research 8:819-824
Heidelberg JF, Heidelberg KB, Colwell RR (2002) Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. Applied and Environmental Microbiology 68:5498-5507
Hellebrust JA (ed) (1974) Extracellular products, Vol. Blackwell Scientific, London
Hollibaugh JT, Wong PS, Murrell MC (2000) Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California. Aquatic Microbial Ecology 21:103-114
Huber JA, Butterfield DA, Baross JA (2003) Bacterial diversity in a subseafloor habitat following a deep-sea volcanic eruption. FEMS Microbiology Ecology 43:393-409
Jackson GA (1987) Simulating chemosensory responses of marine microorganisms. Limnology and Oceanography 32:1253-1266
Jacobsen TR, Azam F (1984) Role of bacteria in copepod fecal pellet decomposition - colonization, growth-rates and mineralization. Bulletin of Marine Science 35:495-502
Jaspers E, Overmann J (2004) Ecological significance of microdiversity: Identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. Applied and Environmental Microbiology 70:4831-4839
Johnson ZI, Zinser ER, Coe A, McNulty NP, Woodward EMS, Chisholm SW (2006) Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science 311:1737-1740
Karner M, Herndl GJ (1992) Extracellular enzymatic-activity and secondary production in free-living and marine-snow-associated bacteria. Marine Biology 113:341-347
Kent AD, Yannarell AC, Rusak JA, Triplett EW, McMahon KD (2007) Synchrony in aquatic microbial community dynamics. The ISME Journal 1:38-47
Kiørboe T, Grossart HP, Ploug H, Tang K (2002) Mechanisms and rates of bacterial colonization of sinking aggregates. Applied and Environmental Microbiology 68:3996-4006
Kiørboe T, Jackson GA (2001) Marine snow, organic solute plumes, and optimal chemosensory behavior of bacteria. Limnology and Oceanography 46:1309-1318
Kirchman D, Yu L, Fuchs BM, Amann R (2001) Structure of bacterial communities in aquatic systems as revealed by filter PCR. Aquatic Microbial Ecology 26:13-22
Knoll S, Zwisler W, Simon M (2001) Bacterial colonization of early stages of limnetic diatom microaggregates. Aquatic Microbial Ecology 25:141-150

Krembs C, Juhl AR, Long RA, Azam F (1998a) Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. Limnology and Oceanography 43:307-314

Krembs C, Juhl AR, Strickler JR (1998b) The spatial information preservation method: Sampling the nanoscale spatial distribution of microorganisms. Limnology and Oceanography 43:298-306

Lambais MR, Crowley DE, Cury JC, Bull RC, Rodrigues RR (2006) Bacterial diversity in tree canopies of the Atlantic forest. Science 312:1917-1917

Lancelot C (1979) Gross excretion rates of natural marine phytoplankton and heterotrophic uptake of excreted products in the southern North Sea, as determined by short-term kinetics. Marine Ecology-Progress Series 1:179-186

Lau WWY, Armbrust EV (2006) Detection of glycolate oxidase gene glcD diversity among cultured and environmental marine bacteria. Environmental Microbiology 8:1688-1702

Li WKW, Harrison WG, Head EJH (2006) Coherent sign switching in multiyear trends of microbial plankton. Science 311:1157-1160

Long RA, Azam F (1996) Abundant protein-containing particles in the sea. Aquatic Microbial Ecology 10:213-221

Long RA, Azam F (2001a) Antagonistic interactions among marine pelagic bacteria. Applied and Environmental Microbiology 67:4975-4983

Long RA, Azam F (2001b) Microscale patchiness of bacterioplankton assemblage richness in seawater. Aquatic Microbial Ecology 26:103-113

Lopez-Lopez A, Bartual SG, Stal L, Onyshchenko O, Rodriguez-Valera F (2005) Genetic analysis of housekeeping genes reveals a deep-sea ecotype of *Alteromonas macleodii* in the Mediterranean Sea. Environmental Microbiology 7:649-659

Maharjan R, Seeto S, Notley-McRobb L, Ferenci T (2006) Clonal adaptive radiation in a constant environment. Science 313:514-517

McCarthy JJ, Goldman JC (1979) Nitrogenous nutrition of marine phytoplankton in nutrient-depleted waters. Science 203:670-672

McGillicuddy DJ, Robinson AR, Siegel DA, Jannasch HW, Johnson R, Dickeys T, McNeil J, Michaels AF, Knap AH (1998) Influence of mesoscale eddies on new production in the Sargasso Sea. Nature 394:263-266

Miller TR, Hnilicka K, Dziedzic A, Desplats P, Belas R (2004) Chemotaxis of *Silicibacter* sp strain TM1040 toward dinoflagellate products. Applied and Environmental Microbiology 70:4692-4701

Mindl B, Sonntag B, Pernthaler J, Vrba J, Psenner R, Posch T (2005) Effects of phosphorus loading on interactions of algae and bacteria: reinvestigation of the 'phytoplankton-bacteria paradox' in a continuous cultivation system. Aquatic Microbial Ecology 38:203-213

Mitchell JG (2002) The energetics and scaling of search strategies in bacteria. American Naturalist 160:727-740

Mitchell JG, Okubo A, Fuhrman JA (1985) Microzones surrounding phytoplankton form the basis for a stratified marine microbial ecosystem. Nature 316:58-59

Mitchell JG, Pearson L, Bonazinga A, Dillon S, Khouri H, Paxinos R (1995) Long lag times and high velocities in the motility of natural assemblages of marine-bacteria. Applied and Environmental Microbiology 61:877-882

Mitchell JG, Pearson L, Dillon S (1996) Clustering of marine bacteria in seawater enrichments. Applied and Environmental Microbiology 62:3716-3721

Moeseneder MM, Herndl GJ (1995) Influence of turbulence on bacterial production in the sea. Limnology and Oceanography 40:1466-1473

Moeseneder MM, Winter C, Herndl GJ (2001) Horizontal and vertical complexity of attached and free-living bacteria of the eastern Mediterranean Sea, determined by 16S rDNA and 16S rRNA fingerprints. Limnology and Oceanography 46:95-107

Moran MA, Buchan A, Gonzalez JM, Heidelberg JF, Whitman WB, Kiene RP, Henriksen JR, King GM, Belas R, Fuqua C, Brinkac L, Lewis M, Johri S, Weaver B, Pai G, Eisen JA, Rahe E, Sheldon WM, Ye WY, Miller TR, Carlton J, Rasko DA, Paulsen IT, Ren QH, Daugherty SC, Deboy RT, Dodson RJ, Durkin AS, Madupu R, Nelson WC, Sullivan SA, Rosovitz MJ, Haft DH, Selengut J, Ward N (2004) Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. Nature 432:910-913

Moran MA, Gonzalez JM, Kiene RP (2003) Linking a bacterial taxon to sulfur cycling in the sea: Studies of the marine *Roseobacter* group. Geomicrobiology Journal 20:375-388

Müller-Niklas G, Agis M, Herndl GJ (1996) Microscale distribution of bacterioplankton in relation to phytoplankton: Results from 100-nl samples. Limnology and Oceanography 41:1577-1582

Mummey DL, Stahl PD (2004) Analysis of soil whole- and inner-microaggregate bacterial communities. Microbial Ecology 48:41-50

Nightingale KK, Lyles K, Ayodele M, Jalan P, Nielsen R, Wiedmann M (2006) Novel method to identify source-associated phylogenetic clustering shows that *Listeria monocytogenes* includes niche-adapted clonal groups with distinct ecological preferences. Journal of Clinical Microbiology 44:3742-3751

Pinhassi J, Sala MM, Havskum H, Peters F, Guadayol O, Malits A, Marrase CL (2004) Changes in bacterioplankton composition under different phytoplankton regimens. Applied and Environmental Microbiology 70:6753-6766

Poindexter JS (1981) Oligotrophy - fast and famine existence. Advances in Microbial Ecology 5:63-89

Polz MF, Hunt DE, Preheim SP, Weinreich DM (2006) Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. Philosophical Transactions of the Royal Society B-Biological Sciences 361:2009-2021

Rainey PB, Travisano M (1998) Adaptive radiation in a heterogeneous environment. Nature 394:69-72

Ramette A, Tiedje JM (2007) Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. Proceedings of the National Academy of Sciences of the United States of America 104:2761-2766

Rhee G-Y (1972) Competition between an alga and an aquatic bacterium for phosphate. Limnology and Oceanography 17:505-514

Riemann L, Winding A (2001) Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. Microbial Ecology 43:290-290

Rocap G, Distel DL, Waterbury JB, Chisholm SW (2002) Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal

transcribed spacer sequences. Applied and Environmental Microbiology 68:1180-1191

Rocap G, Larimer FW, Lamerdin J, Malfatti S, Chain P, Ahlgren NA, Arellano A, Coleman M, Hauser L, Hess WR, Johnson ZI, Land M, Lindell D, Post AF, Regala W, Shah M, Shaw SL, Steglich C, Sullivan MB, Ting CS, Tolonen A, Webb EA, Zinser ER, Chisholm SW (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature 424:1042-1047

Rohwer F, Seguritan V, Azam F, Knowlton N (2002) Diversity and distribution of coral-associated bacteria. Marine Ecology-Progress Series 243:1-10

Schäfer H, Abbas B, Witte H, Muyzer G (2002) Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiology Ecology 42:25-35

Selje N, Simon M (2003) Composition and dynamics of particle-associated and free-living bacterial communities in the Weser estuary, Germany. Aquatic Microbial Ecology 30:221-237

Selje N, Simon M, Brinkhoff T (2004) A newly discovered *Roseobacter* cluster in temperate and polar oceans. Nature 427:445-448

Seymour JR (2005) Microscale spatiotemporal heterogeneity of marine microbial communities. Ph.D., Flinders University

Seymour JR, Mitchell JG, Pearson L, Waters RL (2000) Heterogeneity in bacterioplankton abundance from 4.5 millimetre resolution sampling. Aquatic Microbial Ecology 22:143-153

Seymour JR, Mitchell JG, Seuront L (2004) Microscale heterogeneity in the activity of coastal bacterioplankton communities. Aquatic Microbial Ecology 35:1-16

Seymour JR, Seuront L, Mitchell JG (2005) Microscale and small-scale temporal dynamics of a coastal planktonic microbial community. Marine Ecology-Progress Series 300:21-37

Sikorski J, Nevo E (2007) Patterns of thermal adaptation of *Bacillus simplex* to the microclimatically contrasting slopes of 'Evolution Canyons' I and II, Israel. Environmental Microbiology 9:716-726

Smith DC, Simon M, Alldredge AL, Azam F (1992) Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature 359:139-141

Smith NH, Kremer K, Inwald J, Dale J, Driscoll JR, Gordon SV, van Soolingen D, Hewinson RG, Smith JM (2006) Ecotypes of the *Mycobacterium tuberculosis* complex. Journal of Theoretical Biology 239:220-225

Thompson JR, Pacocha S, Pharino C, Klepac-Ceraj V, Hunt DE, Benoit J, Sarma-Rupavtarm R, Distel DL, Polz MF (2005) Genotypic diversity within a natural coastal bacterioplankton population. Science 307:1311-1313

Thompson JR, Polz MF (2006) Dynamics of vibrio populations and their role in environmental nutrient cycling. In: Thompson FL, Austin B, Swings J (eds) The Biology of *Vibrios*. ASM Press, Washington DC, p 190-203

Thompson JR, Randa MA, Marcelino L, Tomita-Mitchell A, Lim E, Polz MF (2004) Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Applied and Environmental Microbiology 70:4103-4110

Turner JT (2002) Zooplankton fecal pellets, marine snow, and sinking phytoplankton blooms. Aquatic Microbial Ecology 27:57-102

Vogel J, Normand P, Thioulouse J, Nesme X, Grundmann G (2003) Relationship between spacial and genetic distance in *Agrobacterium* spp. in 1 cubic centimeter of soil. Applied and Environmental Microbiology 69:1482-1487

von Mering C, Hugenholtz P, Raes J, Tringe SG, Doerks T, Jensen LJ, Ward N, Bork P (2007) Quantitative phylogenetic assessment of microbial communities in diverse environments. Science 315:1126-1130

Vos M, Velicer GJ (2006) Genetic population structure of the soil bacterium *Myxococcus xanthus* at the centimeter scale. Applied and Environmental Microbiology 72:3615-3625

Ward TJ, Gorski L, Borucki MK, Mandrell RE, Hutchins J, Pupedis K (2004) Intraspecific phylogeny and lineage group identification based on the prfA virulence gene cluster of *Listeria monocytogenes*. Journal of Bacteriology 186:4994-5002

Welch RA, Burland V, Plunkett G, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HLT, Donnenberg MS, Blattner FR (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 99:17020-17024

Wells ML (1998) Marine colloids - A neglected dimension. Nature 391:530-531

Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301:976-978

Worm J, Gustavson K, Garde K, Borch NH, Sondergaard M (2001) Functional similarity of attached and free-living bacteria during freshwater phytoplankton blooms. Aquatic Microbial Ecology 25:103-111

Chapter Two

Adaptation of microdiverse bacterial clusters to distinct marine microenvironments[1]

[1]To be submitted with co-authors: L David, SP Preheim, D Gevers, EJ Alm, MF Polz

ABSTRACT:

How vast numbers of closely related bacteria coexist in the ocean remains poorly understood due to paucity of observations and conflicting theories of ecological speciation. Here, we show spatial and temporal resource partitioning for a group of coastal heterotrophic bacteria (*Vibrionaceae*). Statistical analysis reveals that ecological populations can be recognized as phylogenetic clusters, which primarily correspond to deeply divergent taxa. However, one group (*V. splendidus*) appears to be currently undergoing ecological radiation as evidenced by many microdiverse, and in some cases, nested clades with distinct habitat distributions. Overall, the data suggest that a large number of clades are unevenly distributed between different seasons and lifestyles (free-living, particle-associated and zooplankton-associated) in spite of high potential for population homogenization by genetic recombination and ocean mixing.

INTRODUCTION:

The ocean's microbial communities harbor far greater genetic diversity than previously expected (Acinas et al. 2004, Sogin et al. 2006, Rusch et al. 2007). Although comparative analyses reveal differential distributions of microbial taxa and specific gene families (Giovannoni & Stingl 2005, DeLong et al. 2006), it is poorly understood to what extent the vast co-existing microbial diversity reflects population differentiation (e.g., by resource partitioning) or neutral variation (Giovannoni & Stingl 2005, Polz et al. 2006). First, it has been difficult to determine specific association of bacterial genotypes with

spatio-temporal conditions; second, it remains controversial how ecological differentiation should be manifest genetically. Phylogenetic clusters have been proposed to correspond to ecological populations that arise by neutral diversification following niche-specific selective sweeps (Cohan & Perry 2007). Clusters are indeed observed among closely related isolates [e.g., by multilocus sequence analysis] (Hanage et al. 2006) and in culture-independent analysis of coastal bacterioplankton (Acinas et al. 2004). Yet theoretical studies suggest that clusters can result from neutral evolution (Fraser et al. 2007), and evidence for clusters as ecological populations remains sparse, having been most conclusively demonstrated for cyanobacteria along ocean-scale gradients (Johnson et al. 2006). Further, horizontal gene transfer (HGT) may erode ecological cohesion of populations if adaptive genes are transferred (Doolittle & Papke 2006), and homologous recombination among closely related genomes may obscure the phylogenetic signal in ecologically distinct populations (Retchless & Lawrence 2007). Thus to what extent phylogenetic and ecological differentiation is correlated remains a crucial problem in understanding evolutionary mechanisms of bacterial speciation and ecological differentiation (Fraser et al. 2007).

Here, we ask to what extent closely related genotypes are ecologically differentiated (as evidenced by differential distribution among microhabitats). We focus on heterotrophic bacteria of the family *Vibrionaceae*, which are metabolically and ecologically versatile members of the coastal plankton (Thompson & Polz 2006). The coastal ocean is well suited to test population-level effects of microhabitat selection, since tidal mixing and oceanic circulation ensure high probability of immigration, rendering population differentiation due to endemism unlikely. In the plankton,

heterotrophs may adopt alternate ecological strategies: exploiting either the generally low but more evenly distributed dissolved nutrients or attaching and degrading small, suspended organic particles, originating from algal exopolysaccharides and detritus (Polz et al. 2006). In this dynamic environment, microhabitat preferences may develop since resources are distributed on the same scale as the dispersal range of individuals due to turbulent mixing and active motility (Kiørboe et al. 2002). Particles represent a relatively short-lived resource as the labile components are rapidly utilized (~hours-days) (Pomeroy et al. 1984, Panagiotopoulos et al. 2002), suggesting that particle-colonization is a dynamic process. Moreover, particulate matter may not constitute a uniform resource, changing composition with macroecological conditions (e.g., algal blooms). Zooplankton may provide additional, more stable microhabitats; vibrios attach to chitinous zooplankton exoskeletons (Heidelberg et al. 2002), but may also live in the gut or occupy pathogenic niches. The extent to which microenvironmental preferences contribute to resource partitioning in this complex ecological landscape remains an important question in microbial ecology.

MATERIAL AND METHODS:

Sample collection

Samples were collected at high tide on the marine end of the Plum Island Estuary (NE Massachusetts) (Fig. 1A) on two days representing spring (4/28/06) and fall (9/6/06) conditions in the coastal ocean. Nutrient concentrations, water temperature and chlorophyll levels were measured on both sampling dates (Table 1)

To separate different microhabitats co-existing in the water column, we used sequential filtration with decreasing pore size cutoffs (Fig. 1B). Filtration is commonly used in oceanography to separate particle-associated and free-living populations, although the filter size cut off for collecting particle-associated bacteria varies between 0.8 and 10 μm (Acinas et al. 1999, Crump et al. 1999, Riemann & Winding 2001, Selje & Simon 2003, Eiler et al. 2006). Here, we used sequential gravity filtration to separate particulate and free-living cells by retention of particles on a filter; we collected a total of four size fractions, which are enriched in zooplankton (>63 μm), large (63-5 μm) and small (5-1 μm) particles, and free-living cells (1-0.22 μm) (Fig. 1B). The 5-1 μm size fraction is somewhat ambiguous, likely containing cells attached to small particles, as well as large or dividing cells; however, it provides a firm buffer between obviously particle-associated (>5 μm) and free-living (<1 μm) cells. Zooplankton were enriched by filtering ~100 L through a 63 μm plankton net, which was washed with sterile seawater. Particulate and free-living bacterial populations were collected from quadruplicate water samples, which were pre-filtered through the 63 μm plankton net (to remove the zooplankton-enriched fraction) into 4 L nalgene bottles (Fig. 1B). For each bottle, water was sequentially filtered through 5, 1 and 0.22 μm pore size filters with at least four replicates per size fraction. To avoid disruption of fragile particles, the 63-5 and 5-1 μm fractions were collected on polycarbonate membrane filters (Sterlitech) using gravity filtration followed by washing with 10 ml of sterile (0.22 μm-filtered and Tindalized) seawater to remove free-living bacteria that might have been retained on the filter. The sub-1 μm fraction containing free-living bacteria was collected on 0.22 μm Supor-200 filters (Pall) by applying gentle vacuum pressure.

Once samples were separated, particles and zooplankton were treated before plating since they could contain multiple vibrio cells on a single particle or zooplankter (Fig. 1B). The zooplankton sample was washed with sterile seawater, homogenized using a tissue grinder (VWR Scientific) and vortexed for 20 minutes at low speed before concentration on 0.22 μm Supor-200 filters (Pall); these filters were plated directly on selective media. Similarly, 5 μm and 1 μm filters were placed in 50 ml conical tubes with 50 ml sterile seawater and vortexed at low speed for 20 min to break up particles and detach bacteria from the filters. The supernatant was concentrated on 0.22 μm filters, and both the filters containing the original and supernatant material were placed directly on media to collect isolates.

Strain isolation and identification

Isolates were obtained from TCBS plates (Accumedia or Difo) with 2% NaCl since this media has been shown to yield a similar distribution of isolates as enumerated by qPCR (Thompson et al. 2005). After 2-3 days of growth, colonies were counted and re-streaked a total of three times, alternately on Tryptic Soy Broth (TSB) (Difco) with 2% NaCl and on TCBS media. Purified isolates were grown in marine TSB broth overnight; DNA was extracted using either a tissue DNA kit (Qiagen) or Lyse-N-Go (Pierce). The partial *hsp60* gene sequence was amplified for all isolates as described previously (Goh et al. 1996). For isolates with an *hsp60* sequence differing by more than 2% from an already characterized strain, the 16S rRNA gene was PCR amplified using primers 27F-1492R and sequenced using the 27F primer (Lane 1991). The 16S sequence was used to identify the organism using RDP classifer (Cole et al. 2007) and BLAST (Altschul et al.

1990). For isolates where the *hsp60* gene either failed to amplify or the sequence was highly divergent from other vibrios, 16S rRNA gene sequencing confirmed that these strains largely belonged to the genera *Pseudomonas*, *Shewanella*, *Pseudoalteromonas*, and *Agaravorans* (RDP Classifier) (Cole et al. 2007); these were excluded from further analysis.

To confirm relationships for *V. splendidus,* the most highly represented group among isolates, an additional gene (*mdh*) was sequenced. The partial *mdh* gene was amplified using primers mdh.for (5'- GAY CTD AGY CAY ATC CCW AC -3') and mdh.rev (5'- GCT TCW ACM ACY TCD GTR CCY G -3') (Santos & Ochman 2004). For selected groups of isolates additional housekeeping gene sequences were obtained (*pgi, adk*), using pgi.for (5' –GAC CTW GGY CCW TAC ATG GT - 3')/ pgi.rev (5'- CMG CRC CRT GGA AGT TGT TRT-3') (unpublished data S. Preheim) and adk.for (5'- GTA TTC CAC AAA TYT CTA CTG G-3')/ adk.rev (5'- GCT TCT TTA CCG TAG TA- 3') (Santos & Ochman 2004). All additional genes were amplified using the following PCR conditions: 2 min at 94°C followed by 32 cycles of 1 min each at 94°C, 46°, and 72°C, with a final step of 6 min at 72°C. For the majority of genes high quality bidirectional sequences were obtained from the Bay Paul Center at the Marine Biological Laboratory, Woods Hole MA.

Phylogenetic tree construction and representation

The partial *hsp60* gene sequences yielded an unambiguous alignment of 541 nucleotides. Whereas *mdh, adk* and *pgi* resulting in unambiguous alignments of 422, 372, 395 nucleotides, respectively. Phylogenetic relationships were reconstructed using PhyML

v.2.4.4 (Guindon & Gascuel 2003) with following parameter settings: DNA substitution was modeled using the HKY parameter; the transition/transversion ratio was set to 4.0; PhyML estimated the proportion of invariable nucleotide sites; the gamma distribution parameter was set to 1.0; 4 gamma rate categories were used. Circular trees were drawn using the online iTOL software package (Letunic & Bork 2007).

Identifying phylogenetically related groups

Phylogenetic groups were identified based on the *hsp60* gene tree (Fig 3A) for groups containing at least 10 isolates which were constrained by a node will strong bootstrap support. Within the *V. splendidus* clade very few nodes were well supported by bootstrap values, thus additional phylogenetic clades were identified by eye (Fig 3B). The numbers on both of these trees correspond to identified groups, the data for which is summarized in Figure 6. In order to determine statistical associations with a specific size fraction for each group, the other three size fractions were added together as the "in" group distribution in the specific size fraction was compared to the "out" group consisting of the rest of the isolates using a Fischer's exact 2x2 test (Fig 6C).

Testing for seasonal/ecological association within clusters

To determine whether phylogenetically-related groups were associated with a particular size fraction, we constructed contingency tables to identify association between phylogeny and season/environment for each node (all possible clades) of the *hsp60* tree. To examine specialization in different size fractions, the distribution of strains across the four size fractions (columns) was compared between the clade of interest and the rest

total of the sampled strains (rows) using Fisher's exact test. Results are mapped onto the phylogenetic tree (Fig. 4 and 5): a pie chart indicates significance at the $p<0.001$ level, with the ratio of colors in the pie reflecting the distribution of isolates beneath that node. These p-values are not corrected for multiple hypothesis testing, as they are not independent measurements due to the nested structure of the data.

RESULTS AND DISCUSSION:

We aimed to conservatively identify ecologically coherent groups by examining the distribution of *Vibrionaceae* genotypes among the free-living and associated (with particles and zooplankton) compartments of the planktonic community collected under different macroecological conditions (spring and fall) (Fig. 1, Table 1). Since there is no *a priori* expectation of the level of genetic differentiation at which ecological preferences should emerge, we focused on the entire range of relationships, from identical to ~10% SSU rRNA difference, among co-occurring vibrios (Thompson et al. 2005). Particle-associated and free-living cells were separated into a total of four consecutive size fractions, which are enriched in zooplankton (>63 μm), large (63-5 μm) and small (5-1 μm) particles, and free-living cells (1-0.22 μm) (Fig. 1B). *Vibrionaceae* strains were isolated by plating filters on selective media, previously shown by quantitative PCR to yield good correspondence between genotypes recovered in culture and present in environmental samples (Thompson et al. 2005).

Roughly 1,000 isolates were characterized by partial sequencing of a protein-coding gene (*hsp60*). To confirm relationships, between 1 and 3 additional gene

fragments (*mdh*, *adk* and *pgi*) were sequenced for *V. splendidus*, the dominant taxon during warm water conditions (Thompson et al. 2005). These data allow conservative estimation of ecological differentiation because inadvertent mixing of strains between microhabitats and homologous recombination among strains homogenize rather than create associations. Ecological specialization can be more than simply association of a clade with a given size fraction (Fig. 2A), as single habitats can span multiple size fractions (Fig 2B), or clades may be adapted to multiple microhabitats, each with its own size distribution (Fig 2C). Moreover, significant differences in the relative frequency distributions of genotypes among size fractions can be used to identify habitat differences although the specific microenvironment(s) driving the association remain unidentified.

Visual examination of the isolate phylogeny already reveals differential distribution of clades between both season and size fraction (Fig. 3), suggesting temporal and spatial resource partitioning. Strong seasonal associations are quite apparent in the data and were confirmed with statistical testing (Fig. 4); this extends previously noted correlation of *Vibrio* ribotype abundances with seasonal temperature fluctuation (Thompson et al. 2004, Thompson et al. 2005). Statistical testing also confirms preferential association with specific size fractions across the entire *hsp60* tree (Fig 5). Additionally, this data was tested to ensure that the trends were not just due to clonal expansion on a single filter; removing isolates from the same filter with identical sequences did not change the overall appearance of the tree, suggesting clonal expansion is not the reason for the observed associations. Because the patterns are more complex than for the seasonal data and overview statistics do not reveal the depth and number of ecologically distinct populations, we investigated in greater depth the distribution of

bacterial clades in different microenvironments.

We first sought to robustly identify clades within the *Vibrionaceae* and then tested their possible association with different microenvironments. The strains were initially grouped by subdivision of the *hsp60* tree into clusters, which were well supported by bootstrap values and contained at least 10 members (Fig. 3). For *V. splendidus* isolates, which are highly microdiverse (Thompson et al. 2005) and therefore do not resolve into bootstrap supported clusters, a second housekeeping gene (*mdh*) was used to provide further resolution (2-gene concatenated tree, Fig 3B). Using this additional gene information groups were defined using bootstrap measures and by eye, then tested for differential lifestyle distributions (Fig. 3B). This approach is robust despite 'noise' in the data created by uncertain phylogenetic placement or horizontal gene transfer by homologous recombination since these factors should homogenize rather than falsely create associations.

Twenty phylogenetic groups within co-occurring *Vibrio* isolates were identified, 14 of which exhibited statistically significant associations with one or more microenvironments (Fisher's exact test $p < 0.05$) (Fig 6C). Even within closely-related *V. splendidus*, clusters with distinct preferences were observed. Group 20, exhibited different preferences between spring and fall samples, either this group switches preferences, or these genes do not provide sufficient resolution to separate two ecologically distinct populations.

This data suggests that a single bacterial family resolves into a striking number of populations, which spatially partition resources in the plankton. Ecological specialization appears to be largely driven by association with the zooplankton-enriched and free-living

fractions (Fischer's exact test $p < 0.05$) although representatives of many clades are found on particles (Fig. 6). Vibrios are generally regarded as preferring attached life-styles (Thompson & Polz 2006) so that both the preference for the free-living lifestyle in *Enterovibrio calviensis* (Group 1), *V. ordalii* (Group 4) and two *V. splendidus* groups (13, 20 F) and the paucity of particle specialists provide new facets to the ecological differentiation of this versatile group.

In some cases, paraphyletic clades resolved into clusters with the same habitat preference; this most likely reflects exploitation of different resources within the same size fraction since competitive exclusion would preclude stable maintenance of overlapping preferences over long evolutionary times. Indeed, the deeply divergent clades identified, which largely correspond to broad taxonomic species, appear to be ecologically associated. The notable exception is *V. splendidus*, for which 10 microdiverse clusters with different preferences were observed. Overall, these results suggest that ecological specialization can be identified over a wide range of phylogenetic differentiation, including a group (*V. splendidus*) that may currently be ecologically diversifying, possibly at the expense of other bacterial groups or through increasingly fine scale partitioning of resources.

Current radiation by sympatric resource partitioning among *V. splendidus* is most strongly suggested for several nested clades in which groups of strains differing by as little as a single nucleotide in the *hsp60* tree display distinct ecological preferences (Fig. 7). Such patterns may be the result of recent adaptation to a new microenvironment, which does not affect the sequence of housekeeping genes, but can also be generated if homologous recombination moves alleles into more distantly related (and likely

ecologically distinct) clades. Multilocus sequencing indeed rejects one of the cases (red/blue group) since *hsp60* gene phylogeny is discordant with that of the three other housekeeping genes (Fig. 7B); however, the other cluster contains almost identical alleles for each gene (Fig. 7), supporting ecological differentiation uncoupled from or preceding cluster formation. Such rather abrupt change in ecological preferences of a microdiverse group of organisms may be consistent with acquisition of niche-adaptive genes via horizontal gene transfer (HGT) which allow organisms to exploit new environments (Doolittle & Papke 2006). Such HGT events are thought to generate sequence clusters by local inhibition of homologous recombination leading to genetic isolation, which may propagate through the genome through increasing accumulation of point mutations (Vetsigian & Goldenfeld 2005). Recent genome analysis suggests that *E. coli* and *Salmonella* have diverged according to this model (Retchless & Lawrence 2007); the nested clades identified here, are so closely related that they may present an opportunity to identify the genes responsible for ecological differentiation. Additionally, sequencing of multiple housekeeping genes in the *V. alginolyticus /parahaemolyticus* clade, which was well mixed between size fractions did not result in further separation into fraction-based clusters, indicating that generalist clades can be adapted to resources which exist on a number of spatial scales (Fig 7).

The strong microenvironmental associations observed here may have important implications for population biology in the bacterioplankton. As recently suggested (Fraser et al. 2007), the effective population size (N_e) of particle-associated bacteria can be much smaller than the census size since colonization provides a population bottleneck. On the contrary, in exclusively free-living clades, N_e may be closer to the census size.

Because N_e determines the effect of selection and drift, attached and free-living populations may evolve under different population constraints. Attachment can also structure bacterial populations through differential rates of predation and DNA exchange (Pernthaler & Amann 2005, Polz et al. 2006). For example, chitin was recently shown to induce competence in *V. cholerae* (Meibom et al. 2005). If chitin-induced competence is a common characteristic among vibrios it could dramatically enhance rates of recombination and lateral gene transfer among zooplankton-associated populations.

While it has recently been suggested that phylogenetic lineages remain specific to macroenvironments over long evolutionary times (von Mering et al. 2007), this study demonstrates relatively frequent microenvironmental switches within a bacterial family and even within *V. splendidus* which share 99% 16S rRNA gene identity (Thompson et al. 2005). Ecologically adapted groups are likely further subdivided than is apparent from this relatively crude sampling scheme, since increased spatial and temporal resolution sampling may yield additional differentiation, and groups with few representatives in the dataset were excluded. This level of resource subdivision is particularly surprising since vibrios are a relatively small fraction of the total planktonic community in this environment (Thompson et al. 2005) although they may reach high densities on zooplankton (Heidelberg et al. 2002), etc. How other microbial taxa are partitioned in marine microenvironments is yet to be determined. However, we have recently shown that a bacterioplankton community is structured into ~500 microdiverse ribotype clusters; such clusters may constitute ecologically-differentiated populations, the question is now along which resource axes (Acinas et al. 2004). We note that this study confirms ecological differentiation for relatively divergent taxa and suggests that

ecological associations in the plankton remain stable at least over millions of years. The important exception is *V. splendidus*, for which many populations were identified. Relatively deeply diverging ecological populations contain considerable neutral sequence variation (Giovannoni & Stingl 2005). Neutral divergence was recently suggested as the explanation for many co-occurring genotypes within *V. splendidus*, each with such low average concentrations that unique traits may be ecologically (nearly) neutral (Thompson et al. 2005); however, this large genome diversity may serve as a genetic reservoir for adaptive change.

LITERATURE CITED:

Acinas SG, Anton J, Rodriguez-Valera F (1999) Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Applied and Environmental Microbiology 65:514-522

Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL, Polz MF (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430:551-554

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410

Cohan FM, Perry EB (2007) A systematics for discovering the fundamental units of bacterial diversity. Current Biology 17:R373-R386

Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM, Bandela AM, Cardenas E, Garrity GM, Tiedje JM (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Research 35:D169-D172

Crump BC, Armbrust EV, Baross JA (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. Applied and Environmental Microbiology 65:3192-3204

DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, Frigaard NU, Martinez A, Sullivan MB, Edwards R, Brito BR, Chisholm SW, Karl DM (2006) Community genomics among stratified microbial assemblages in the ocean's interior. Science 311:496-503

Doolittle WF, Papke RT (2006) Genomics and the bacterial species problem. Genome Biology 7

Eiler A, Johansson M, Bertilsson S (2006) Environmental influences on *Vibrio* populations in northern temperate and boreal coastal waters (Baltic and Skagerrak Seas). Applied and Environmental Microbiology 72:6004-6011

Fraser C, Hanage WP, Spratt BG (2007) Recombination and the nature of bacterial speciation. Science 315:476-480
Giovannoni SJ, Stingl U (2005) Molecular diversity and ecology of microbial plankton. Nature 437:343-348
Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, Chow AW (1996) HSP60 gene sequences as universal targets for microbial species identification: Studies with coagulase-negative staphylococci. Journal of Clinical Microbiology 34:818-823
Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52:696-704
Hanage WP, Fraser C, Spratt BG (2006) Sequences, sequence clusters and bacterial species. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1917-1927
Heidelberg JF, Heidelberg KB, Colwell RR (2002) Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. Applied and Environmental Microbiology 68:5498-5507
Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining Chlorophylls a, b, c1 and c2 in higher-plants, algae and natural phytoplankton. Biochemie Und Physiologie Der Pflanzen 167:191-194
Johnson ZI, Zinser ER, Coe A, McNulty NP, Woodward EMS, Chisholm SW (2006) Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science 311:1737-1740
Kiørboe T, Grossart HP, Ploug H, Tang K (2002) Mechanisms and rates of bacterial colonization of sinking aggregates. Applied and Environmental Microbiology 68:3996-4006
Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons Ltd., p 115-147
Letunic I, Bork P (2007) Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23:127-128
Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK (2005) Chitin induces natural competence in *Vibrio cholerae*. Science 310:1824-1827
Panagiotopoulos C, Sempere R, Obernosterer I, Striby L, Goutx M, Van Wambeke F, Gautier S, Lafont R (2002) Bacterial degradation of large particles in the southern Indian Ocean using in vitro incubation experiments. Organic Geochemistry 33:985-1000
Pernthaler J, Amann R (2005) Fate of heterotrophic microbes in pelagic habitats: Focus on populations. Microbiology and Molecular Biology Reviews 69:440-+
Polz MF, Hunt DE, Preheim SP, Weinreich DM (2006) Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. Philosophical Transactions of the Royal Society B-Biological Sciences 361:2009-2021
Pomeroy LR, Hanson RB, McGillivary PA, Sherr BF, Kirchman D, Deibel D (1984) Microbiology and chemistry of fecal products of pelagic tunicates - rates and fates. Bulletin of Marine Science 35:426-439
Retchless AC, Lawrence JG (2007) Temporal fragmentation of speciation in bacteria. Science 317:1093-1096

Riemann L, Winding A (2001) Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. Microbial Ecology 43:290-290

Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S, Wu DY, Eisen JA, Hoffman JM, Remington K, Beeson K, Tran B, Smith H, Baden-Tillson H, Stewart C, Thorpe J, Freeman J, Andrews-Pfannkoch C, Venter JE, Li K, Kravitz S, Heidelberg JF, Utterback T, Rogers YH, Falcon LI, Souza V, Bonilla-Rosso G, Eguiarte LE, Karl DM, Sathyendranath S, Platt T, Bermingham E, Gallardo V, Tamayo-Castillo G, Ferrari MR, Strausberg RL, Nealson K, Friedman R, Frazier M, Venter JC (2007) The Sorcerer II Global Ocean Sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. PLOS Biology 5:398-431

Santos SR, Ochman H (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. Environmental Microbiology 6:754-759

Selje N, Simon M (2003) Composition and dynamics of particle-associated and free-living bacterial communities in the Weser estuary, Germany. Aquatic Microbial Ecology 30:221-237

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences of the United States of America 103:12115-12120

Thompson JR, Pacocha S, Pharino C, Klepac-Ceraj V, Hunt DE, Benoit J, Sarma-Rupavtarm R, Distel DL, Polz MF (2005) Genotypic diversity within a natural coastal bacterioplankton population. Science 307:1311-1313

Thompson JR, Polz MF (2006) Dynamics of vibrio populations and their role in environmental nutrient cycling. In: Thompson FL, Austin B, Swings J (eds) The Biology of *Vibrios*. ASM Press, Washington DC, p 190-203

Thompson JR, Randa MA, Marcelino L, Tomita-Mitchell A, Lim E, Polz MF (2004) Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Applied and Environmental Microbiology 70:4103-4110

Vetsigian K, Goldenfeld N (2005) Global divergence of microbial genome sequences mediated by propagating fronts. Proceedings of the National Academy of Sciences of the United States of America 102:7332-7337

von Mering C, Hugenholtz P, Raes J, Tringe SG, Doerks T, Jensen LJ, Ward N, Bork P (2007) Quantitative phylogenetic assessment of microbial communities in diverse environments. Science 315:1126-1130

TABLE AND FIGURES:

Table 1 Physical and nutrient conditions of bulk samples

	Temperature °C	Chlorophyll a[1] µg/L	DOC[2] mg C/L	TDN mg N/L	NO_3+NO_2 µg N/L	NH_4 µg N/L	TDP µg P/L	PO_4 µg P/L
Spring (4/28/06)	**11**	**4.07**	**2.11**	**0.17**	**9**	**189**	**18**	**14**
Fall (9/6/06)	**16**	**6.03**	**2.28**	**0.27**	**5**	**144**	**24**	**25**

[1] measured using overnight extraction in 90% acetone (Jeffrey & Humphrey 1975)
[2] DOC= dissolved organic carbon, TDN= Total Dissolved Nitrogen, TDP= total dissolved phosphorous, all chemicals analyses were measured at the University of New Hampshire, Durham, NH

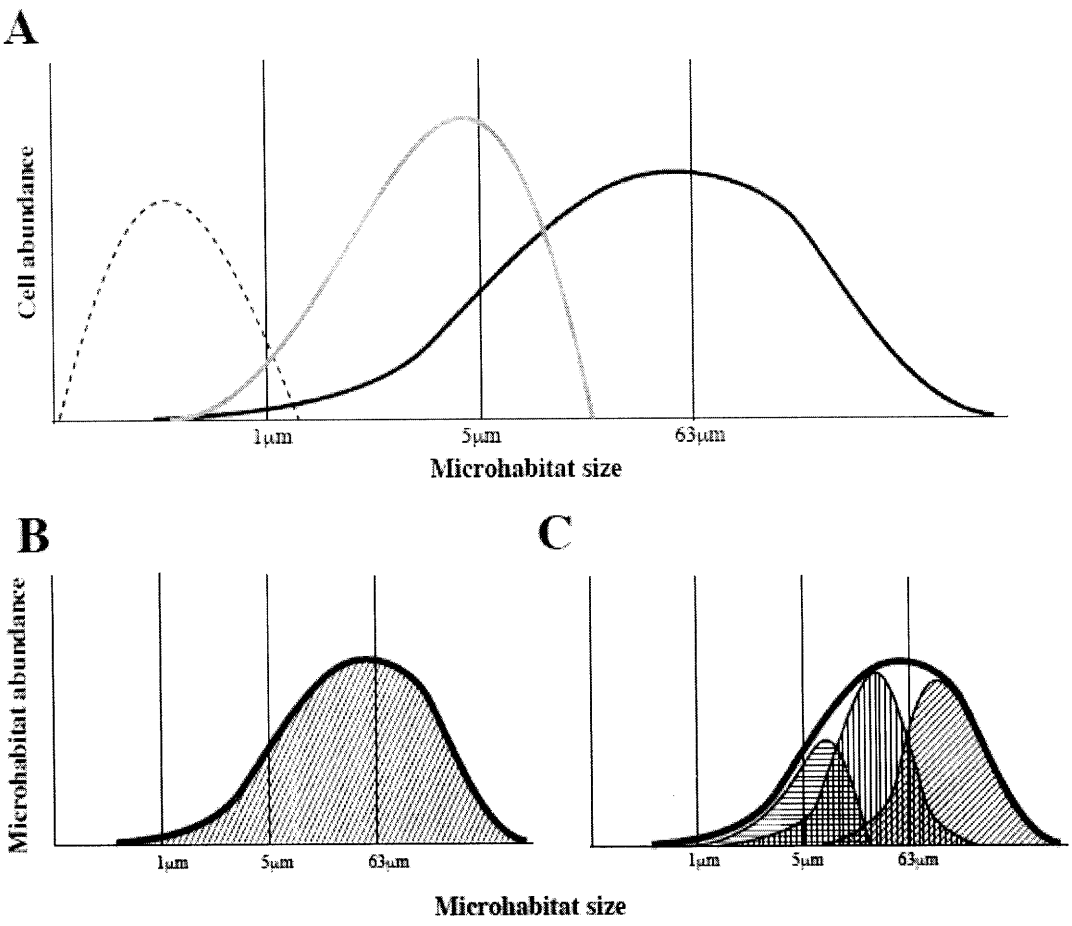


Figure 1. Alternate explanations for the distribution of bacterial clades between seawater size fractions

a The distribution of bacterial clades between size fractions indicates fraction-specific clades (dashed line) or a single clade spread over several size fractions (bold line)

b. The distribution of a specialist clade (bold) due to association with a single microhabitat that spans multiple size fractions (hatched)

c. The distribution of a generalist clade (bold) reflects adaptation to several different microhabitats of different sizes.

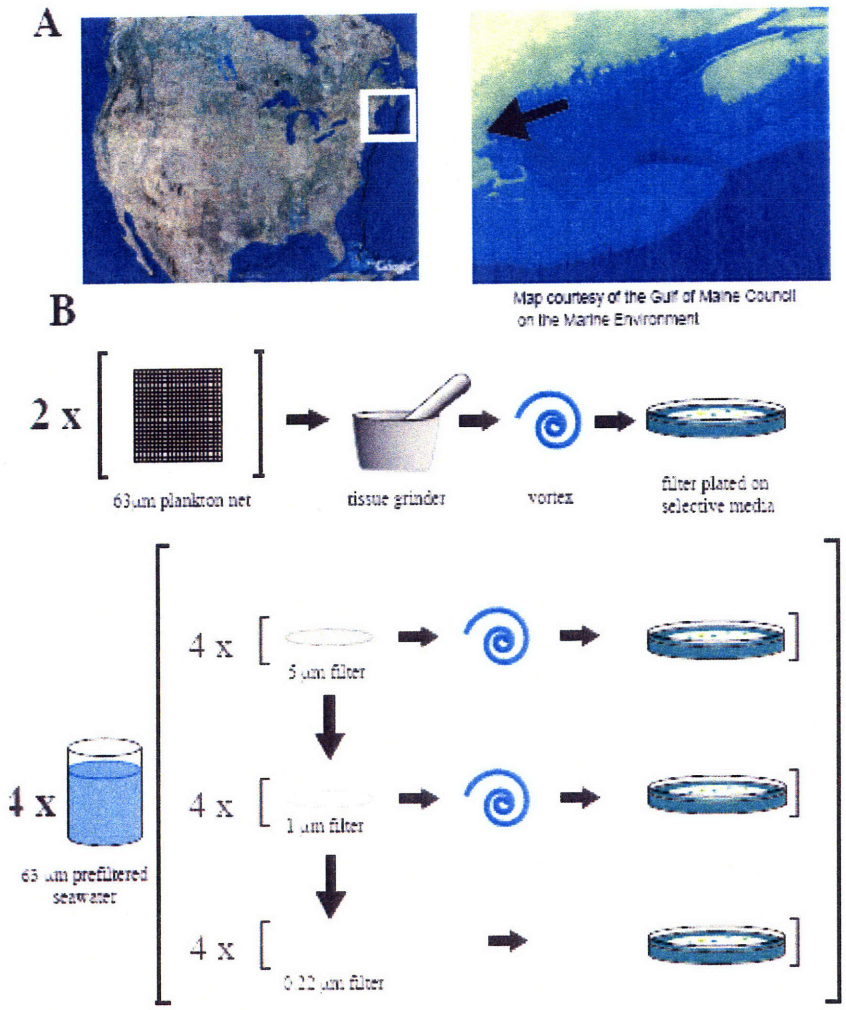


Figure 2 Depiction of site and method of *in situ* sampling of bacterial microenvironmental association.

a. Sampling location on a map of North America (left) with a white box depicting the bounds of the picture at right, the Gulf of Maine. The arrow indicates the sampling location, Plum Island Sound, MA.

b. Protocol for obtaining size fractionated bacterial seawater isolates using sequential filtration.

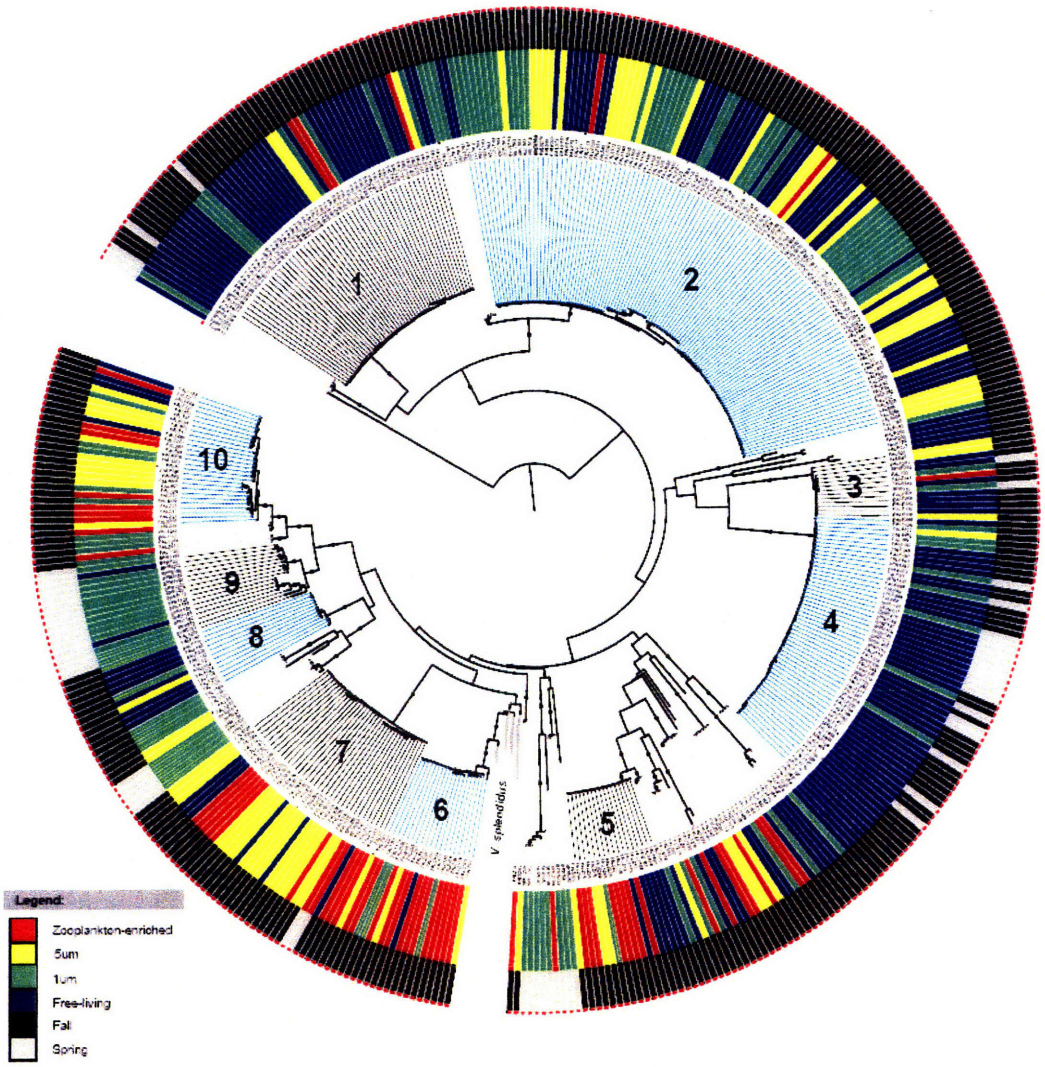
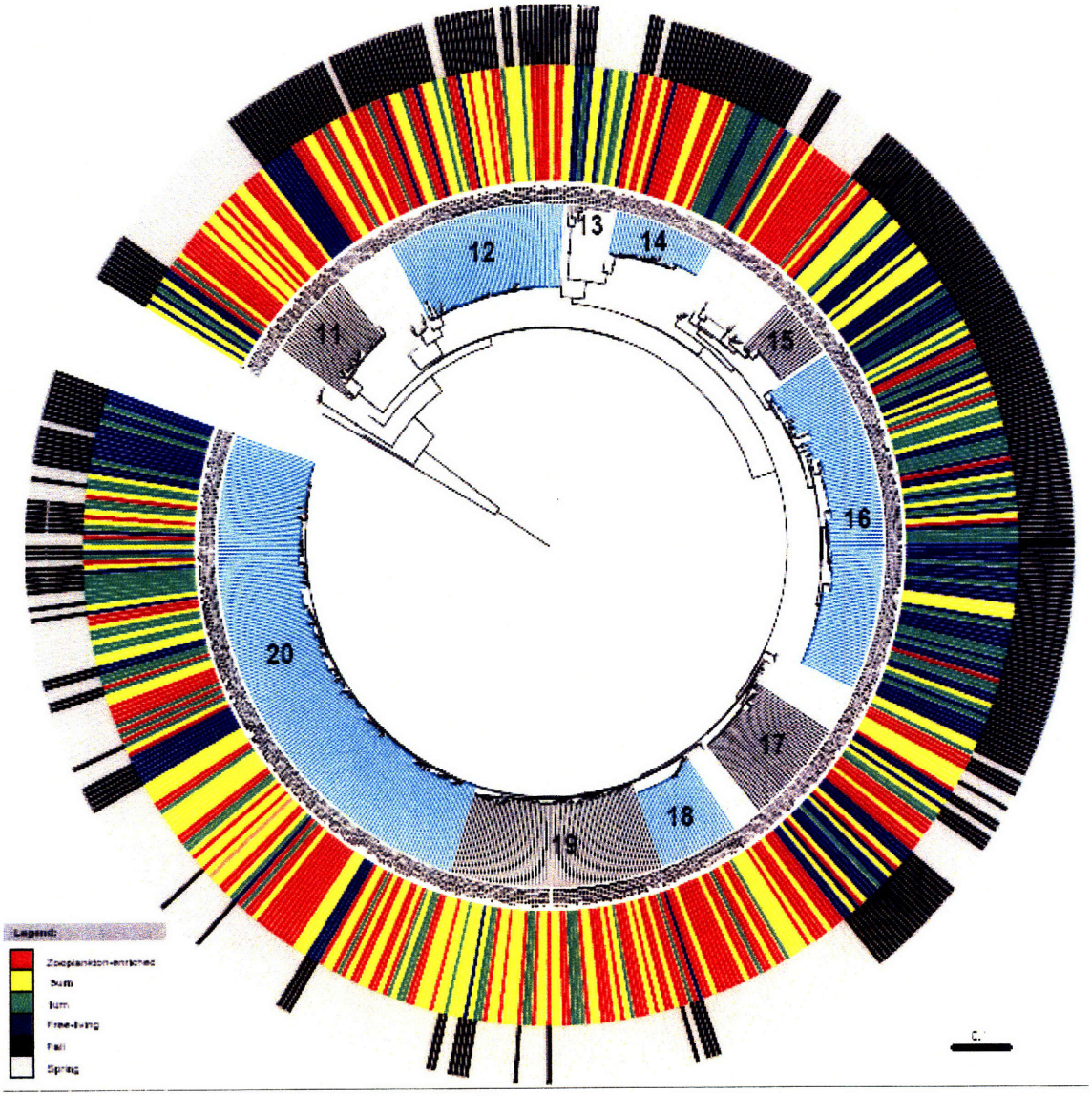


Figure 3. Phylogenetic relationships of *Vibrionaceae* isolates

a. Maximum likelihood tree based on the partial sequence of *hsp60*. Inner ring colors correspond to the size fraction of isolation, outer ring colors correspond to the season of isolation. Diamonds on the branches reflect nodes supported by >70/100 bootstrap replicates. Collapsed branches correspond to *V. splendidus* isolates which are presented in Figure 3B. Numbered and highlighted regions correspond to phylogenetic groups with strong bootstrap support (largely named species).



b. Maximum likelihood tree based on the concatenation of partial sequences of *hsp60* and *mdh*. Inner ring colors correspond to the size fraction of isolation, outer ring colors correspond to the season of isolation. Diamonds on the branchs reflect nodes supported by >70/100 bootstrap replicates. Numbered and highlighted regions correspond to phylogenetic groups identified by eye as appearing to be ecologically or phylogenetically distinct.

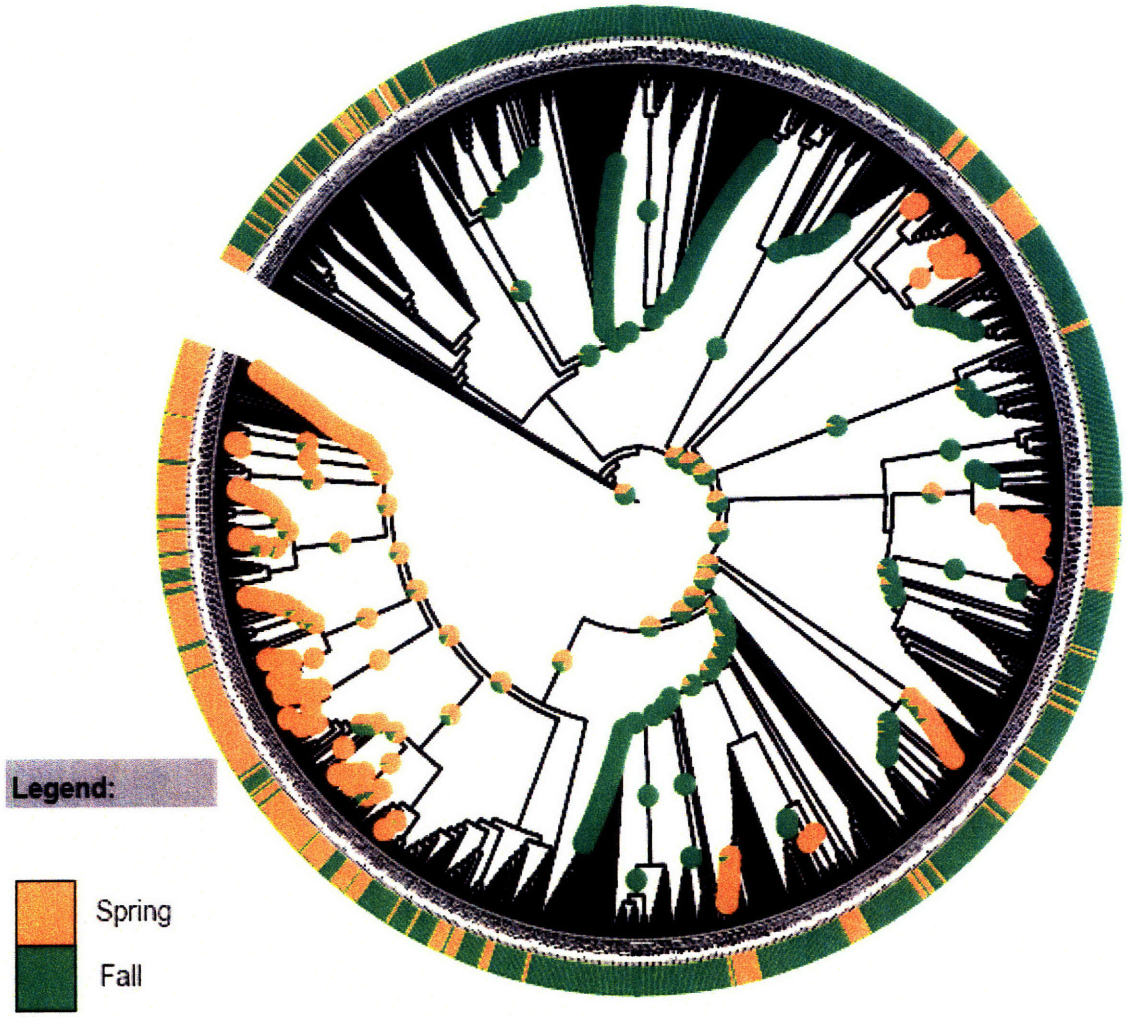


Figure 4. Testing clades for seasonal association. Fisher's exact test was used to test whether the leaves in each subtree have a distribution of seasons distinct from that the rest of the tree. spring (orange), fall (green). Nodes corresponding to significant distributions ($p<0.001$) are labeled with a pie chart showing the distribution of seasons associated with its leaves. Branch lengths are adjusted to aid readability and do not represent accurate evolutionary distances.

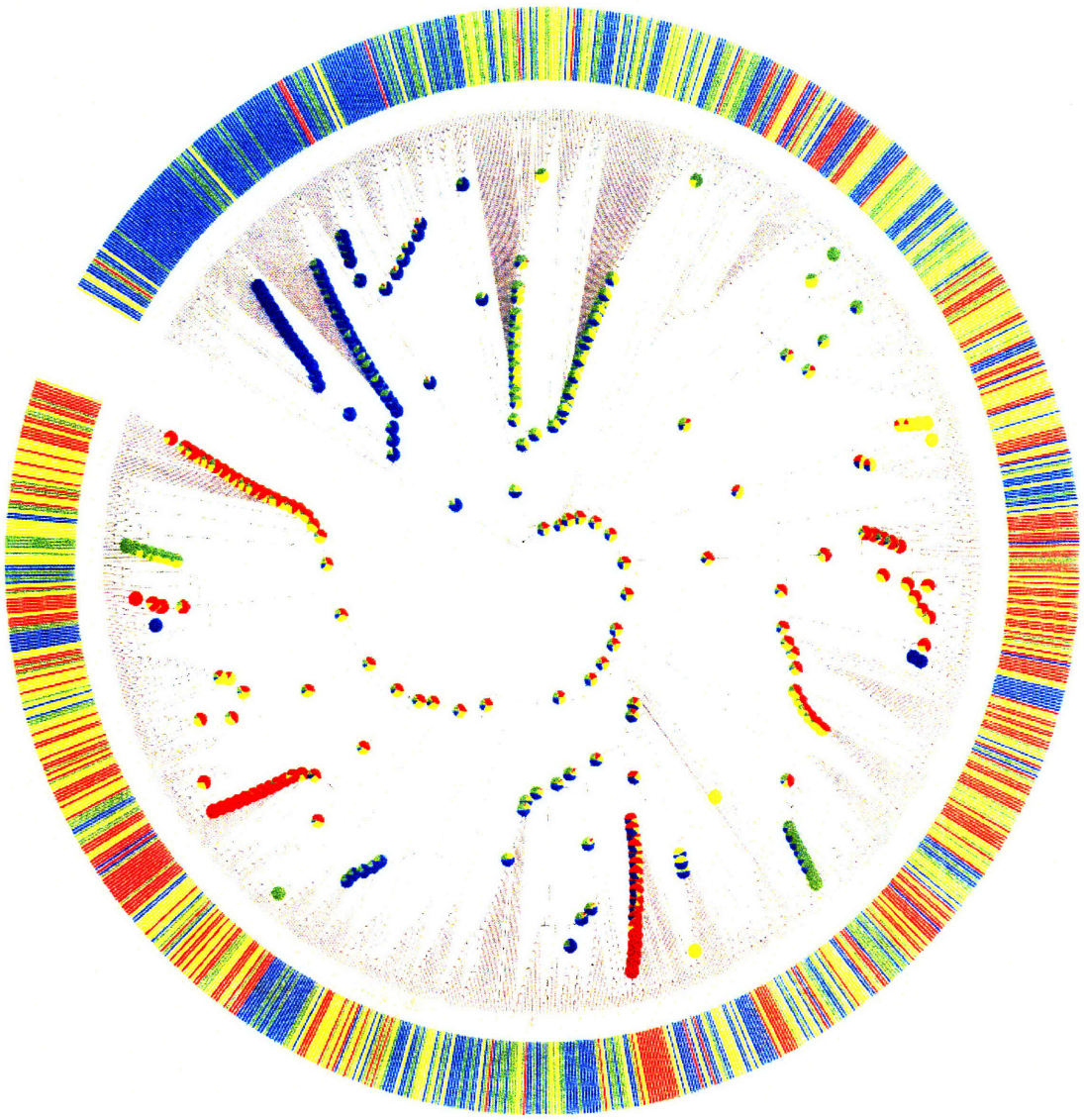


Figure 5 Testing clades for ecological association. Fisher's exact test was used to test whether the leaves in each subtree have a distribution of size fractions distinct from that the rest of the tree. Nodes corresponding to significant distribution ($p<0.001$) are labeled with a pie chart showing the distribution of size fractions associated with its leaves. Branch lengths are adjusted to aid readability and do not represent accurate evolutionary distances.

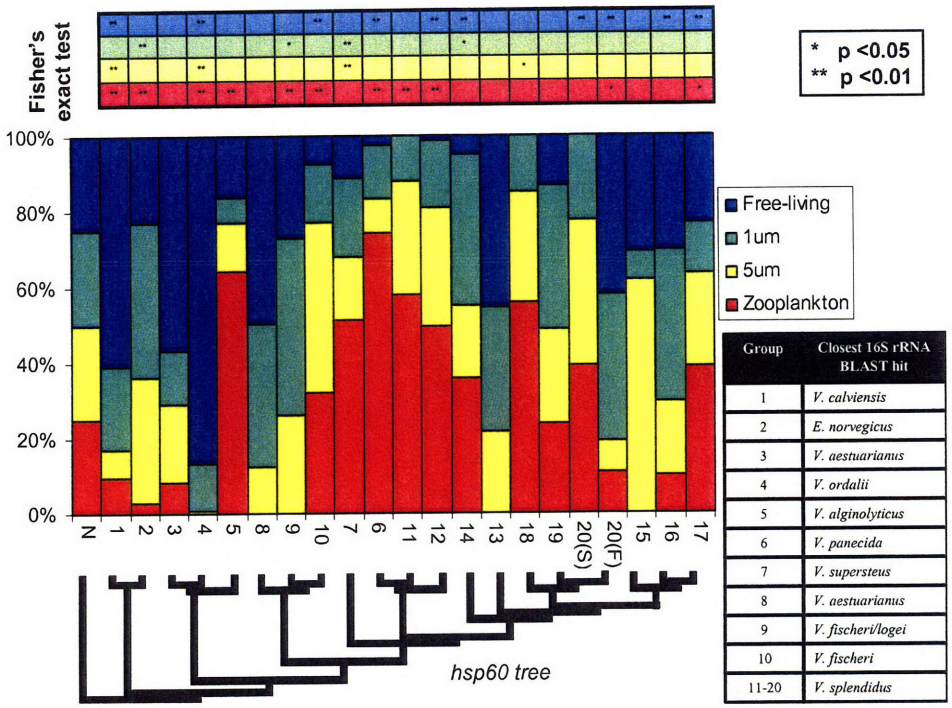


Figure 6. Summary of vibrio clades and associations with microenvironments

a. normalized graph showing the distribution of clades between different size fractions

b. Neighbor Joining ultrametric trees showing *hsp60* phylogenetic relationships between clades.

c. Fisher's exact test results for over (+) or under(-) representation in a clade. P values <0.01 are indicated by "**" while p value <0.05 and >0.01 are designated by "*".

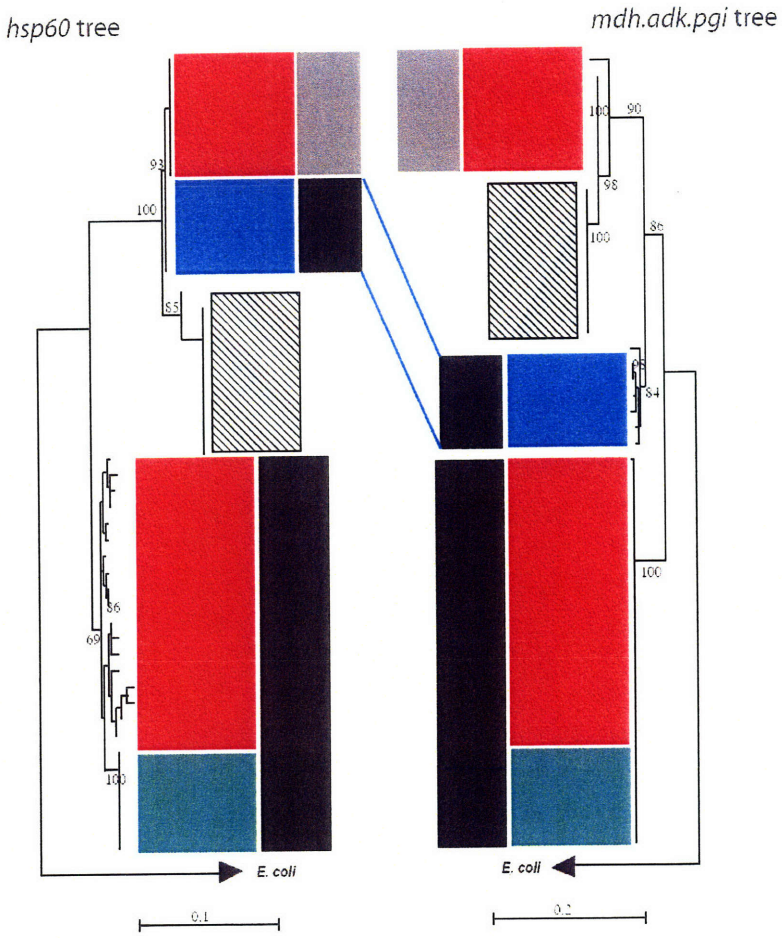


Figure 7 Comparison of phylogenies for closely related isolates.

a. Maximum likelihood tree based on partial *hsp60* gene sequence. Numbers indicate nodes with support from >70/100 bootstrap replicates

b. Maximum likelihood tree based on concatenation of partial *pgi*, *mdh* and *adk* gene sequence. Numbers indicate nodes with support from >70/100 bootstrap replicates Lines point out the bounds of discordant phylogenies in the blue/red group relative to the outgroup (hatched).

Chapter Three

Conservation of the chitin utilization pathway in the *Vibrionaceae* [1]

[1] In press *Applied and Environmental Microbiology*

with coauthors Dirk Gevers, Nisha M. Vahora, Martin F. Polz

ABSTRACT

The *Vibrionaceae* are regarded as important marine chitin degraders, and attachment to chitin regulates important biological functions; yet the degree of chitin pathway conservation in the *Vibrionaceae* is unknown. Here, a core chitin degradation pathway is proposed based on comparison of 19 *Vibrio* and *Photobacterium* genomes with a detailed metabolic map assembled for *V. cholerae* from published biochemical, genomic and transcriptomic results. Further, to assess whether chitin degradation is a conserved property of the *Vibrionaceae*, a set 54 strains from 32 taxa were tested for their ability to grow on various forms of chitin. All strains grew on N-acetylglucosamine (GlcNAc), the monomer of chitin. The majority of isolates grew on α (crab shell) and β (squid pen) chitin, and contained chitinase A (*chiA*) genes. ChiA sequencing and phylogenetic analysis suggests that this gene is a good indicator of chitin metabolism but appears subject to horizontal gene transfer and duplication. Overall, chitin metabolism appears to be a core function of the *Vibrionaceae*, but individual pathway components exhibit dynamic evolutionary histories.

INTRODUCTION

Chitin is the second most abundant biopolymer after cellulose and, particularly in the marine environment, may comprise an important source of organic carbon and nitrogen (McCarthy et al. 1997, Aluwihare et al. 2005). Chitin is composed of chains of *N*-acetylglucosamine (GlcNAc) residues arranged in antiparallel (α) or parallel (β)

configurations. Both forms are found in the marine environment: β-chitin is produced by diatoms and is a major component of squid pens while the more recalcitrant α form makes up crustacean shells. While the ability to grow on the chitin monomer GlcNAc is thought to be widespread among bacteria (Riemann & Azam 2002),likely because it is a component of peptidoglycan, chitinoclastic ability is limited to a number of bacterial groups within the Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Cottrell et al. 2000).

We focus here on bacteria of the family *Vibrionaceae*, which includes *Vibrio* and *Photobacterium* as its primary genera, since they have been extensively studied with respect to growth on chitin. Vibrios are ubiquitous and easily cultivatable members of the coastal marine bacterioplankton community; as obligate heterotrophs, they can utilize a wide range of carbon sources for energy (Thompson & Polz 2006). Moreover, there has been growing interest in the effect of chitin on pathogenicity and the regulation of gene expression in the vibrios (Meibom et al. 2004). Attachment of pathogenic *V. cholerae* to chitinous zooplankton may not only provide a nutrient-rich habitat (Heidelberg et al. 2002), but could play a role in enhancing human disease transmission (Colwell 1996, Huq et al. 2005). Chitin has been shown to change the physiology of the vibrios by inducing competence (Meibom et al. 2005), upregulating attachment/ colonization proteins involved in pathogenesis (Kirn et al. 2005, Reguera & Kolter 2005), and increasing survival during temperature stress and exposure to stomach acid (Nalin et al. 1979, Amako et al. 1987). Thus chitin has a strong influence on the growth and physiology of vibrios. It is estimated that chitin can support up to 10% of marine bacterial production (Kirchman & White 1999) and it has been speculated that the

ubiquity of the vibrios can be explained by their ability to degrade chitin (Riemann & Azam 2002).

Chitin degradation is achieved by a complex pathway including multiple chitinases (Svitil et al. 1997); however, most studies of chitinase diversity in the aquatic environment focus on the distribution of the extracellular endochitinase 'chitinase A' (*chiA*) since this gene is thought to be conserved in both Proteobacteria and Firmicutes (Cottrell et al. 2000, LeCleir et al. 2004). Additionally, for organisms with multiple chitinases, *chiA* appears to have the highest expression and activity in response to crab shell chitin (Svitil et al. 1997, Orikoshi et al. 2005), suggesting that it may be the most active in the environment and thus is a potentially useful indicator of chitinoclastic ability.

In this study, we propose a chitin degradation pathway for *V. cholerae* by incorporating bioinformatic predictions, biochemical studies and expression data. We then ask how this pathway maps onto sequenced *Vibrio* and *Photobacterium* genomes to determine whether there is a conserved chitin degradation core. Second, we evaluate how widespread chitin metabolism is among *Vibrionaceae* isolates, which cover the co-existing diversity in temperate coastal waters (Thompson et al. 2004, Thompson et al. 2005b), by assaying growth on different forms of chitin (α or β). Third, we explore conservation of the chitin degradation pathway in strains using the *chiA* gene, and we evaluate its evolutionary dynamics in the *Vibrionaceae*.

MATERIALS AND METHODS

In silico analysis of the chitin pathway

The annotated protein and DNA sequences from *Vibrionaceae* genome sequences (complete and unfinished) were obtained from the National Center for Biotechnology Information (NCBI) Web site on May 10, 2007; *Shewanella oneidensis* MR-1 was included as an outgroup. A list of genomes and their accession numbers is contained in Table S1. OrthoMCL (Li et al. 2003) was used to identify orthologous groups (families) in the sequenced genomes. This program takes an all-against-all BLASTp as input, and defines putative pairs of orthologs or recent paralogs based on reciprocal best BLAST hit. Recent paralogs are identified as genes within the same genome that are more similar to each other than any sequence from another genome. OrthoMCL then converts the reciprocal BLASTp values to a normalized similarity matrix that is analyzed by a Markov Cluster algorithm (MCL). In return, the MCL yields a set of clusters, with each cluster containing a set of orthologs and/or recent paralogs. OrthoMCL was run with a BLAST e-value cut-off of 1e-6, and an inflation parameter of 1.5 . Families related to chitin metabolism were obtained from the chitin pathway defined in *V. cholerae* (Fig. 1) and by using a keyword search for "chitin" in the annotated genomes. For each of the chitin-related families, the orthologous genes were identified for all *Vibrionaceae* genomes with OrthoMCL; and a presence / absence profile was constructed. A complete list of locus tags and gene locations are provided in Table S2.

Vibrionaceae genome phylogeny

A "whole genome phylogeny" was generated for the annotated genomes by taking 100 randomly selected, single-copy genes present in all genomes. These were aligned in

MUSCLE (Edgar 2004) and poorly aligned regions were removed; this concatenated alignment was used to estimate maximum likelihood phylogenetic tree using the PhyML program with 100 bootstrap replicates (Guindon & Gascuel 2003) with options "0 i 1 100 GTR e e 4 e BIONJ y y".

Growth assays

Vibrionaceae strains were tested for growth on GlcNAc, α, and β chitin as the nitrogen and carbon nutrient sources (Table 1). Cultures were grown over night in 0.25x 2216 medium (Difco) and diluted 1:100 in minimal media containing chitin substrates. The minimal medium was derived from that used in Meibom et al. (2005): 234 mmoles /L (brackish) or 428 mmoles/L (marine) NaCl, 27.5 mmoles/L $MgSO_4$, 4.95 mmoles/L $CaCl_2$, 5.15 mmoles/L KCl, 0.07 mmoles/L $Na_2B_4O_7$, 0.187 mmoles/L K_2HPO_4, 1x "K" trace metals (Keller et al. 1987), 50 mmoles/L HEPES, pH 7.4 and supplemented with a filter-sterilized vitamin mixture (Newman et al. 1997). β chitin was isolated from squid pen (*Loligo pealeii*) by treatment with 1 mol/L NaOH for 5 hours to remove protein followed by extensive washing to remove residual base (Chaussard & Domard 2004). Tubes containing media (15 ml) were supplemented with 25 mmoles/L GlcNAc, or 0.05 g of either crab shell α-chitin (Sigma) or β-chitin. Strains were grown at room temperature (~22°C) with shaking at 150 rpm, and growth was assessed every two days. A starting OD_{600} value of less than or equal to 0.01 that increased to a value of at least 0.1 by day 30 was scored as positive.

PCR amplification and phylogeny of chiA

PCR primers designed to target all known proteobacterial *chiA* genes were used to amplify and sequence this gene in vibrio isolates: chiAf (GGN GGN TGG CAN YTN WSN GAY CCN TT) (Cottrell et al. 2000) and chiAr (ATR TCN CCR TTR TCN GCR TC) (LeCleir et al. 2004). DNA was obtained using a DNA extraction kit (Gentra) or Lyse'N Go (Pierce). The PCR mixture contained 1 μmol/L final concentration of chiAf and chiAr, 0.75 U Jumpstart Taq (Sigma), 200 μmol/L dNTPs, and 1x buffer. The PCR reactions were thermocycled as follows: 3 min at 94°C, followed by 35 cycles of (1 min at 94°C, 1 min at 50°C, 2 min 72°C) with a final 6 min extension at 72°C. Alternate primers targeting *P. profundum chiA*-family sequences were designed based on the sequences of strains SS9 and 3TCK and contain all codon degeneracies. Primers Pprof_chiAf (AAR CAY TTY CCN GAR ATG GCN GC) and Pprof_chiAr (TCR TTR TCN ACD ATR TAY TGN GC) were amplified as above.

An alignment, including *chiA* gene sequences from diverse isolates, previously analyzed taxa (LeCleir et al. 2004) and whole genomes, was prepared using Clustal and refined manually. Ambiguously aligned regions were excluded, yielding an alignment of 603 nucleotide positions. The maximum likelihood tree was constructed using PHYML under the GTR model with estimation of all parameters and generation of 100 bootstraps (Guindon & Gascuel 2003).

Additional gene sequencing

The partial 16S rRNA gene was amplified as described (Thompson et al. 2005b) and identified based on similarity to database sequences (Altschul et al. 1990). For a

limited subset of isolates adenylate kinase (*adk*) and malate dehydrogenase (*mdh*) sequences were amplified as described previously (Santos & Ochman 2004).

RESULTS AND DISCUSSION

The chitinolytic pathway in *V. cholerae*

The chitinolytic system in the vibrios channels chitin monomers into the central metabolism as fructose-6-P, acetate, and ammonium (Keyhani & Roseman 1999). We refine previous representations of the chitinolytic pathway (Park et al. 2002b) by incorporating literature data on biochemical experiments, microarray expression data, and bioinformatic predictions to fill gaps in the pathway related to chitobiose metabolism and identify a core set of genes which are responsible for chitin degradation in vibrios.

Figure 1 depicts the proposed chitin catabolic cascade in *V. cholerae* beginning with the break down of chitin polymer into oligomers by extracellular chitinases, labeled **1**. These genes are assumed to have differential activity or regulation and act collectively to degrade chitin into $(GlcNAc)_{n>2}$ oligosaccharides (Svitil et al. 1997, Orikoshi et al. 2005), which are transported into the periplasmic space via a specific porin **2** (Keyhani et al. 2000). The monomer GlcNAc and dimer *N,N'* diacetylchitobiose are thought to enter the periplasm by non-specific porins. Once in the periplasm, chitin oligosaccharides are degraded by periplamic chitinodextrinases **3** (Keyhani & Roseman 1996b) and β-*N*-acetylglucosaminidases **4** (Keyhani & Roseman 1996a) to $(GlcNAc)_{1,2}$. $(GlcNAc)_2$ is transported across the inner membrane by **5** an ABC-type transporter (Li & Roseman 2004), whereas GlcNAc can be transported into the cytosol and phosphorylated via **8,** a

PTS transporter (Bouma & Roseman 1996). In the cytosol, $(GlcNAc)_2$ is converted into 2(GlcNAc-6-P) by **6** a *N,N'*-diacetylchitobiose phosphorylase (Park et al. 2000) , **7** a GlcNAc-1P-mutase (Li & Roseman 2004), and a predicted GlcNAc-specific ATP-dependent kinase (gene not identified) (Bassler et al. 1991). The GlcNAc-6-P generated either during uptake by the PTS or by the *N,N'*-deacetylchitobiose phosphorylase pathway is converted into fructose-6-P via the action of **9** a N-acetylglucosamine-6-phosphate deacetylase and **10** a glucosamine -6-phosphate deaminase (Heidelberg et al. 2000).

Complete degradation of chitin must also take into account the assimilation of deacetylated residues (GlcN), which can comprise up to a sixth of the residues in natural forms of chitin (Muzzarelli 1973). Here, we propose a mechanism by which GlcN could be incorporated into the chitin catabolic cascade. Recently a set of genes annotated as a cellobiose PTS transporter **12** (VC1281-VC1286) was demonstrated to transport $(GlcN)_2$ into the cytosol (Meibom et al. 2004). An adjacent gene (VC1280) was also upregulated upon addition of $(GlcN)_2$ and has a predicted deacetylase function **11**, suggesting it converts GlcN-GlcNAc to $(GlcN)_2$. Once in the cytoplasm the β1-4 linkage between the glucosamine residues could be broken by enzyme **13** currently characterized as a cellobiase (Park et al. 2002a). We reannotate this gene as a chitobiase as *V. cholerae* does not grow on cellobiose and both substrates consist of β1-4 linked glucose. Further, this gene is upregulated by growth on chitin (Meibom et al. 2004) and is adjacent to components of the chitin metabolic pathway. The cytoplasmic GlcN can be phosphorylated by **14** an ATP-dependent glucosamine kinase (Park et al. 2002b) and converted to fructose-6-phosphate **10**. The proposed chitin utilization scheme described

above identifies a predicted chitin degradation core; the question is how well conserved is this pathway in the vibrios?

Distribution of chitin pathway genes in *Vibrionaceae* genomes

The conservation of the chitin degradation pathway in the sequenced *Vibrionaceae* genomes suggests that chitin metabolism is an ancestral feature of the vibrios (Fig. 2). In Figure 2, the left panel depicts the phylogenetic relationships of the sequenced genomes, which demonstrates that gene presence/absence in the right panel has a phylogenetic context (e.g. the second copy of chitinodextrinase is shared among all *V. cholerae* genomes, but not other isolates). The chitin degradation genes identified in *V. cholerae* (Fig. 1), appear to be almost universally conserved with homologs identified for 91% of core gene matrix positions in sequenced genomes (Fig. 2). The genes in *V. cholerae,* which are not well conserved in other genomes, include the second copy of a GlcNAc-6-P deacetylase, an alternative chitinase (VC1952) and the $(GlcN)_2$ PTS transporter. We note that the *V. angustum* S14 whole genome phylogeny and 16S rRNA gene sequence place this strain within the genus *Photobacterium*, and it is included in this group for subsequent analyses. Gene families annotated with chitin-related functions and present in at least two genomes are also shown in Fig. 2; these genes have a spotty distribution in the *Vibrionaceae* genomes, suggesting that outside of the chitin degradation core, there is tremendous gene content flexibility.

There is also evidence for several gene duplications. For example, the chitodextrinases (labeled **3** in Fig. 1) contain two orthologous copies in all *V. cholerae* genomes and in *P. profundum* SS9; although one of the copies (VC1073) is not

upregulated in the presence of chitin (Meibom et al. 2004), suggesting that this gene may no longer be active in chitin degradation. Additionally, the *chiA* gene family has two copies in *Photobacterium* sp. SKA34 and *V. angustum* one of which clusters with the vibrios while the second is more closely related to other Proteobacteria (Fig. 3). However, multiple copies of PTS genes (**8** &**12** in Fig. 1) may reflect similarities between transporters for different substrates rather than multiple copies of the same gene.

Growth of *Vibrionaceae* environmental isolates on chitin substrates

Although the genome analysis suggests chitin utilization is a universal characteristic among the *Vibrionaceae*, a previous study had indicated that growth on chitin was spottily distributed among *Vibrionaceae* isolates (Ramaiah et al. 2000). Therefore, more diverse set of isolates was tested for growth on α and β chitin as well as GlcNAc, the monomer of chitin. Growth on GlcNAc is common among marine bacteria, even among those not capable of metabolizing chitin (Yang et al. 2006). Indeed, all 54 *Vibrionaceae* strains assayed grew on GlcNAc as the sole nitrogen and carbon nutrient, including the few strains which did not grow on chitin and appeared to lack *chiA* genes (e.g. *V. halioticoli, V. hispanicus*) (Table 1). This suggests that growth on GlcNAc is not a good indicator of chitin metabolism and is consistent with the previously suggested GlcNAc uptake by the PTS system, which is independent of chitin degradation (Fig. 1).

The majority of isolates also grew on both α and β chitin, although ten strains grew only on the more enzymatically accessible β form. Overall, the broad distribution of chitin metabolism suggests that chitin degradation is indeed an ancestral capability of the vibrios. However, several isolates were incapable of chitin degradation (Table 1),

corroborating that it is not a universally conserved characteristic within the vibrios, and that strains within a family may have alternate lifestyles. However, the fraction of isolates which displayed growth on chitin was much higher than reported in the previous study (Ramaiah et al. 2000), and we attribute this to more complete media containing trace metals and vitamins. Several isolates, including both *V. ordalii* strains, *V. ichthyoenteri* and *V. calviensis*, produced a yellow pigment when attached to chitin but not when grown on rich media, glucose or GlcNAc, indicating that chitin or perhaps biofilm growth regulated pigment production.

Diversity of chitinase A among *Vibrionaceae*

The *chiA* gene fragments amplified from stains in Table 1 were sequenced and found to be highly divergent, with a maximum nucleotide divergence of 55% within the genus *Vibrio* and compared to 22% for *recA* (Thompson et al. 2005a) and ~10% for the 16S rRNA gene within the *Vibrionaceae* (Kita-Tsukamoto et al. 1993). The photobacterial *chiA* sequences are even more diverse with the second copy of the strain S14 and SKA34 *chiA* genes grouping with non-vibrio Proteobacteria (Fig. 3); while the *P. profundum chiA* family genes share only ~30% amino acid identity with other vibrio sequences. The majority of the *Vibrionaceae* strains form a large clade albeit without strong bootstrap-support (Fig. 3); although, the *Enterovibrio* and *V. fischeri* sequences are distinct from this large cluster.

A positive *chiA* PCR assay was a good predictor of chitin metabolism; however several photobacteria and vibrio strains gave negative PCR results but still grew on chitin. Indeed, the *P. profundum* genomes harbor highly divergent sequences (Table S2),

which are distinct from the other vibrio *chiA* sequences but contain the conserved catalytic site motif suggesting chitinase activity (LeCleir et al. 2004). Because the "universal" proteobacterial *chiA* primers do not match these *P. profundum chiA* sequences, new primers were designed for the divergent *chiA* genes (Table S2). However, these new primers did not capture additional *chiA* sequences in strains positive for growth on chitin; suggesting that *chiA* is either not necessary for chitin degradation or more diverse than previously anticipated. The second possibility is supported by phylogenetic analysis using additional genes (*hsp60, mdh* and *adk*) for five *Vibrionaceae* isolates, which grew on chitin but had negative PCR results for *chiA*. Four of the strains, with 16S rRNA gene sequences most similar to *P. damselae* and *P. phosphoreum,* formed two deep clades within the photobacteria distinct from the sequenced genomes (Fig. S1). Given that the sequenced photobacteria genomes contain divergent *chiA* sequences these additional clades (Fig. S1) may harbor highly differentiated *chiA* genes. This is an indication that even apparently core chitin-degradation genes are subject to duplication and transfer.

The use of *chiA* to identify chitin degraders (Cottrell et al. 2000, Ramaiah et al. 2000, LeCleir et al. 2004) is problematic; as even within a single bacterial family, the *chiA* gene family is too divergent to capture with PCR primers. Additionally, there is evidence for lateral gene transfer (LGT) or duplication of this gene, which will make developing relationships with the organismal phylogeny difficult (Cottrell et al. 2000). The phylogeny of the *chiA* gene suggests several other instances of LGT (Fig 3); the most obvious is the placement of alpha proteobacterial sequences in a node within the *Enterovibrio* group that has a well-supported bootstrap value. While some alpha

Proteobacteria strains contain the pathways to assimilate GlcNAc, chitinase-like sequences have not been observed thus far in sequenced genomes (Yang et al. 2006). Moreover, Cottrell et al (2000) found that the *chiA*-containing alpha Proteobacteria isolates did not grow on chitin, suggesting a non-functional chitinoclastic pathway, potentially a hallmark of LGT into a strain without a complete metabolic pathway. Perhaps the chitinase gene in these strains has taken on another role, such as serving as a chitin attachment protein. Less well supported evidence of gene transfer, includes the presence of a second *chiA* family gene in *Photobacterium* SKA34 and *V. angustum* S14 more closely related to non-vibrio Proteobacteria; and several gamma proteobacterial sequences that cluster within the vibrios (Fig. 2 and 3). Although *chiA* appears subject to lateral transfer and/or duplication, there is no other gene that serves as a good indicator of growth on chitin, alternate exochitinases are either not present in all sequenced genomes (Fig. 2) or are not upregulated in the presence of chitin (Fig. 1). Additional genome sequencing in the photobacteria may reveal alternate genes/pathways of chitin metabolism.

Sequences were submitted to Genbank with accession numbers EU177043-EU177094.

Acknowledgements:

Squid pens were provided by the Marine Resources Center of the Marine Biological Laboratory, Woods Hole MA. Thanks to S.P. Preheim who sequenced additional housekeeping genes. This research was funded by the NSF-NIEHS co-sponsored Woods Hole Center for Ocean and Human Health, NSF-OCE, the DOE Genomes to Life

program, and the Moore Foundation. DEH also acknowledges graduate fellowships from NSF and the Whitaker Foundation. DG is indebted to the Fund for Scientific Research – Flanders (Belgium) for a position as postdoctoral fellow and research funding.

Name (BLAST identity)[1]	Strain	chiA PCR[2]	Growth assays[3]			Source[4]
			GlcNAc	α-chitin	β-chitin	
P. damselae (99%)	12H05	-	+	+	+	(37)
P. kishitanii (95%)	14H04	-	+	+	+	(37)
P. phosphoreum (96%)	14E11	-	+	+	+	(37)
P. profundum (96%)	14G04	-	+	+	+	(37)
P. profundum (99%)	7A02	-	+	-	-	(37)
V. aestuarianus (99%)	12C03	+	+	+	+	(37)
V. alginolyticus	PWH3a	+	+	-	+	
V. alginolyticus (99%)	14C03	+	+	+	+	(37)
V. alginolyticus (99%)	12G01	+	+	+	+	(37)
V. anguillarum	ATCC 14181	+	+	+	+	KB
V. calviensis (99%)	FALF182	+	+	+	+	IBYC
V. cholerae	0395	+	+	+	+	JM
V. cholerae	VO-146	+	+	-	+	JM
V. cholerae (99%)	OP3D	+	+	+	+	OP
V. cholerae (99%)	OP7F	+	+	+	+	OP
V. cholerae 569B	ATCC 25870	+	+	+	+	JM
V. cholerae E7946	ATCC 55056	+	+	+	+	JM
V. fischeri (98%)	14A09	+	+	+	+	(37)
V. fischeri (98%)	14A08	+	+	-	-	(37)
V. fischeri (99%)	14C05	+	+	+	+	(37)
V. fischeri (99%)	7H01	-	+	-	-	(37)
V. fortis (99%)	12F11	+	+	+	+	(37)
V. furnissi (99%)	12F04	+	+	+	+	(37)
V. halioticoli (97%)	7A03	-	+	-	-	(37)
V. halioticoli (97%)	7H03	-	+	-	-	(37)
V. halioticoli (98%)	1C10	-	+	-	-	(37)
V. halioticoli (99%)	1A06	-	+	-	-	(37)
V. halioticoli (99%)	1A07	-	+	-	-	JM
V. harveyi	B392	+	+	+	+	KB
V. hispanicus (98%)	FALF230	-	+	-	-	IBYC
V. ichthyoenteri (94%)	FALF124	+	+	+	+	IBYC
V. lentus (98%)	12B10	+	+	-	+	(37)
V. logei	ATCC 35077	+	+	-	+	KB
V. logei (99%)	7A08	-	+	-	+	(37)
V. metschnikovii (99%)	OP5F	+	+	-	+	OP
V. mytili (98%)	1B04	+	+	+	+	(37)
V. natriegens	ATCC 14048	+	+	-	+	KB
V. neptunius (98%)	FALF109	+	+	+	+	IBYC
V. ordalii	ATCC 33509	+	+	+	+	KB
V. ordalii (100%)	14C08	+	+	+	+	(37)
V. orientalis	ATCC 33434	+	+	+	+	KB
V. parahaemolyticus	ATCC 17802	+	+	+	+	ATCC
V. parahaemolyticus (97%)	1A02	-	+	-	-	(37)
V. ponticus (97%)	12D02	-	+	+	+	(37)
V. rumoiensis (95%)	1C01	+	+	-	-	(37)
V. shiloni (99%)	12F08	+	+	+	+	(37)
V. sp.	MED222	+	+	+	+	JP
V. splendidus (97%)	14F04	+	+	-	+	(37)
V. splendidus (99%)	12B01	+	+	+	+	(37)
V. splendidus biovar 2 (99%)	1C05	+	+	-	+	(37)
V. tasmaniensis (98%)	13B08	+	+	-	+	(37)
V. tubiashi	ATCC 19105	+	+	+	+	KB
V. vulnificus	"kathy"	+	+	+	+	KB
V. vulnificus	ATCC 27562	+	+	+	+	ATCC

Table 1 Growth of *Vibrio* isolates on different forms of chitin and GlcNAc

[1] Environmental isolates were named using the best BLAST (Altschul et al. 1990) hit for the partial 16S rRNA gene, the percentage identity is given in parentheses.

[2] "+"a PCR band of the correct size was amplified, "-"two or more PCR reactions failed to amplify a band of the correct size.

[3] "+" an OD_{600} value of >0.1 was reached by day 30, "-" no observable growth on the chitin substrate.

[4] Type strains used to assay chitin growth and amplify chitinase A sequences were obtained from ATCC (American Type Culture Collection), KB (Laboratory of Kathy Boetcher, University of Maine), JM (Laboratory of John Mekalanos, Harvard Medical School) JP (Laboratory of Jarone Pinhassi, Kalmar University). Environmental isolates were obtained from a previous study (Thompson et al. 2005b), and new strains were isolated from OP (Oyster Pond, Falmouth, MA) and the IBYC (Ipswich Bay Yacht Club, Ipswich, MA) as described (Thompson et al. 2005b).

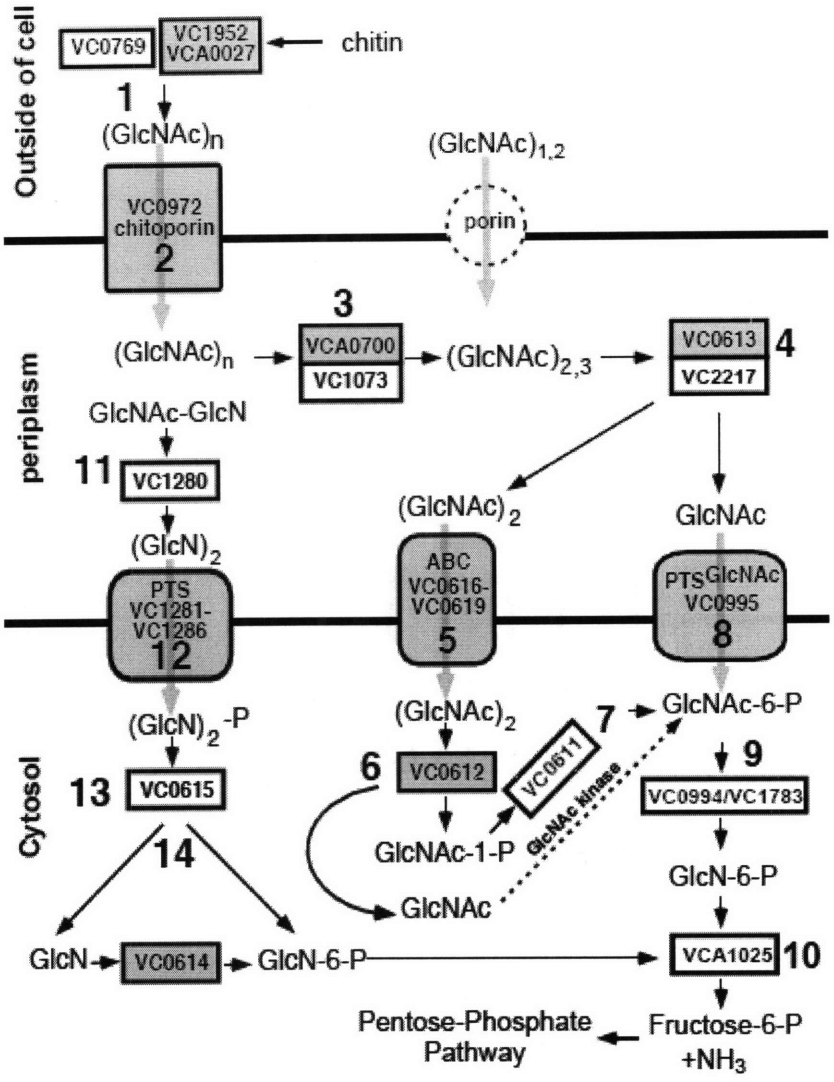


Figure 1. Schematic of the chitin catabolic cascade in *V. cholerae*, expanded from (Park et al. 2002b). Enzymes and transporters are given gene identifiers from *V. cholerae* N16961 when possible. The boxes around gene identifiers denote how functions were predicted with grey shading = biochemical evidence in the vibrios, thick outline = microarray expression data (Meibom et al. 2004), thin lines = bioinformatic prediction only and dashed lines = predicted functions based on experimental evidence.

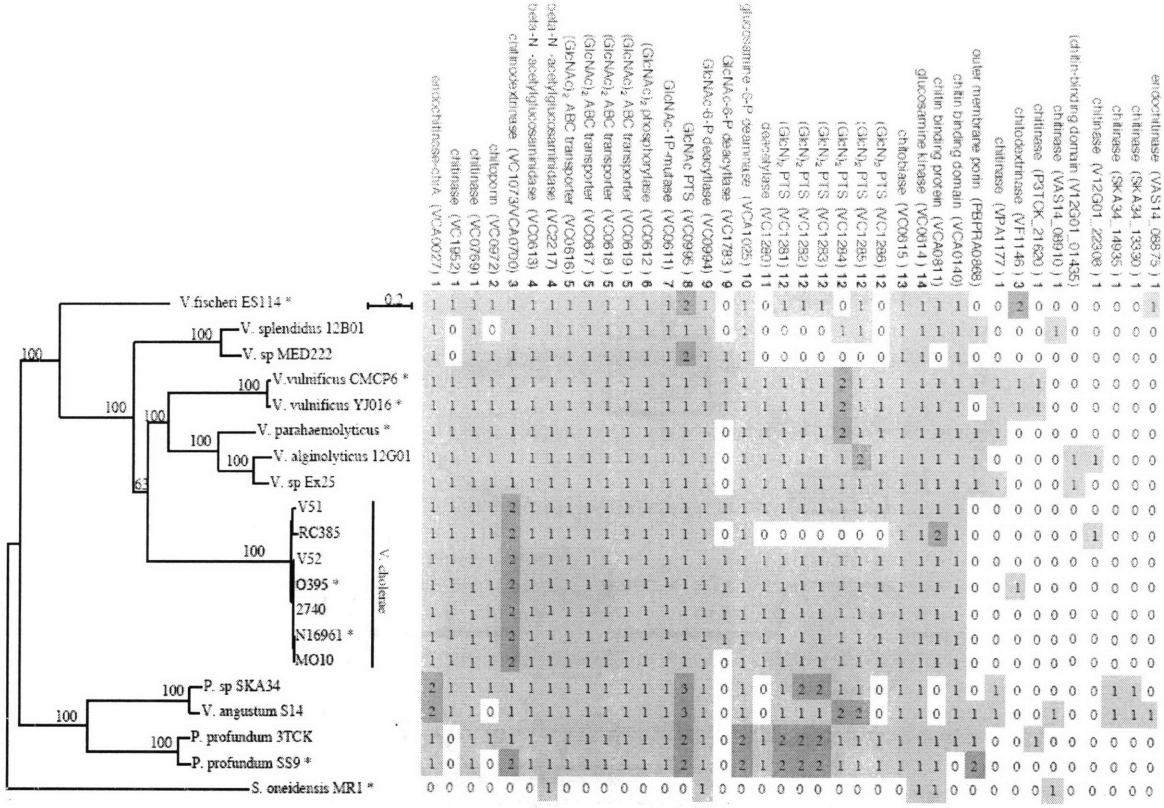


Figure 2. Distribution of predicted chitin pathway genes among *Vibrionaceae* genomes. The phylogenetic relationship is based on maximum likelihood analysis of a concatenation of 100 shared genes. Numbers at nodes represent values based on 100 bootstrap replicates. Each of the columns corresponds to a chitin-metabolism related gene family, with the family name indicating the predicted function and the number indicating the reaction or transport mechanism identifier in Figure 1, with a representative gene designation in parenthesis. The number within the box indicated the number of copies of that gene family in the corresponding genome, which is further indicated by light grey shading for one gene copy while dark grey shading indicates the presence two or more genes in that family. An * indicates a completed genome sequence.

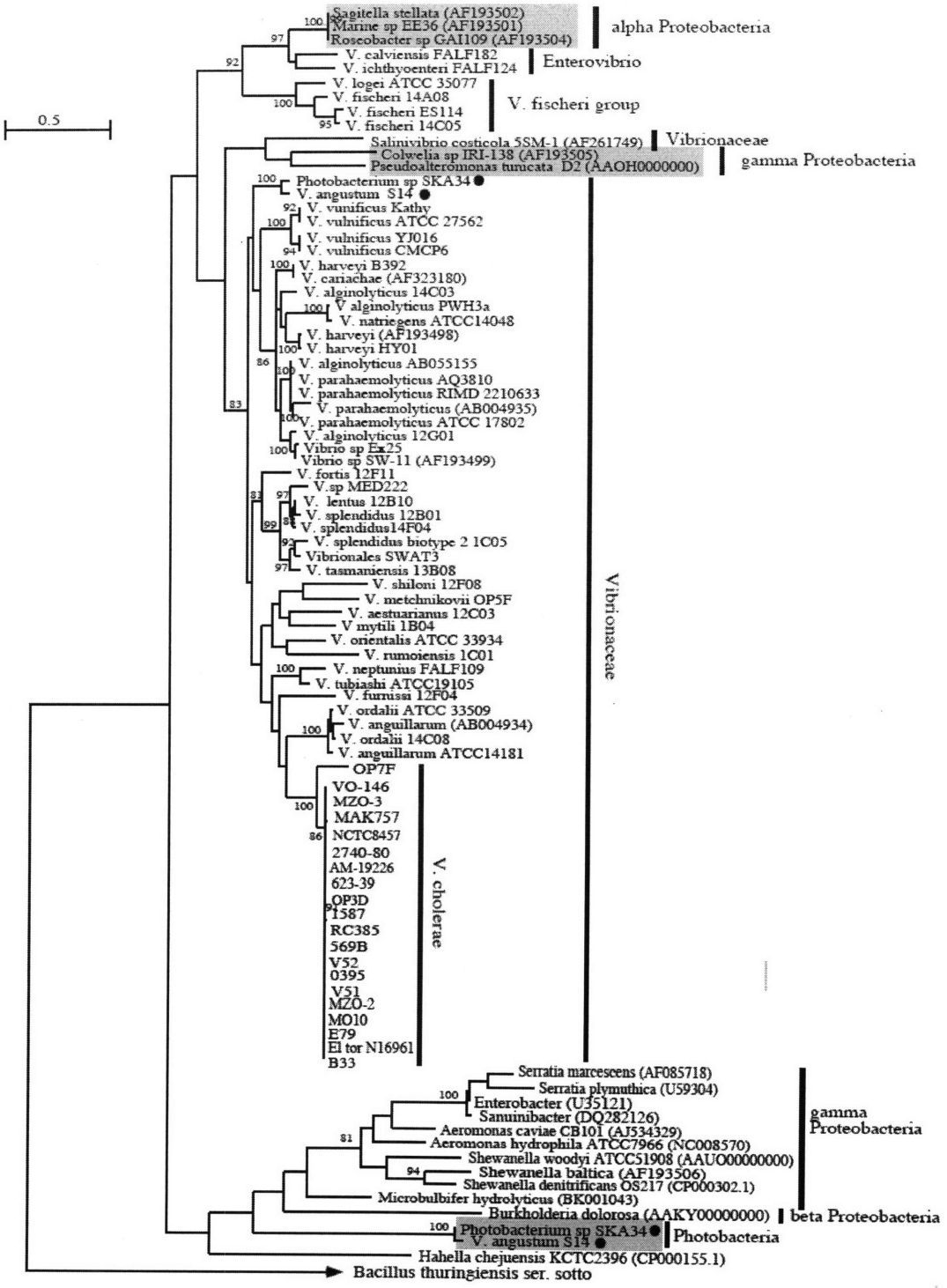

Sagitella stellata (AF193502)
Marine sp EE36 (AF193501)
Roseobacter sp GAI109 (AF193504)
alpha Proteobacteria
V. calviensis FALF182
V. ichthyoenteri FALF124
Enterovibrio
V. logei ATCC 35077
V. fischeri 14A08
V. fischeri ES114
V. fischeri 14C05
V. fischeri group
0.5
Salinivibrio costicola 5SM-1 (AF261749)
Vibrionaceae
Colwelia sp IRI-138 (AF193505)
Pseudoalteromonas tunicata D2 (AAOH0000000)
gamma Proteobacteria
Photobacterium sp SKA34
V. angustum S14
V. vunificus Kathy
V. vulnificus ATCC 27562
V. vulnificus YJ016
V. vulnificus CMCP6
V. harveyi B392
V. cariachae (AF323180)
V. alginolyticus 14C03
V alginolyticus PWH3a
V. natriegens ATCC14048
V. harveyi (AF193498)
V. harveyi HY01
V. alginolyticus AB055155
V. parahaemolyticus AQ3810
V. parahaemolyticus RIMD 2210633
V. parahaemolyticus (AB004935)
V. parahaemolyticus ATCC 17802
V. alginolyticus 12G01
Vibrio sp Ex25
Vibrio sp SW-11 (AF193499)
V. fortis 12F11
V.sp MED222
V. lentus 12B10
V. splendidus 12B01
V. splendidus14F04
V. splendidus biotype 2 1C05
Vibrionales SWAT3
V. tasmaniensis 13B08
V. shiloni 12F08
V. metchnikovii OP5F
V. aestuarianus 12C03
V mytili 1B04
V. orientalis ATCC 33934
V. rumoiensis 1C01
V. neptunius FALF109
V. tubiashi ATCC19105
V. furnissi 12F04
V. ordalii ATCC 33509
V. anguillarum (AB004934)
V. ordalii 14C08
V. anguillarum ATCC14181
OP7F
VO-146
MZO-3
MAK757
NCTC8457
2740-80
AM-19226
623-39
OP3D
P1587
RC385
569B
V52
0395
V51
MZO-2
MO10
E79
El tor N16961
B33
V. cholerae
Vibrionaceae
Serratia marcescens (AF085718)
Serratia plymuthica (U59304)
Enterobacter (U35121)
Sanuinibacter (DQ282126)
Aeromonas caviae CB101 (AJ534329)
Aeromonas hydrophila ATCC7966 (NC008570)
Shewanella woodyi ATCC51908 (AAUO00000000)
Shewanella baltica (AF193506)
Shewanella denitrificans OS217 (CP000302.1)
Microbulbifer hydrolyticus (BK001043)
gamma Proteobacteria
Burkholderia dolorosa (AAKY00000000)
beta Proteobacteria
Photobacterium sp SKA34
V. angustum S14
Photobacteria
Hahella chejuensis KCTC2396 (CP000155.1)
Bacillus thuringiensis ser. sotto

Figure 3. Phylogenetic relationships of partial *chiA* gene sequences from *Vibrionaceae* and related organisms based on maximum likelihood analysis. Numbers shown at nodes represent values based on 100 bootstrap replicates, only nodes >80 are shown. Branch length to the outgroup is truncated, as indicated by arrow. Genbank accession numbers are given for previously sequenced genes. Grey boxes indicate potential instances of lateral gene transfer. Round circles indicate the two copies of *chiA* family genes in *Photobacteria.* Grey boxes indicate potential lateral gene transfer events.

Table S1 Names and accession numbers of genome sequences analyzed in this study

Accession number	organism/replicon
NC_004459	Vibrio vulnificus CMCP6 chromosome I
NC_004460	Vibrio vulnificus CMCP6 chromosome II
NC_005139	Vibrio vulnificus YJ016 chromosome I
NC_005140	Vibrio vulnificus YJ016 chromosome II
NC_006840	Vibrio fischeri ES114 chromosome I
NC_006841	Vibrio fischeri ES114 chromosome II
NC_004603	Vibrio parahaemolyticus RIMD 2210633 chromosome I
NC_004605	Vibrio parahaemolyticus RIMD 2210633 chromosome II
NC_002505	Vibrio cholerae O1 biovar eltor str. N16961 chromosome I
NC_002506	Vibrio cholerae O1 biovar eltor str. N16961 chromosome II
NC_006371	Photobacterium profundum SS9 chromosome 2
NC_006370	Photobacterium profundum SS9 chromosome 1
NZ_AAKG00000000	Vibrio cholerae O395
NZ_AAUT00000000	Vibrio cholerae 2740-80
NZ_AAKJ00000000	Vibrio cholerae V52
NZ_AAKF00000000	Vibrio cholerae MO10
NZ_AAKH00000000	Vibrio cholerae RC385
NZ_AAKI00000000	Vibrio cholerae V51
NZ_AAPS00000000	Vibrio alginolyticus 12G01
NZ_AAOJ00000000	Vibrio angustum S14
NZ_AAMR00000000	Vibrio splendidus 12B01
NZ_AAKK00000000	Vibrio sp. Ex25
NZ_AAND00000000	Vibrio sp. MED222
NZ_AAPH00000000	Photobacterium profundum 3TCK
NZ_AAOU00000000	Photobacterium sp. SKA34

Table S2 Detailed list of genes present in each chitin-related family

Strain	Gene Family[1]	Locus	Replicon Accession Number	Start Position
Photobacterium profundum 3TCK	32	P3TCK_21928	NZ_AAPH01000059	6836
Photobacterium profundum SS9	32	PBPRA2198	NC_006370	2527805
Photobacterium profundum SS9	32	PBPRA2199	NC_006370	2529673
Photobacterium sp. SKA34	32	SKA34_12615	NZ_AAOU01000006	123369
Vibrio alginolyticus 12G01	32	V12G01_08775	NZ_AAPS01000011	1277
Vibrio angustum S14	32	VAS14_04313	NZ_AAOJ01000002	381585
Vibrio cholerae 2740-80	32	VC274080_0207	NZ_AAUT01000001	59687
Vibrio cholerae 2740-80	32	VC274080_1133	NZ_AAUT01000020	30671
Vibrio cholerae MO10	32	VchoM_02000663	NZ_AAKF02000005	47124
Vibrio cholerae MO10	32	VchoM_02002017	NZ_AAKF02000025	41570
Vibrio cholerae O1	32	VC1073	NC_002505	1139646
Vibrio cholerae O1	32	VCA0700	NC_002506	638828
Vibrio cholerae O395	32	VchoO_01000531	NZ_AAKG01000001	631355
Vibrio cholerae O395	32	VchoO_01003200	NZ_AAKG01000002	699339
Vibrio cholerae RC385	32	VchoR_02000694	NZ_AAKH02000017	28677
Vibrio cholerae RC385	32	VchoR_02000712	NZ_AAKH02000018	20080
Vibrio cholerae V51	32	VchoV5_02000739	NZ_AAKI02000011	9909
Vibrio cholerae V51	32	VchoV5_02001150	NZ_AAKI02000021	13627
Vibrio cholerae V52	32	VCV52_A0652	NZ_AAKJ02000001	131964
Vibrio cholerae V52	32	VCV52_1029	NZ_AAKJ02000004	14803
Vibrio fischeri ES114	32	VF0986	NC_006840	1083554
Vibrio parahaemolyticus RIMD 2210633	32	VPA0832	NC_004605	865637
Vibrio sp. Ex25	32	VEx2w_02000997	NZ_AAKK02000008	13551
Vibrio sp. MED222	32	MED222_07578	NZ_AAND01000001	11186
Vibrio splendidus 12B01	32	V12B01_15311	NZ_AAMR01000019	8940
Vibrio vulnificus CMCP6	32	VV2_0213	NC_004460	240955
Vibrio vulnificus YJ016	32	VVA0720	NC_005140	816901
Photobacterium profundum 3TCK	33	P3TCK_04531	NZ_AAPH01000001	487589
Photobacterium profundum 3TCK	33	P3TCK_15999	NZ_AAPH01000008	69566
Photobacterium profundum SS9	33	PBPRA1032	NC_006370	1145725
Photobacterium profundum SS9	33	PBPRB0263	NC_006371	299761
Photobacterium sp. SKA34	33	SKA34_02639	NZ_AAOU01000010	75450
Photobacterium sp. SKA34	33	SKA34_14250	NZ_AAOU01000024	26847
Photobacterium sp. SKA34	33	SKA34_04125	NZ_AAOU01000047	23477
Vibrio alginolyticus 12G01	33	V12G01_15715	NZ_AAPS01000014	87036
Vibrio angustum S14	33	VAS14_11539	NZ_AAOJ01000001	479892
Vibrio angustum S14	33	VAS14_02456	NZ_AAOJ01000003	392967
Vibrio angustum S14	33	VAS14_17651	NZ_AAOJ01000004	247083
Vibrio cholerae 2740-80	33	VC274080_1061	NZ_AAUT01000026	26481
Vibrio cholerae MO10	33	VchoM_02002450	NZ_AAKF02000035	27198
Vibrio cholerae O1	33	VC0995	NC_002505	1061324
Vibrio cholerae O395	33	VchoO_01000457	NZ_AAKG01000001	552103
Vibrio cholerae RC385	33	VchoR_02001601	NZ_AAKH02000061	13474
Vibrio cholerae V51	33	VchoV5_02002527	NZ_AAKI02000093	146
Vibrio cholerae V52	33	VCV52_0955	NZ_AAKJ02000035	15165

Vibrio fischeri ES114	33	VF0808	NC_006840	887972
Vibrio fischeri ES114	33	VFA0438	NC_006841	501572
Vibrio parahaemolyticus RIMD 2210633	33	VP0831	NC_004603	861813
Vibrio sp. Ex25	33	VEx2w_02002989	NZ_AAKK02000048	29669
Vibrio sp. MED222	33	MED222_20594	NZ_AAND01000041	2106
Vibrio sp. MED222	33	MED222_20249	NZ_AAND01000045	4512
Vibrio splendidus 12B01	33	V12B01_21359	NZ_AAMR01000050	1149
Vibrio vulnificus CMCP6	33	VV1_0179	NC_004459	166310
Vibrio vulnificus YJ016	33	VV1012	NC_005139	1010486
Photobacterium profundum 3TCK	174	P3TCK_12296	NZ_AAPH01000007	52479
Photobacterium profundum 3TCK	174	P3TCK_15994	NZ_AAPH01000008	68509
Photobacterium profundum SS9	174	PBPRA1031	NC_006370	1144668
Photobacterium profundum SS9	174	PBPRB0360	NC_006371	405081
Photobacterium sp. SKA34	174	SKA34_02634	NZ_AAOU01000010	74393
Vibrio alginolyticus 12G01	174	V12G01_03961	NZ_AAPS01000001	3553
Vibrio angustum S14	174	VAS14_17646	NZ_AAOJ01000004	246026
Vibrio cholerae 2740-80	174	VC274080_A1073	NZ_AAUT01000019	1128
Vibrio cholerae MO10	174	VchoM_02000006	NZ_AAKF02000001	6626
Vibrio cholerae O1	174	VCA1025	NC_002506	972427
Vibrio cholerae O395	174	VchoO_01002812	NZ_AAKG01000002	251817
Vibrio cholerae RC385	174	VchoR_02001737	NZ_AAKH02000073	3896
Vibrio cholerae V51	174	VchoV5_02000500	NZ_AAKI02000006	58048
Vibrio cholerae V52	174	VCV52_A0973	NZ_AAKJ02000008	50374
Vibrio fischeri ES114	174	VF2357	NC_006840	2652754
Vibrio parahaemolyticus RIMD 2210633	174	VPA0038	NC_004605	31871
Vibrio sp. Ex25	174	VEx2w_02000665	NZ_AAKK02000005	36002
Vibrio sp. MED222	174	MED222_10678	NZ_AAND01000001	732712
Vibrio splendidus 12B01	174	V12B01_18786	NZ_AAMR01000004	196747
Vibrio vulnificus CMCP6	174	VV2_1200	NC_004460	1298917
Vibrio vulnificus YJ016	174	VVA0028	NC_005140	26168
Photobacterium profundum 3TCK	195	P3TCK_06297	NZ_AAPH01000005	94086
Photobacterium profundum SS9	195	PBPRB0541	NC_006371	630462
Photobacterium sp. SKA34	195	SKA34_06660	NZ_AAOU01000002	230639
Photobacterium sp. SKA34	195	SKA34_07004	NZ_AAOU01000021	14512
Vibrio alginolyticus 12G01	195	V12G01_03881	NZ_AAPS01000021	57751
Vibrio angustum S14	195	VAS14_09224	NZ_AAOJ01000001	20859
Vibrio angustum S14	195	VAS14_08870	NZ_AAOJ01000006	113283
Vibrio cholerae 2740-80	195	VC274080_A0063	NZ_AAUT01000004	31221
Vibrio cholerae MO10	195	VchoM_02000528	NZ_AAKF02000004	31009
Vibrio cholerae O1	195	VCA0027	NC_002506	33035
Vibrio cholerae O395	195	VchoO_01002710	NZ_AAKG01000002	117139
Vibrio cholerae RC385	195	VchoR_02000559	NZ_AAKH02000013	15088
Vibrio cholerae V51	195	VchoV5_02000270	NZ_AAKI02000003	22197
Vibrio cholerae V52	195	VCV52_A0050	NZ_AAKJ02000031	9789
Vibrio fischeri ES114	195	VF1598	NC_006840	1795291
Vibrio parahaemolyticus RIMD 2210633	195	VPA0055	NC_004605	45762
Vibrio sp. Ex25	195	VEx2w_02000681	NZ_AAKK02000005	49888
Vibrio sp. MED222	195	MED222_10778	NZ_AAND01000001	749938
Vibrio splendidus 12B01	195	V12B01_18891	NZ_AAMR01000048	17472
Vibrio vulnificus CMCP6	195	VV2_1217	NC_004460	1314417

Vibrio vulnificus YJ016	195	VVA0044	NC_005140	40806
Photobacterium profundum 3TCK	646	P3TCK_16004	NZ_AAPH01000008	71209
Photobacterium profundum SS9	646	PBPRA1033	NC_006370	1147302
Photobacterium sp. SKA34	646	SKA34_02644	NZ_AAOU01000010	77111
Shewanella oneidensis MR-1	646	SO3509	NC_004347	3659904
Vibrio alginolyticus 12G01	646	V12G01_15345	NZ_AAPS01000014	2468
Vibrio angustum S14	646	VAS14_17656	NZ_AAOJ01000004	248745
Vibrio cholerae 2740-80	646	VC274080_2241	NZ_AAUT01000007	33820
Vibrio cholerae MO10	646	VchoM_02000881	NZ_AAKF02000007	39269
Vibrio cholerae O1	646	VC2217	NC_002505	2371485
Vibrio cholerae O395	646	VchoO_01001675	NZ_AAKG01000001	1924830
Vibrio cholerae RC385	646	VchoR_02000082	NZ_AAKH02000002	37040
Vibrio cholerae V51	646	VchoV5_02000419	NZ_AAKI02000005	40164
Vibrio cholerae V52	646	VCV52_2186	NZ_AAKJ02000007	41063
Vibrio fischeri ES114	646	VFA1010	NC_006841	1134909
Vibrio parahaemolyticus RIMD 2210633	646	VP0755	NC_004603	791330
Vibrio sp. Ex25	646	VEx2w_02002877	NZ_AAKK02000044	30027
Vibrio sp. MED222	646	MED222_03428	NZ_AAND01000007	125562
Vibrio splendidus 12B01	646	V12B01_05195	NZ_AAMR01000020	2233
Vibrio vulnificus CMCP6	646	VV1_0241	NC_004459	233680
Vibrio vulnificus YJ016	646	VV0942	NC_005139	941943
Photobacterium profundum 3TCK	913	P3TCK_01409	NZ_AAPH01000010	2812
Photobacterium profundum SS9	913	PBPRA0497	NC_006370	524884
Photobacterium sp. SKA34	913	SKA34_07803	NZ_AAOU01000001	12886
Vibrio alginolyticus 12G01	913	V12G01_14109	NZ_AAPS01000065	3304
Vibrio angustum S14	913	VAS14_15489	NZ_AAOJ01000001	1387645
Vibrio angustum S14	913	VAS14_08585	NZ_AAOJ01000006	54116
Vibrio cholerae 2740-80	913	VC274080_1356	NZ_AAUT01000011	55998
Vibrio cholerae MO10	913	VchoM_02002143	NZ_AAKF02000028	9320
Vibrio cholerae O1	913	VC1284	NC_002505	1359014
Vibrio cholerae O395	913	VchoO_01000832	NZ_AAKG01000001	917473
Vibrio cholerae V51	913	VchoV5_02002326	NZ_AAKI02000074	14178
Vibrio cholerae V52	913	VCV52_1235	NZ_AAKJ02000060	14794
Vibrio parahaemolyticus RIMD 2210633	913	VP2634	NC_004603	2785068
Vibrio parahaemolyticus RIMD 2210633	913	VPA1695	NC_004605	1813073
Vibrio sp. Ex25	913	VEx2w_02001894	NZ_AAKK02000019	59862
Vibrio splendidus 12B01	913	V12B01_01217	NZ_AAMR01000007	128680
Vibrio vulnificus CMCP6	913	VV1_1485	NC_004459	1472806
Vibrio vulnificus CMCP6	913	VV2_1050	NC_004460	1130136
Vibrio vulnificus YJ016	913	VV2898	NC_005139	2958366
Vibrio vulnificus YJ016	913	VVA1565	NC_005140	1711690
Photobacterium profundum 3TCK	997	P3TCK_15989	NZ_AAPH01000008	67355
Photobacterium profundum SS9	997	PBPRA1030	NC_006370	1143515
Photobacterium sp. SKA34	997	SKA34_02629	NZ_AAOU01000010	73238
Shewanella oneidensis MR-1	997	SO3505	NC_004347	3656532
Vibrio alginolyticus 12G01	997	V12G01_15710	NZ_AAPS01000014	85368
Vibrio angustum S14	997	VAS14_17641	NZ_AAOJ01000004	244871
Vibrio cholerae 2740-80	997	VC274080_1060	NZ_AAUT01000026	24876
Vibrio cholerae MO10	997	VchoM_02002449	NZ_AAKF02000035	25551
Vibrio cholerae O1	997	VC0994	NC_002505	1059719

Vibrio cholerae O395	997	VchoO_01000456	NZ_AAKG01000001	550456
Vibrio cholerae RC385	997	VchoR_02001602	NZ_AAKH02000061	15508
Vibrio cholerae V51	997	VchoV5_02003095	NZ_AAKI02000196	2
Vibrio cholerae V52	997	VCV52_0954	NZ_AAKJ02000035	17205
Vibrio fischeri ES114	997	VF0807	NC_006840	886433
Vibrio parahaemolyticus RIMD 2210633	997	VP0829	NC_004603	860187
Vibrio sp. Ex25	997	VEx2w_02002988	NZ_AAKK02000048	28043
Vibrio sp. MED222	997	MED222_20599	NZ_AAND01000041	4141
Vibrio splendidus 12B01	997	V12B01_21369	NZ_AAMR01000050	3185
Vibrio vulnificus CMCP6	997	VV1_0180	NC_004459	168373
Vibrio vulnificus YJ016	997	VV1011	NC_005139	1008857
Photobacterium profundum 3TCK	1108	P3TCK_13490	NZ_AAPH01000022	7609
Photobacterium profundum SS9	1108	PBPRA0519	NC_006370	547536
Photobacterium sp. SKA34	1108	SKA34_18219	NZ_AAOU01000003	7894
Shewanella oneidensis MR-1	1108	SO3507	NC_004347	3658778
Vibrio alginolyticus 12G01	1108	V12G01_21658	NZ_AAPS01000041	9878
Vibrio angustum S14	1108	VAS14_07849	NZ_AAOJ01000007	130246
Vibrio cholerae 2740-80	1108	VC274080_0701	NZ_AAUT01000043	10171
Vibrio cholerae MO10	1108	VchoM_02002785	NZ_AAKF02000045	22245
Vibrio cholerae O1	1108	VC0614	NC_002505	648783
Vibrio cholerae O395	1108	VchoO_01000128	NZ_AAKG01000001	139569
Vibrio cholerae RC385	1108	VchoR_02000903	NZ_AAKH02000025	18647
Vibrio cholerae V51	1108	VchoV5_02001626	NZ_AAKI02000037	7147
Vibrio cholerae V52	1108	VCV52_0581	NZ_AAKJ02000039	11205
Vibrio fischeri ES114	1108	VF2145	NC_006840	2404475
Vibrio parahaemolyticus RIMD 2210633	1108	VP2485	NC_004603	2617251
Vibrio sp. Ex25	1108	VEx2w_02003985	NZ_AAKK02000109	2071
Vibrio sp. MED222	1108	MED222_21846	NZ_AAND01000034	31212
Vibrio splendidus 12B01	1108	V12B01_22865	NZ_AAMR01000051	20858
Vibrio vulnificus CMCP6	1108	VV1_1667	NC_004459	1642166
Vibrio vulnificus YJ016	1108	VV2740	NC_005139	2785209
Photobacterium profundum 3TCK	1627	P3TCK_01404	NZ_AAPH01000010	2010
Photobacterium profundum SS9	1627	PBPRA0498	NC_006370	526260
Photobacterium sp. SKA34	1627	SKA34_07798	NZ_AAOU01000001	12071
Vibrio alginolyticus 12G01	1627	V12G01_21218	NZ_AAPS01000010	90980
Vibrio alginolyticus 12G01	1627	V12G01_14104	NZ_AAPS01000065	2530
Vibrio angustum S14	1627	VAS14_15484	NZ_AAOJ01000001	1386830
Vibrio angustum S14	1627	VAS14_08580	NZ_AAOJ01000006	53349
Vibrio cholerae 2740-80	1627	VC274080_1357	NZ_AAUT01000011	55217
Vibrio cholerae MO10	1627	VchoM_02002144	NZ_AAKF02000028	10665
Vibrio cholerae O1	1627	VC1285	NC_002505	1360359
Vibrio cholerae O395	1627	VchoO_01000833	NZ_AAKG01000001	918818
Vibrio cholerae V51	1627	VchoV5_02002325	NZ_AAKI02000074	13397
Vibrio cholerae V52	1627	VCV52_1236	NZ_AAKJ02000060	14013
Vibrio fischeri ES114	1627	VF1341	NC_006840	1489662
Vibrio parahaemolyticus RIMD 2210633	1627	VP2633	NC_004603	2784294
Vibrio sp. Ex25	1627	VEx2w_02001895	NZ_AAKK02000019	61203
Vibrio splendidus 12B01	1627	V12B01_01222	NZ_AAMR01000007	130119
Vibrio vulnificus CMCP6	1627	VV1_1486	NC_004459	1474136
Vibrio vulnificus YJ016	1627	VV2897	NC_005139	2957588

Photobacterium profundum 3TCK	1628	P3TCK_03231	NZ_AAPH01000001	200144
Photobacterium profundum 3TCK	1628	P3TCK_26215	NZ_AAPH01000018	55703
Photobacterium profundum SS9	1628	PBPRA2778	NC_006370	3223560
Photobacterium profundum SS9	1628	PBPRB2005	NC_006371	2210272
Photobacterium sp. SKA34	1628	SKA34_08148	NZ_AAOU01000001	95681
Photobacterium sp. SKA34	1628	SKA34_02020	NZ_AAOU01000032	22052
Vibrio alginolyticus 12G01	1628	V12G01_14114	NZ_AAPS01000065	4639
Vibrio angustum S14	1628	VAS14_08590	NZ_AAOJ01000006	55411
Vibrio cholerae 2740-80	1628	VC274080_1355	NZ_AAUT01000011	57302
Vibrio cholerae MO10	1628	VchoM_02002142	NZ_AAKF02000028	9003
Vibrio cholerae O1	1628	VC1283	NC_002505	1358697
Vibrio cholerae O395	1628	VchoO_01000831	NZ_AAKG01000001	917156
Vibrio cholerae V51	1628	VchoV5_02002865	NZ_AAKI02000143	20
Vibrio cholerae V52	1628	VCV52_1234	NZ_AAKJ02000060	16098
Vibrio fischeri ES114	1628	VF0607	NC_006840	667014
Vibrio parahaemolyticus RIMD 2210633	1628	VP2635	NC_004603	2786401
Vibrio sp. Ex25	1628	VEx2w_02001893	NZ_AAKK02000019	59498
Vibrio vulnificus CMCP6	1628	VV1_1484	NC_004459	1472488
Vibrio vulnificus YJ016	1628	VV2899	NC_005139	2959698
Photobacterium profundum 3TCK	1629	P3TCK_03241	NZ_AAPH01000001	202089
Photobacterium profundum 3TCK	1629	P3TCK_01414	NZ_AAPH01000010	4183
Photobacterium profundum SS9	1629	PBPRA0496	NC_006370	523486
Photobacterium profundum SS9	1629	PBPRB2007	NC_006371	2212219
Photobacterium sp. SKA34	1629	SKA34_08163	NZ_AAOU01000001	97290
Photobacterium sp. SKA34	1629	SKA34_02010	NZ_AAOU01000032	19192
Vibrio alginolyticus 12G01	1629	V12G01_14119	NZ_AAPS01000065	5002
Vibrio angustum S14	1629	VAS14_08595	NZ_AAOJ01000006	55821
Vibrio cholerae 2740-80	1629	VC274080_1354	NZ_AAUT01000011	57684
Vibrio cholerae MO10	1629	VchoM_02002141	NZ_AAKF02000028	7625
Vibrio cholerae O1	1629	VC1282	NC_002505	1357310
Vibrio cholerae O395	1629	VchoO_01000830	NZ_AAKG01000001	915778
Vibrio cholerae V51	1629	VchoV5_02002866	NZ_AAKI02000143	402
Vibrio cholerae V52	1629	VCV52_1233	NZ_AAKJ02000060	16480
Vibrio fischeri ES114	1629	VF0603	NC_006840	662898
Vibrio parahaemolyticus RIMD 2210633	1629	VP2636	NC_004603	2786762
Vibrio sp. Ex25	1629	VEx2w_02001891	NZ_AAKK02000019	58144
Vibrio vulnificus CMCP6	1629	VV1_1483	NC_004459	1471103
Vibrio vulnificus YJ016	1629	VV2900	NC_005139	2960060
Photobacterium profundum 3TCK	1722	P3TCK_21840	NZ_AAPH01000002	422584
Photobacterium profundum SS9	1722	PBPRA2181	NC_006370	2508155
Photobacterium sp. SKA34	1722	SKA34_08218	NZ_AAOU01000001	110253
Vibrio alginolyticus 12G01	1722	V12G01_12910	NZ_AAPS01000009	80809
Vibrio angustum S14	1722	VAS14_15829	NZ_AAOJ01000001	1474012
Vibrio cholerae 2740-80	1722	VC274080_0850	NZ_AAUT01000002	8359
Vibrio cholerae MO10	1722	VchoM_02000256	NZ_AAKF02000002	35835
Vibrio cholerae O1	1722	VC0769	NC_002505	823377
Vibrio cholerae O395	1722	VchoO_01000270	NZ_AAKG01000001	314112
Vibrio cholerae RC385	1722	VchoR_02000957	NZ_AAKH02000028	290
Vibrio cholerae V51	1722	VchoV5_02000334	NZ_AAKI02000004	8804
Vibrio cholerae V52	1722	VCV52_0735	NZ_AAKJ02000002	95275

Vibrio fischeri ES114	1722	VF1390	NC_006840	1539840
Vibrio parahaemolyticus RIMD 2210633	1722	VP0619	NC_004603	646391
Vibrio sp. Ex25	1722	VEx2w_02000923	NZ_AAKK02000007	37790
Vibrio sp. MED222	1722	MED222_01587	NZ_AAND01000003	62628
Vibrio splendidus 12B01	1722	V12B01_09271	NZ_AAMR01000016	57466
Vibrio vulnificus CMCP6	1722	VV1_0417	NC_004459	409923
Vibrio vulnificus YJ016	1722	VV0777	NC_005139	783897
Photobacterium profundum 3TCK	1747	P3TCK_13515	NZ_AAPH01000022	13294
Photobacterium profundum SS9	1747	PBPRA0524	NC_006370	553220
Photobacterium sp. SKA34	1747	SKA34_18244	NZ_AAOU01000003	13651
Vibrio alginolyticus 12G01	1747	V12G01_21683	NZ_AAPS01000041	15631
Vibrio angustum S14	1747	VAS14_07824	NZ_AAOJ01000007	124383
Vibrio cholerae 2740-80	1747	VC274080_0706	NZ_AAUT01000043	15885
Vibrio cholerae MO10	1747	VchoM_02002780	NZ_AAKF02000045	16429
Vibrio cholerae O1	1747	VC0619	NC_002505	654497
Vibrio cholerae O395	1747	VchoO_01000133	NZ_AAKG01000001	145283
Vibrio cholerae RC385	1747	VchoR_02000898	NZ_AAKH02000025	12831
Vibrio cholerae V51	1747	VchoV5_02001631	NZ_AAKI02000037	12861
Vibrio cholerae V52	1747	VCV52_0586	NZ_AAKJ02000039	16919
Vibrio fischeri ES114	1747	VF2140	NC_006840	2398688
Vibrio parahaemolyticus RIMD 2210633	1747	VP2480	NC_004603	2611402
Vibrio sp. Ex25	1747	VEx2w_02003738	NZ_AAKK02000086	3330
Vibrio sp. MED222	1747	MED222_21821	NZ_AAND01000034	25291
Vibrio splendidus 12B01	1747	V12B01_22840	NZ_AAMR01000051	14952
Vibrio vulnificus CMCP6	1747	VV1_1672	NC_004459	1647902
Vibrio vulnificus YJ016	1747	VV2735	NC_005139	2779377
Photobacterium profundum 3TCK	1748	P3TCK_13510	NZ_AAPH01000022	12263
Photobacterium profundum SS9	1748	PBPRA0523	NC_006370	552189
Photobacterium sp. SKA34	1748	SKA34_18239	NZ_AAOU01000003	12614
Vibrio alginolyticus 12G01	1748	V12G01_21678	NZ_AAPS01000041	14603
Vibrio angustum S14	1748	VAS14_07829	NZ_AAOJ01000007	125372
Vibrio cholerae 2740-80	1748	VC274080_0705	NZ_AAUT01000043	14857
Vibrio cholerae MO10	1748	VchoM_02002781	NZ_AAKF02000045	17418
Vibrio cholerae O1	1748	VC0618	NC_002505	653469
Vibrio cholerae O395	1748	VchoO_01000132	NZ_AAKG01000001	144255
Vibrio cholerae RC385	1748	VchoR_02000899	NZ_AAKH02000025	13820
Vibrio cholerae V51	1748	VchoV5_02001630	NZ_AAKI02000037	11833
Vibrio cholerae V52	1748	VCV52_0585	NZ_AAKJ02000039	15891
Vibrio fischeri ES114	1748	VF2141	NC_006840	2399677
Vibrio parahaemolyticus RIMD 2210633	1748	VP2481	NC_004603	2612391
Vibrio sp. Ex25	1748	VEx2w_02003737	NZ_AAKK02000086	2302
Vibrio sp. MED222	1748	MED222_21826	NZ_AAND01000034	26280
Vibrio splendidus 12B01	1748	V12B01_22845	NZ_AAMR01000051	15941
Vibrio vulnificus CMCP6	1748	VV1_1671	NC_004459	1646874
Vibrio vulnificus YJ016	1748	VV2736	NC_005139	2780366
Photobacterium profundum 3TCK	1749	P3TCK_13505	NZ_AAPH01000022	11292
Photobacterium profundum SS9	1749	PBPRA0522	NC_006370	551218
Photobacterium sp. SKA34	1749	SKA34_18234	NZ_AAOU01000003	11643
Vibrio alginolyticus 12G01	1749	V12G01_21673	NZ_AAPS01000041	13617
Vibrio angustum S14	1749	VAS14_07834	NZ_AAOJ01000007	126409

Vibrio cholerae 2740-80	1749	VC274080_0704	NZ_AAUT01000043	13871
Vibrio cholerae MO10	1749	VchoM_02002782	NZ_AAKF02000045	18446
Vibrio cholerae O1	1749	VC0617	NC_002505	652483
Vibrio cholerae O395	1749	VchoO_01000131	NZ_AAKG01000001	143269
Vibrio cholerae RC385	1749	VchoR_02000900	NZ_AAKH02000025	14884
Vibrio cholerae V51	1749	VchoV5_02001629	NZ_AAKI02000037	10847
Vibrio cholerae V52	1749	VCV52_0584	NZ_AAKJ02000039	14905
Vibrio fischeri ES114	1749	VF2142	NC_006840	2400704
Vibrio parahaemolyticus RIMD 2210633	1749	VP2482	NC_004603	2613419
Vibrio sp. Ex25	1749	VEx2w_02003736	NZ_AAKK02000086	1316
Vibrio sp. MED222	1749	MED222_21831	NZ_AAND01000034	27308
Vibrio splendidus 12B01	1749	V12B01_22850	NZ_AAMR01000051	16969
Vibrio vulnificus CMCP6	1749	VV1_1670	NC_004459	1645888
Vibrio vulnificus YJ016	1749	VV2737	NC_005139	2781394
Photobacterium profundum 3TCK	1750	P3TCK_13500	NZ_AAPH01000022	10267
Photobacterium profundum SS9	1750	PBPRA0521	NC_006370	550194
Photobacterium sp. SKA34	1750	SKA34_18229	NZ_AAOU01000003	10617
Vibrio alginolyticus 12G01	1750	V12G01_21668	NZ_AAPS01000041	12574
Vibrio angustum S14	1750	VAS14_07839	NZ_AAOJ01000007	127411
Vibrio cholerae 2740-80	1750	VC274080_0703	NZ_AAUT01000043	12833
Vibrio cholerae MO10	1750	VchoM_02002783	NZ_AAKF02000045	19472
Vibrio cholerae O1	1750	VC0616	NC_002505	651445
Vibrio cholerae O395	1750	VchoO_01000130	NZ_AAKG01000001	142231
Vibrio cholerae RC385	1750	VchoR_02000901	NZ_AAKH02000025	15874
Vibrio cholerae V51	1750	VchoV5_02001628	NZ_AAKI02000037	9809
Vibrio cholerae V52	1750	VCV52_0583	NZ_AAKJ02000039	13867
Vibrio fischeri ES114	1750	VF2143	NC_006840	2401710
Vibrio parahaemolyticus RIMD 2210633	1750	VP2483	NC_004603	2614450
Vibrio sp. Ex25	1750	VEx2w_02003735	NZ_AAKK02000086	272
Vibrio sp. MED222	1750	MED222_21836	NZ_AAND01000034	28379
Vibrio splendidus 12B01	1750	V12B01_22855	NZ_AAMR01000051	18040
Vibrio vulnificus CMCP6	1750	VV1_1669	NC_004459	1644855
Vibrio vulnificus YJ016	1750	VV2738	NC_005139	2782415
Photobacterium profundum 3TCK	1751	P3TCK_13495	NZ_AAPH01000022	8489
Photobacterium profundum SS9	1751	PBPRA0520	NC_006370	548416
Photobacterium sp. SKA34	1751	SKA34_18224	NZ_AAOU01000003	8771
Vibrio alginolyticus 12G01	1751	V12G01_21663	NZ_AAPS01000041	10771
Vibrio angustum S14	1751	VAS14_07844	NZ_AAOJ01000007	128517
Vibrio cholerae 2740-80	1751	VC274080_0702	NZ_AAUT01000043	11052
Vibrio cholerae MO10	1751	VchoM_02002784	NZ_AAKF02000045	20524
Vibrio cholerae O1	1751	VC0615	NC_002505	649664
Vibrio cholerae O395	1751	VchoO_01000129	NZ_AAKG01000001	140450
Vibrio cholerae RC385	1751	VchoR_02000902	NZ_AAKH02000025	16926
Vibrio cholerae V51	1751	VchoV5_02001627	NZ_AAKI02000037	8028
Vibrio cholerae V52	1751	VCV52_0582	NZ_AAKJ02000039	12086
Vibrio fischeri ES114	1751	VF2144	NC_006840	2402754
Vibrio parahaemolyticus RIMD 2210633	1751	VP2484	NC_004603	2615509
Vibrio sp. Ex25	1751	VEx2w_02003984	NZ_AAKK02000109	329
Vibrio sp. MED222	1751	MED222_21841	NZ_AAND01000034	29488
Vibrio splendidus 12B01	1751	V12B01_22860	NZ_AAMR01000051	19134

Vibrio vulnificus CMCP6	1751	VV1_1668	NC_004459	1643058
Vibrio vulnificus YJ016	1751	VV2739	NC_005139	2783486
Photobacterium profundum 3TCK	1752	P3TCK_13485	NZ_AAPH01000022	5668
Photobacterium profundum SS9	1752	PBPRA0518	NC_006370	545595
Photobacterium sp. SKA34	1752	SKA34_18214	NZ_AAOU01000003	5956
Vibrio alginolyticus 12G01	1752	V12G01_21653	NZ_AAPS01000041	7950
Vibrio angustum S14	1752	VAS14_07854	NZ_AAOJ01000007	131140
Vibrio cholerae 2740-80	1752	VC274080_0700	NZ_AAUT01000043	8261
Vibrio cholerae MO10	1752	VchoM_02002786	NZ_AAKF02000045	23126
Vibrio cholerae O1	1752	VC0613	NC_002505	646873
Vibrio cholerae O395	1752	VchoO_01000127	NZ_AAKG01000001	137659
Vibrio cholerae RC385	1752	VchoR_02000904	NZ_AAKH02000025	19528
Vibrio cholerae V51	1752	VchoV5_02001625	NZ_AAKI02000037	5237
Vibrio cholerae V52	1752	VCV52_0580	NZ_AAKJ02000039	9295
Vibrio fischeri ES114	1752	VF2146	NC_006840	2405368
Vibrio parahaemolyticus RIMD 2210633	1752	VP2486	NC_004603	2618150
Vibrio sp. Ex25	1752	VEx2w_02003986	NZ_AAKK02000109	2970
Vibrio sp. MED222	1752	MED222_21851	NZ_AAND01000034	32109
Vibrio splendidus 12B01	1752	V12B01_22870	NZ_AAMR01000051	21755
Vibrio vulnificus CMCP6	1752	VV1_1666	NC_004459	1640237
Vibrio vulnificus YJ016	1752	VV2741	NC_005139	2786109
Photobacterium profundum 3TCK	1753	P3TCK_13480	NZ_AAPH01000022	3223
Photobacterium profundum SS9	1753	PBPRA0517	NC_006370	543150
Photobacterium sp. SKA34	1753	SKA34_18209	NZ_AAOU01000003	3509
Vibrio alginolyticus 12G01	1753	V12G01_21648	NZ_AAPS01000041	5457
Vibrio angustum S14	1753	VAS14_07859	NZ_AAOJ01000007	133109
Vibrio cholerae 2740-80	1753	VC274080_0699	NZ_AAUT01000043	5737
Vibrio cholerae MO10	1753	VchoM_02002787	NZ_AAKF02000045	25158
Vibrio cholerae O1	1753	VC0612	NC_002505	644349
Vibrio cholerae O395	1753	VchoO_01000126	NZ_AAKG01000001	135135
Vibrio cholerae RC385	1753	VchoR_02000905	NZ_AAKH02000025	21560
Vibrio cholerae V51	1753	VchoV5_02001624	NZ_AAKI02000037	2715
Vibrio cholerae V52	1753	VCV52_0579	NZ_AAKJ02000039	6771
Vibrio fischeri ES114	1753	VF2147	NC_006840	2407314
Vibrio parahaemolyticus RIMD 2210633	1753	VP2487	NC_004603	2620154
Vibrio sp. Ex25	1753	VEx2w_02003987	NZ_AAKK02000109	4974
Vibrio sp. MED222	1753	MED222_21856	NZ_AAND01000034	34182
Vibrio splendidus 12B01	1753	V12B01_22875	NZ_AAMR01000051	23863
Vibrio vulnificus CMCP6	1753	VV1_1665	NC_004459	1637741
Vibrio vulnificus YJ016	1753	VV2742	NC_005139	2788113
Photobacterium profundum 3TCK	1754	P3TCK_13475	NZ_AAPH01000022	1746
Photobacterium profundum SS9	1754	PBPRA0516	NC_006370	541673
Photobacterium sp. SKA34	1754	SKA34_18204	NZ_AAOU01000003	2034
Vibrio alginolyticus 12G01	1754	V12G01_21643	NZ_AAPS01000041	3919
Vibrio angustum S14	1754	VAS14_07864	NZ_AAOJ01000007	135577
Vibrio cholerae 2740-80	1754	VC274080_0698	NZ_AAUT01000043	4202
Vibrio cholerae MO10	1754	VchoM_02002788	NZ_AAKF02000045	27686
Vibrio cholerae O1	1754	VC0611	NC_002505	642814
Vibrio cholerae O395	1754	VchoO_01000125	NZ_AAKG01000001	133600
Vibrio cholerae RC385	1754	VchoR_02000906	NZ_AAKH02000025	24088

Vibrio cholerae V51	1754	VchoV5_02001623	NZ_AAKI02000037	1180
Vibrio cholerae V52	1754	VCV52_0578	NZ_AAKJ02000039	5236
Vibrio fischeri ES114	1754	VF2148	NC_006840	2409800
Vibrio parahaemolyticus RIMD 2210633	1754	VP2488	NC_004603	2622669
Vibrio sp. Ex25	1754	VEx2w_02004027	NZ_AAKK02000117	3908
Vibrio sp. MED222	1754	MED222_21861	NZ_AAND01000034	36662
Vibrio splendidus 12B01	1754	V12B01_22880	NZ_AAMR01000051	26341
Vibrio vulnificus CMCP6	1754	VV1_1664	NC_004459	1636257
Vibrio vulnificus YJ016	1754	VV2743	NC_005139	2790560
Photobacterium profundum 3TCK	1855	P3TCK_10273	NZ_AAPH01000052	13451
Photobacterium profundum SS9	1855	PBPRB0312	NC_006371	355697
Shewanella oneidensis MR-1	1855	SO1072	NC_004347	1112703
Vibrio alginolyticus 12G01	1855	V12G01_04871	NZ_AAPS01000001	218690
Vibrio cholerae 2740-80	1855	VC274080_A0854	NZ_AAUT01000081	9383
Vibrio cholerae MO10	1855	VchoM_02000194	NZ_AAKF02000001	222921
Vibrio cholerae O1	1855	VCA0811	NC_002506	755480
Vibrio cholerae O395	1855	VchoO_01003002	NZ_AAKG01000002	468183
Vibrio cholerae RC385	1855	VchoR_02003034	NZ_AAKH02000364	992
Vibrio cholerae RC385	1855	VchoR_02003222	NZ_AAKH02000466	706
Vibrio cholerae V51	1855	VchoV5_02002877	NZ_AAKI02000145	2932
Vibrio cholerae V52	1855	VCV52_A0765	NZ_AAKJ02000083	8565
Vibrio fischeri ES114	1855	VFA0143	NC_006841	160610
Vibrio parahaemolyticus RIMD 2210633	1855	VPA1598	NC_004605	1696131
Vibrio sp. Ex25	1855	VEx2w_02000370	NZ_AAKK02000003	3
Vibrio splendidus 12B01	1855	V12B01_11400	NZ_AAMR01000015	98474
Vibrio vulnificus CMCP6	1855	VV2_0044	NC_004460	44286
Vibrio vulnificus YJ016	1855	VVA0551	NC_005140	622897
Photobacterium profundum 3TCK	1892	P3TCK_11419	NZ_AAPH01000014	50296
Photobacterium sp. SKA34	1892	SKA34_06425	NZ_AAOU01000002	189907
Vibrio alginolyticus 12G01	1892	V12G01_03776	NZ_AAPS01000021	37366
Vibrio angustum S14	1892	VAS14_09469	NZ_AAOJ01000001	68185
Vibrio cholerae 2740-80	1892	VC274080_A0168	NZ_AAUT01000044	29427
Vibrio cholerae MO10	1892	VchoM_02001121	NZ_AAKF02000010	303
Vibrio cholerae O1	1892	VCA0140	NC_002506	153872
Vibrio cholerae O395	1892	VchoO_01003542	NZ_AAKG01000002	1105876
Vibrio cholerae RC385	1892	VchoR_02002216	NZ_AAKH02000131	197
Vibrio cholerae V51	1892	VchoV5_02002855	NZ_AAKI02000141	2
Vibrio cholerae V52	1892	VCV52_A0154	NZ_AAKJ02000077	13464
Vibrio fischeri ES114	1892	VFA0013	NC_006841	13291
Vibrio parahaemolyticus RIMD 2210633	1892	VPA0092	NC_004605	84564
Vibrio sp. Ex25	1892	VEx2w_02000719	NZ_AAKK02000005	90835
Vibrio sp. MED222	1892	MED222_15644	NZ_AAND01000013	65757
Vibrio splendidus 12B01	1892	V12B01_04783	NZ_AAMR01000013	36051
Vibrio vulnificus CMCP6	1892	VV2_1258	NC_004460	1377423
Vibrio vulnificus YJ016	1892	VVA0086	NC_005140	97037
Photobacterium profundum 3TCK	2006	P3TCK_03246	NZ_AAPH01000001	203474
Photobacterium profundum 3TCK	2006	P3TCK_26225	NZ_AAPH01000018	57387
Photobacterium profundum SS9	2006	PBPRA2776	NC_006370	3221897
Photobacterium profundum SS9	2006	PBPRB2008	NC_006371	2213604
Photobacterium sp. SKA34	2006	SKA34_02005	NZ_AAOU01000032	18763

Vibrio alginolyticus 12G01	2006	V12G01_14124	NZ_AAPS01000065	6425
Vibrio angustum S14	2006	VAS14_08600	NZ_AAOJ01000006	57364
Vibrio cholerae 2740-80	2006	VC274080_1353	NZ_AAUT01000011	59103
Vibrio cholerae MO10	2006	VchoM_02002140	NZ_AAKF02000028	7265
Vibrio cholerae O1	2006	VC1281	NC_002505	1356926
Vibrio cholerae O395	2006	VchoO_01000829	NZ_AAKG01000001	915418
Vibrio cholerae V51	2006	VchoV5_02002867	NZ_AAKI02000143	1824
Vibrio cholerae V52	2006	VCV52_1232	NZ_AAKJ02000060	17899
Vibrio fischeri ES114	2006	VF0604	NC_006840	664286
Vibrio parahaemolyticus RIMD 2210633	2006	VP2637	NC_004603	2788183
Vibrio sp. Ex25	2006	VEx2w_02001890	NZ_AAKK02000019	57818
Vibrio vulnificus CMCP6	2006	VV1_1482	NC_004459	1470754
Vibrio vulnificus YJ016	2006	VV2901	NC_005139	2961480
Photobacterium profundum 3TCK	2405	P3TCK_15185	NZ_AAPH01000004	133897
Photobacterium sp. SKA34	2405	SKA34_01930	NZ_AAOU01000032	5972
Vibrio alginolyticus 12G01	2405	V12G01_15365	NZ_AAPS01000014	9579
Vibrio cholerae 2740-80	2405	VC274080_1038	NZ_AAUT01000026	3048
Vibrio cholerae MO10	2405	VchoM_02002429	NZ_AAKF02000035	3723
Vibrio cholerae O1	2405	VC0972	NC_002505	1037891
Vibrio cholerae O395	2405	VchoO_01000436	NZ_AAKG01000001	528617
Vibrio cholerae RC385	2405	VchoR_02002579	NZ_AAKH02000206	2792
Vibrio cholerae V51	2405	VchoV5_02002838	NZ_AAKI02000138	1367
Vibrio cholerae V52	2405	VCV52_0932	NZ_AAKJ02000035	39131
Vibrio fischeri ES114	2405	VF1889	NC_006840	2128307
Vibrio parahaemolyticus RIMD 2210633	2405	VP0760	NC_004603	798857
Vibrio sp. Ex25	2405	VEx2w_02002873	NZ_AAKK02000044	24039
Vibrio sp. MED222	2405	MED222_20719	NZ_AAND01000041	28740
Vibrio vulnificus CMCP6	2405	VV1_0238	NC_004459	228819
Vibrio vulnificus YJ016	2405	VV0946	NC_005139	948403
Photobacterium sp. SKA34	2527	SKA34_12285	NZ_AAOU01000006	53178
Vibrio alginolyticus 12G01	2527	V12G01_22303	NZ_AAPS01000002	76031
Vibrio angustum S14	2527	VAS14_04668	NZ_AAOJ01000002	465167
Vibrio cholerae 2740-80	2527	VC274080_1994	NZ_AAUT01000039	30119
Vibrio cholerae MO10	2527	VchoM_02001371	NZ_AAKF02000013	44654
Vibrio cholerae O1	2527	VC1952	NC_002505	2104179
Vibrio cholerae O395	2527	VchoO_01001430	NZ_AAKG01000001	1657274
Vibrio cholerae RC385	2527	VchoR_02002351	NZ_AAKH02000158	2
Vibrio cholerae V51	2527	VchoV5_02001398	NZ_AAKI02000028	27690
Vibrio cholerae V52	2527	VCV52_1918	NZ_AAKJ02000028	37836
Vibrio fischeri ES114	2527	VF0655	NC_006840	718813
Vibrio parahaemolyticus RIMD 2210633	2527	VP2338	NC_004603	2448556
Vibrio sp. Ex25	2527	VEx2w_02002007	NZ_AAKK02000022	10942
Vibrio vulnificus CMCP6	2527	VV1_1833	NC_004459	1828917
Vibrio vulnificus YJ016	2527	VV2578	NC_005139	2604228
Photobacterium profundum 3TCK	2808	P3TCK_01399	NZ_AAPH01000010	740
Photobacterium profundum SS9	2808	PBPRA0499	NC_006370	527273
Vibrio alginolyticus 12G01	2808	V12G01_14099	NZ_AAPS01000065	1427
Vibrio cholerae 2740-80	2808	VC274080_1358	NZ_AAUT01000011	54094
Vibrio cholerae MO10	2808	VchoM_02002145	NZ_AAKF02000028	11524
Vibrio cholerae O1	2808	VC1286	NC_002505	1361218

Vibrio cholerae O395	2808	VchoO_01000834	NZ_AAKG01000001	919677
Vibrio cholerae V51	2808	VchoV5_02002324	NZ_AAKI02000074	12274
Vibrio cholerae V52	2808	VCV52_1237	NZ_AAKJ02000060	12890
Vibrio parahaemolyticus RIMD 2210633	2808	VP2632	NC_004603	2783178
Vibrio sp. Ex25	2808	VEx2w_02001896	NZ_AAKK02000019	62064
Vibrio vulnificus CMCP6	2808	VV1_1487	NC_004459	1474945
Vibrio vulnificus YJ016	2808	VV2896	NC_005139	2956497
Photobacterium profundum 3TCK	2809	P3TCK_01424	NZ_AAPH01000010	6184
Photobacterium profundum SS9	2809	PBPRA0494	NC_006370	521400
Vibrio alginolyticus 12G01	2809	V12G01_14129	NZ_AAPS01000065	6978
Vibrio cholerae 2740-80	2809	VC274080_1352	NZ_AAUT01000011	59651
Vibrio cholerae MO10	2809	VchoM_02002139	NZ_AAKF02000028	5694
Vibrio cholerae O1	2809	VC1280	NC_002505	1355388
Vibrio cholerae O395	2809	VchoO_01000828	NZ_AAKG01000001	913847
Vibrio cholerae V51	2809	VchoV5_02002868	NZ_AAKI02000143	2372
Vibrio cholerae V52	2809	VCV52_1231	NZ_AAKJ02000060	18447
Vibrio parahaemolyticus RIMD 2210633	2809	VP2638	NC_004603	2788736
Vibrio sp. Ex25	2809	VEx2w_02001889	NZ_AAKK02000019	56227
Vibrio vulnificus CMCP6	2809	VV1_1481	NC_004459	1469157
Vibrio vulnificus YJ016	2809	VV2902	NC_005139	2961865
Photobacterium profundum 3TCK	3444	P3TCK_15175	NZ_AAPH01000004	131325
Photobacterium profundum SS9	3444	PBPRA0868	NC_006370	957471
Photobacterium profundum SS9	3444	PBPRA0872	NC_006370	962809
Vibrio alginolyticus 12G01	3444	V12G01_15545	NZ_AAPS01000014	46320
Vibrio angustum S14	3444	VAS14_16941	NZ_AAOJ01000004	103265
Vibrio parahaemolyticus RIMD 2210633	3444	VP0802	NC_004603	835874
Vibrio sp. Ex25	3444	VEx2w_02002964	NZ_AAKK02000048	3629
Vibrio splendidus 12B01	3444	V12B01_21494	NZ_AAMR01000050	27333
Vibrio vulnificus CMCP6	3444	VV1_0205	NC_004459	193703
Vibrio cholerae 2740-80	3745	VC274080_1835	NZ_AAUT01000003	76869
Vibrio cholerae O1	3745	VC1783	NC_002505	1931750
Vibrio cholerae O395	3745	VchoO_01001281	NZ_AAKG01000001	1484920
Vibrio cholerae V51	3745	VchoV5_02000226	NZ_AAKI02000002	98735
Vibrio cholerae V52	3745	VCV52_1756	NZ_AAKJ02000025	4757
Vibrio sp. MED222	3745	MED222_20144	NZ_AAND01000054	361
Vibrio vulnificus CMCP6	3745	VV2_0736	NC_004460	788281
Vibrio vulnificus YJ016	3745	VVA1206	NC_005140	1325234
Photobacterium sp. SKA34	4210	SKA34_19559	NZ_AAOU01000004	146128
Vibrio angustum S14	4210	VAS14_06318	NZ_AAOJ01000002	858048
Vibrio parahaemolyticus RIMD 2210633	4210	VPA1177	NC_004605	1245266
Vibrio sp. Ex25	4210	VEx2w_02000124	NZ_AAKK02000001	173786
Vibrio vulnificus CMCP6	4210	VV2_0549	NC_004460	602769
Vibrio vulnificus YJ016	4210	VVA1099	NC_005140	1224598
Vibrio cholerae O395	4637	VchoO_01000530	NZ_AAKG01000001	630422
Vibrio fischeri ES114	4637	VF1146	NC_006840	1272372
Vibrio fischeri ES114	4637	VFA0715	NC_006841	804689
Vibrio vulnificus CMCP6	4637	VV2_0820	NC_004460	876874
Vibrio vulnificus YJ016	4637	VVA1285	NC_005140	1413818
Photobacterium profundum 3TCK	6025	P3TCK_21620	NZ_AAPH01000002	370817
Vibrio vulnificus CMCP6	6025	VV1_2342	NC_004459	2368764

Vibrio vulnificus YJ016	6025	VV1997	NC_005139	1996169
Shewanella oneidensis MR-1	6081	SO4085	NC_004347	4238538
Vibrio angustum S14	6081	VAS14_08910	NZ_AAOJ01000006	126030
Vibrio splendidus 12B01	6081	V12B01_26059	NZ_AAMR01000002	213365
Vibrio alginolyticus 12G01	6498	V12G01_01435	NZ_AAPS01000007	98312
Vibrio sp. Ex25	6498	VEx2w_02000061	NZ_AAKK02000001	70226
Vibrio alginolyticus 12G01	6541	V12G01_22308	NZ_AAPS01000002	77654
Vibrio cholerae RC385	6541	VchoR_02003282	NZ_AAKH02000506	311
Photobacterium sp. SKA34	6774	SKA34_14935	NZ_AAOU01000018	77261
Vibrio angustum S14	6774	VAS14_01801	NZ_AAOJ01000003	245327
Photobacterium sp. SKA34	6798	SKA34_13330	NZ_AAOU01000017	4582
Vibrio angustum S14	6798	VAS14_03518	NZ_AAOJ01000002	218976
Vibrio angustum S14	7412	VAS14_08875	NZ_AAOJ01000006	116063
Vibrio fischeri ES114	7412	VF1059	NC_006840	1171809

[1] Gene family number as determined by OrthoMCL, genes with the same number are from the same family

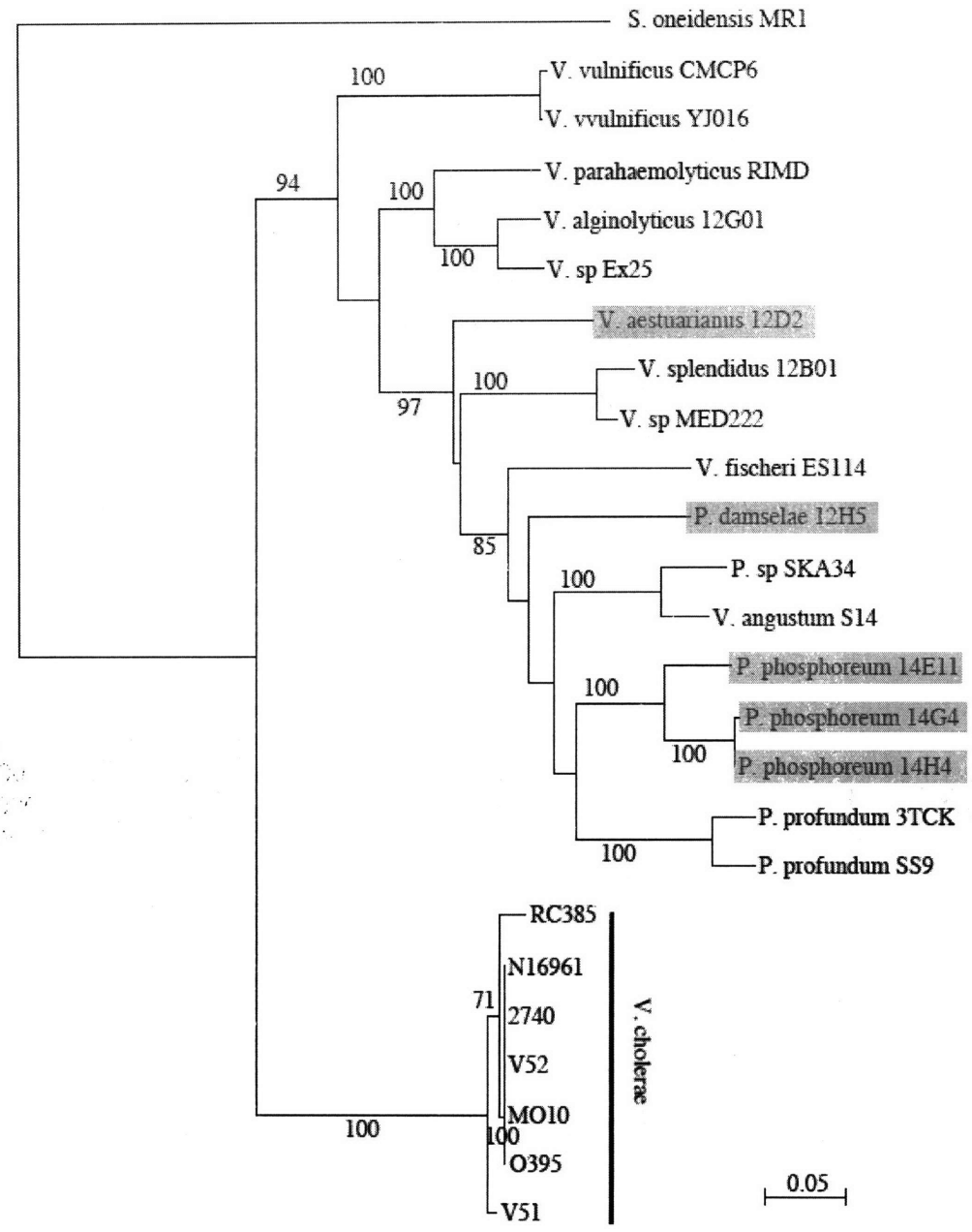


Figure S1 Phylogenetic relationship of vibrio isolates and sequenced genomes based on concatenated *mdh, adk* and *hsp60* genes. Isolates from this study are highlighted in grey.

WORKS CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410

Aluwihare LI, Repeta DJ, Pantoja S, Johnson CG (2005) Two chemically distinct pools of organic nitrogen accumulate in the ocean. Science 308:1007-1010

Amako K, Shimodori S, Imoto T, Miake S, Umeda A (1987) Effects of chitin and its soluble derivatives on survival of *Vibrio cholerae* O1 at low temperature. Applied and Environmental Microbiology 53:603-605

Bassler BL, Yu C, Lee YC, Roseman S (1991) Chitin utilization by marine bacteria - Degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. Journal of Biological Chemistry 266:24276-24286

Bouma CL, Roseman S (1996) Sugar transport by the marine chitinolytic bacterium *Vibrio furnissii* - Molecular cloning and analysis of the glucose and N-acetylglucosamine permeases. Journal of Biological Chemistry 271:33457-33467

Chaussard G, Domard A (2004) New aspects of the extraction of chitin from squid pens. Biomacromolecules 5:559-564

Colwell RR (1996) Global climate and infectious disease: The cholera paradigm. Science 274:2025-2031

Cottrell MT, Wood DN, Yu LY, Kirchman DL (2000) Selected chitinase genes in cultured and uncultured marine bacteria in the alpha- and gamma-subclasses of the proteobacteria. Applied and Environmental Microbiology 66:1195-1201

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5:1-19

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52:696-704

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin HY, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406:477-483

Heidelberg JF, Heidelberg KB, Colwell RR (2002) Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. Applied and Environmental Microbiology 68:5498-5507

Huq A, Sack RB, Nizam A, Longini IM, Nair GB, Ali A, Morris JG, Khan MNH, Siddique AK, Yunus M, Albert MJ, Sack DA, Colwell RR (2005) Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. Applied and Environmental Microbiology 71:4645-4654

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. Journal of Phycology 23:633-638

Keyhani NO, Li XB, Roseman S (2000) Chitin catabolism in the marine bacterium *Vibrio furnissii* - Identification and molecular cloning of a chitoporin. Journal of Biological Chemistry 275:33068-33076

Keyhani NO, Roseman S (1996a) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii* - Molecular cloning, isolation, and characterization of a periplasmic beta-N-acetylglucosaminidase. Journal of Biological Chemistry 271:33425-33432

Keyhani NO, Roseman S (1996b) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii* - Molecular cloning, isolation, and characterization of a periplasmic chitodextrinase. Journal of Biological Chemistry 271:33414-33424

Keyhani NO, Roseman S (1999) Physiological aspects of chitin catabolism in marine bacteria. Biochimica Et Biophysica Acta-General Subjects 1473:108-122

Kirchman DL, White J (1999) Hydrolysis and mineralization of chitin in the Delaware Estuary. Aquatic Microbial Ecology 18:187-196

Kirn TJ, Jude BA, Taylor RK (2005) A colonization factor links *Vibrio cholerae* environmental survival and human infection. Nature 438:863-866

Kita-Tsukamoto K, Oyaizu H, Nanba K, Simidu U (1993) Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae,* determined on the basis of 16S rRNA sequences. International Journal of Systematic Bacteriology 43:8-19

LeCleir GR, Buchan A, Hollibaugh JT (2004) Chitinase gene sequences retrieved from diverse aquatic habitats reveal environment-specific distributions. Applied and Environmental Microbiology 70:6977-6983

Li L, Stoeckert CJ, Roos DS (2003) OrthoMCL: Identification of ortholog groups for eukaryotic genomes. Genome Research 13:2178-2189

Li XB, Roseman S (2004) The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. Proceedings of the National Academy of Sciences of the United States of America 101:627-631

McCarthy M, Pratum T, Hedges J, Benner R (1997) Chemical composition of dissolved organic nitrogen in the ocean. Nature 390:150-154

Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK (2005) Chitin induces natural competence in *Vibrio cholerae*. Science 310:1824-1827

Meibom KL, Li XBB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK (2004) The *Vibrio cholerae* chitin utilization program. Proceedings of the National Academy of Sciences of the United States of America 101:2524-2529

Muzzarelli RAA (1973) Natural chelating polymers; alginic acid, chitin and chitosan, Vol. Pergamon press, Oxford

Nalin DR, Daya V, Reid A, Levine MM, Cisneros L (1979) Adsorption and growth of Vibrio cholerae on chitin. Infection and Immunity 25:768-770

Newman DK, Kennedy EK, Coates JD, Ahmann D, Ellis DJ, Lovley DR, Morel FMM (1997) Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. Archives of Microbiology 168:380-388

Orikoshi H, Nakayama S, Miyamoto K, Hanato C, Yasuda M, Inamori Y, Tsujibo H (2005) Roles of four chitinases (ChiA, ChiB, ChiC, and ChiD) in the chitin degradation system of marine bacterium *Alteromonas* sp strain O-7. Applied and Environmental Microbiology 71:1811-1815

Park JK, Keyhani NO, Roseman S (2000) Chitin catabolism in the marine bacterium *Vibrio furnissii* - Identification, molecular cloning, and characterization of a N-N

'-diacetylchitobiose phosphorylase. Journal of Biological Chemistry 275:33077-33083

Park JK, Wang LX, Patel HV, Roseman S (2002a) Molecular cloning and characterization of a unique beta-glucosidase from *Vibrio cholerae*. Journal of Biological Chemistry 277:29555-29560

Park JK, Wang LX, Roseman S (2002b) Isolation of a glucosamine-specific kinase, a unique enzyme of *Vibrio cholerae*. Journal of Biological Chemistry 277:15573-15578

Ramaiah N, Hill RT, Chun J, Ravel J, Matte MH, Straube WL, Colwell RR (2000) Use of a *chiA* probe for detection of chitinase genes in bacteria from the Chesapeake Bay. FEMS Microbiology Ecology 34:63-71

Reguera G, Kolter R (2005) Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. Journal of Bacteriology 187:3551-3555

Riemann L, Azam F (2002) Widespread *N*-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. Applied and Environmental Microbiology 68:5554-5562

Santos SR, Ochman H (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. Environmental Microbiology 6:754-759

Svitil AL, Chadhain SMN, Moore JA, Kirchman DL (1997) Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Applied and Environmental Microbiology 63:408-413

Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J (2005a) Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Applied and Environmental Microbiology 71:5107-5115

Thompson JR, Pacocha S, Pharino C, Klepac-Ceraj V, Hunt DE, Benoit J, Sarma-Rupavtarm R, Distel DL, Polz MF (2005b) Genotypic diversity within a natural coastal bacterioplankton population. Science 307:1311-1313

Thompson JR, Polz MF (2006) Dynamics of vibrio populations and their role in environmental nutrient cycling. In: Thompson FL, Austin B, Swings J (eds) The Biology of *Vibrios*. ASM Press, Washington DC, p 190-203

Thompson JR, Randa MA, Marcelino L, Tomita-Mitchell A, Lim E, Polz MF (2004) Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Applied and Environmental Microbiology 70:4103-4110

Yang C, Rodionov DA, Li XQ, Laikova ON, Gelfand MS, Zagnitko OP, Romine MF, Obraztsova AY, Nealson KH, Osterman AL (2006) Comparative genomics and experimental characterization of N-acetylglucosamine utilization pathway of *Shewanella oneidensis*. Journal of Biological Chemistry 281:29872-29885

Chapter Four

Microscale spatial coupling of phytoplankton and bacteria in the coastal ocean[1]

[1] To be submitted with co-authors: JR Seymour, D Veneziano, R Stocker, and MF Polz

ABSTRACT

Phytoplankton, as the most important primary producers in the oceans, control the activity of heterotrophic bacteria. It has been speculated that bacteria cluster around photosynthesizing phytoplankton to enhance their uptake of released organic matter. Here, by counting microscale seawater samples over a seasonal cycle, we show that bacterial patchiness increases with eukaryotic cell abundance. Additionally, a positive correlation was observed between the numbers of eukaryotic and prokaryotic cells in individual microscale samples both within and between months, suggesting that bacteria could be clustering around phytoplankton. Using a model system to investigate this clustering further, the marine diatom *Thalassiosira weissflogii* was shown to elicit a chemotactic response in a *Roseobacter* strain to both photosynthetic exudate and dead algal cells. A bacterial motility mutant was found to be deficient in colonization of dead *T. weissflogii*, suggesting that motility is an important factor in utilization of phytoplankton resources. This research presents the first evidence for *in situ* clustering of bacteria around phytoplankton; aggregation around nutrient point sources such as algae may be an important lifestyle in aquatic bacteria.

INTRODUCTION:

Although the coupled action of phytoplankton and bacteria drive biogeochemical cycling in the world's oceans, very little is know about *in situ* spatial interactions between primary producers and heterotrophic bacteria. Coupling of photosynthesis and bacterial production is well established in bulk samples from the photic zone, where

bacterial abundance and production are positively correlated with chlorophyll levels (Gasol & Duarte 2000, Li et al. 2006, Kent et al. 2007). Up to 60% of photoassimilated carbon can be leaked or released by phytoplankton (Lancelot 1979), although the amount can be considerably less (Granum et al. 2002). Such released photosynthate is thought to be a major source of organic matter for bacterioplankton (Lancelot 1979, Azam & Cho 1987). In order to gain better access to released photosynthate, bacteria may cluster around phytoplankton; Bell and Mitchell (1972) first suggested the importance of this "phycosphere", the region surrounding a photosynthesizing alga analogous to the rhizosphere of plants.

The overall importance of bacterial clustering around algae remains controversial: some models predict no clustering (Jackson 1989) or clustering only in special low-turbulence regions (Mitchell et al. 1985), while others estimate that up to 20% of chemotactic bacteria reside in the phycosphere (Bowen et al. 1993). Work with cultured isolates has shown that some bacteria are chemotactic to algal exudates (Bell & Mitchell 1972) and can track motile algae (Barbara & Mitchell 2003). However, the few direct observations of bacterial clustering around live algae were unable to differentiate aerotaxis (to released oxygen) from chemotaxis (to released photosynthate) (Blackburn et al. 1998, Barbara & Mitchell 2003).

If the extent of bacterial clustering is condition-dependent it may be difficult to observe in seawater samples. In order for chemotaxis-driven bacterial patchiness to occur, several conditions likely have to be met: the bacterial population has to be actively motile, chemo-effectors have to be spatially localized, and bacteria must be chemotactic to these substrates. Further, the experimental design must sample at the

appropriate spatial scale to capture bacterial dynamics. Failure in one or more of these conditions may have prevented observation of clustering in previous experiments. In a mesocosm experiment, there was no observed bacterial clustering around phytoplankton in either natural or algae-amended (to 1000 cells/ml of *Chaetoceros muelleri*) seawater samples (Müller-Niklas et al. 1996). Possibly the bacterial cells were non-motile or the algae were not a good chemoattractant. A lack of bacterial motility could have limited clustering in another study, bacterial patchiness was observed only several hours after amendment with lysed diatoms and was not associated with the distribution of algal cells (Krembs et al. 1998). Here, algal nutrients may have indirectly stimulated patchiness by up-regulating motility or shifting the bacterial population to more motile phylotypes (Krembs et al. 1998). Moreover, observations of bacterial patchiness have sampled volumes much larger than the size predicted for chemotaxis-driven patches (Seymour et al. 2000, Daubin et al. 2003, Seymour et al. 2004), suggesting that these studies may not capture bacterial aggregation but rather mixing of water bodies or suspension of sediment/particles. While bacteria can cluster around phytoplankton in artificial systems, the importance of this association is not known in natural waters, with clustering potentially occurring only under specific conditions.

Interactions between heterotrophic bacteria and algae are likely much more complex than the classical view of fixed carbon release by algae and remineralization of nutrients by bacteria. Although heterotrophs depend on carbon fixed by phytoplankton, they concurrently compete with them for macronutrients such as nitrogen and phosphorous. In nutrient-limited culture, bacteria out-compete algae for phosphorous (Rhee 1972), resulting in P-starved algae that release more DOM (Guerrini et al. 1998,

Mindl et al. 2005). Over time, heterotrophs become co-limited by phosphorus and carbon, thus limiting their abundance relative to the algae (Mindl et al. 2005). Algae may also be dependent on bacteria. Vitamin B_{12}-requiring phytoplankton have recently been shown to obtain this co-factor from bacteria (Croft et al. 2005). Additionally, it is difficult to culture phytoplankton axenically, although the function bacteria serve is unknown (Grossart 1999). Moreover, algae may influence bacterial physiology through the release of cAMP, a metabolic regulator that increases the production of catabolic enzymes; their action may release nutrients that can be taken up by algal cells (Azam & Ammerman 1984). While large-scale coupling of bacteria and phytoplankton is driven by carbon fixation, finer scale interactions may include elements of mutualism, commensalism and parasitism.

MATERIALS AND METHODS

Field sampling and counting:

Field samples were collected roughly monthly on the rising tide from the marine end of Plum Island Sound (Ipswitch, MA) over a seasonal cycle. Nutrients were quantified by the Marine Biological Laboratory. To measure environmental variability at multiple scales seawater volumes ranging five orders of magnitude from 1 µl to 10 mls were sampled. Samples were taken at larger distances as sample volume increased, parallel to the shoreline (Figure 1); and greater numbers of samples were taken for smaller volumes (with >30 replicates taken for 1 µl volume). Seawater for cell counts was pipetted into individual sterile containers, fixed in 3.7% formaldehyde (final concentration) and stored

at -20°C until staining. Both prokaryotic and eukaryotic cells were counted by staining with 4', 6 diamidino-2-phenylindole (DAPI) and viewed with epifluorescence microscopy (Porter & Feig 1980). Eukaryotic cells were identified by a visible nucleus. For 1 μl samples of seawater all cells were counted; for all other sample volumes, samples were filtered such that at least 20 cells were present per field and 20 fields were counted. In order to assess experimental variability in the sampling method, natural seawater was filtered through a 1 μm pore-size filter to remove particles and eukaryotic cells before homogenization by vortexing. This water was subsampled and counted following the same protocol used for the *in situ* seawater experiments.

Culture strains and conditions:

Phytoplankton cultures of *Thalassiosira pseudonanna* CCMP 1336 and *Synnechococcus elongatus* were obtained from the Provosoli Guillard Center for Culture of Marine Phytoplankton and cultured in F/2 (Guillard 1975). *Roseobacter* strain TM1040 along with the non-motile mutant TM2014 (TM1040 with Tn5-transposon insertion in *flaA*) was obtained from Robert Belas, (University of Maryland Biotechnology Institute) and cultured in 0.5x 2216 medium (Difco) (Miller et al. 2004, Miller & Belas 2006). *Roseobacter* strain Y4I and motility mutant Y4I1AA7 (Tn5-transposon insertion in histidine sensor kinase) were obtained from Alison Buchan (University of Tennessee, Knoxville) and grown on YTSS medium (yeast extract 4 g/L, tryptone 2.5 g/ L with a seawater base).

Microfluidic chemotaxis testing

Chemotaxis of bacteria to exudates in phytoplankton culture spent media was assessed using a microfluidic device. The microfluidic device (described in detail in Seymour et al, in preparation) consists of a 25 mm long, 3 mm wide by 50 μm deep channel, with two in-line inlet points, used to separately introduce bacteria and potential attractants (e.g., algal exudates) via individual glass syringes (Figure 2). Bacteria and substrates were simultaneously added to the channel at a flow rate of 240 μm s^{-1} using a PHD 2000 syringe pump (Harvard Apparatus). The substrate inlet introduced the potential chemoattractant as a 300 μm band in the center of the microchannel with fluid containing bacterial cells on both sides of the substrate layer. Chemotaxis of bacteria was assessed by stopping the flow of fluid in the channel (T=0), allowing diffusion of the substrate and free swimming by the bacteria. The positions and swimming paths of individual cells at mid-depth in the channel were obtained at 2 minute intervals for 8 minutes by recording sequences of 200-400 frames at 32 frames per second using a 1600x1200 pixel, 14 bit, cooled CCD camera (PCO 1600, Cooke). Chemotaxis to phytoplankton exudates was assessed by qualitatively comparing bacterial swimming tracks in spent and fresh F/2 media, ensuring that the nutrients in the fresh media did not serve as an important attractant for the bacteria.

Chemotaxis experiments

For chemotaxis experiments, bacteria were grown to exponential phase in wide mouth flasks at room temperature (~23°C) at 175 rpm. Cells were pelleted using low speed centrifugation 2,500 x g and washed several times in artificial seawater or F/2 to remove traces of media. Dead algae (*T. weissflogii*) were prepared by pelleting algae

(3,500 x g) and heat shocking them at 80°C for 3 minutes. Bacterial cultures and dead algae were placed in a Secure-Seal chamber (Sigma-Aldrich) and interactions were observed under the microscope. The locations of algal cells were positively identified using chlorophyll fluorescence.

RESULTS AND DISCUSSION

Microscale samples revealed patchy bacterial cell numbers which appeared to be related to eukaryotic cell counts. Examining bulk cell abundance over an entire year revealed the lowest total numbers in late winter and the highest numbers in early summer, overall ranging over an order of magnitude from 5×10^5 to 5×10^6 per ml, as is typically observed in temperate coastal environments. Beyond these seasonal changes, the number of bacteria among individual microscale samples varied up to 8-fold within a single sampling date. This is consistent with previous observations of up to 16-fold variation in microscale cell counts (Seymour et al. 2000). The greatest variability was observed for the 1 µl samples; however, 10 µl and larger samples, which were counted for the initial time points, up to 4-fold variability was observed. Averages of cell counts obtained from 1 µl samples were in good agreement with those of larger scale samples (Table 1), suggesting that the environment was well sampled by this method. Because the 1 µl volume corresponds to the size at which biologically driven interactions are projected to occur (clustering, particle attachment etc) (Blackburn et al. 1998), the remainder of the paper focuses on this sampling scale.

The patchiness of cell counts is reported using the coefficient of variation (CV), which normalizes the standard deviation by the mean thus reducing the effect of overall

shifts in cell numbers (Figure 3). Figure 3 reveals variation in patchiness peaks in April and September samples, corresponding to predicted phytoplankton blooms in the estuary; and lows during the winter months when algal and bacteria cell concentration is minimal (Figure 3, Table 1). This peak in CV was repeated in April of the following year, suggesting a common seasonal trigger (Figure 3). Alternate drivers of these observed peaks in patchiness were investigated. One possible cause examined were low bulk nutrients which made chemotaxis or attachment more energetically favorable. However, patchy bacteria could also be clustering around algae or attached to particles, which are unevenly distributed in the environment.

The idea that clustering might be induced in response to a limiting nutrient was tested by comparing the patchiness to the concentration of various nutrients (Figure 4). However, there was no obvious relationship between the extent of patchiness and any of the bulk water column nutrients measured (DOC, TDN, TDP, nitrate, ammonia, and phosphate). It is possible that either nutrient limitation does not stimulate clustering or attachment or that the range of concentration present in the coastal ocean always limits bacterial productivity.

Another potential explanation for the observed bacterial patchiness is attachment to particles. The fraction of bacteria attached to particles was estimated two ways, by counting the number of cells either retained on a 1 μm filter or those visibly attached to DAPI stained particles in 1 μl samples. Both measures reported that ~10% of cells were particle attached. Thus, particle attachment does not seem to contribute the observed bacterioplankton patchiness, with the possible exception of the September 2003 time

point, which showed a positive trend between the number of visibly attached cells and total cell counts in 1 μl samples.

A comparison of the average number of eukaryotic cells and versus the CV yielded a positive correlation (Figure 5). There appear to be two separate trend lines within the data, representing variation with an unknown variable; however without further sampling it may not be possible to determine if these are valid distinct trends or represent scatter in the data (Figure 5). Prokaryotic cells were never observed attached to individual eukaryotic cells, suggesting attachment to phytoplankton is not the source of patchiness in this study. This data supports the idea that bacteria may cluster around phytoplankton *in situ*. Previous studies may have failed to note this effect since it only becomes apparent at high phytoplankton concentrations or blooms (Müller-Niklas et al. 1996).

The relationship between prokaryotic and eukaryotic cells within individual 1 μl samples, was examined to determine if bacteria indeed cluster around algae or if high algal concentrations merely stimulate bacterial motility, allowing cells to cluster around another marine point source. To reduce the influence of particle attachment on patchiness, the number of free-living prokaryotic cells was plotted against the number of eukaryotic cells observed in the same 1 μl sample (Figure 6). A strong positive trend was observed in the data between the number of prokaryotic and eukaryotic cells in each sample. In order to fit a line to this trend the data was binned by averaging prokaryotic cell numbers for a given number of observed eukaryotic cells; the error bars indicate one standard deviation of the raw data (Figure 6). The binned data reveals a strong

relationship across all samples between prokaryotic and eukaryotic cell numbers. The equation for an empirically fitted line was obtained:

Prokaryotic cells= 1550 +35(Eukaryotes) –exp(-Eukaryotes*0.5)

Thus there is a background of bacteria in all samples and an additional ~35 prokaryotic cells are present per eukaryotic cell, suggesting a consistent spatial relationship that may be due to clustering. However, alternate explanations are that algae and bacteria chemotax to a common nutrient source or that patchiness reflects a process other than clustering such as suspension of high cell density sediment. Although peaks of patchiness are observed in the spring and the fall corresponding to phytoplankton bloom periods, there is a linear trend between the number of eukaryotic cells and CV over the entire season (Figure 5). Clustering could thus be occurring even at low phytoplankton cell numbers; however, there may be an experimental threshold concentration of algae for which it is no longer possible to detect clustering (e.g. <1 per μl in the winter months).

A model system of motile *Roseobacter* and the diatom *T. weissflogii* was developed to test the energetic benefits and potential limits of chemotactic clustering in greater detail. This approach allows testing if (i) bacteria can sense and cluster around small point sources like leaking algae, and (ii) the energetics of clustering are dependent on the density of the point sources (i.e. does the cost of swimming outweigh the benefit obtained from clustering at some threshold of low algal abundance?). Constant swimming by bacteria may be a poor strategy when nutrient point sources (such as algae) are sparse. In fact, the percentage of motile bacteria has been shown to vary on seasonal cycles with the lowest numbers in winter (Fenchel 2001), suggesting that either the type

of bacteria changes or that swimming is down-regulated in motile bacteria. We can develop a simple cost-benefit model of cell energetics to better understand this concept:

$$\mathbf{E_{cell} = E_{background} + E_{point\ sources} - E_{motility}}$$

The energy level of the cell (E_{cell}) is the energy derived from background nutrient concentrations ($E_{background}$) plus the energy derived from clustering around point sources ($E_{point\ sources}$) minus the cost of swimming ($E_{motility}$). According to this model cells should swim (and cluster) as long as the encounter rate of point sources nets more energy than the cost of motility.

This simple model was tested using the two heterotrophic *Roseobacter* strains TM1040 and Y41 and the diatom *Thalassiosira weissflogii*; alpha Proteobacteria and *Roseobacter* in particular appear tuned to make use of phytoplankton products: they are abundant in phytoplankton culture (Grossart et al. 2005), colonize particles in algal blooms (Riemann et al. 2000), exhibit chemotaxis towards algal products (Miller et al. 2004) and degrade the algal osmolite dimethylsulfoniopropionate (Moran et al. 2003, Moran et al. 2004). Thus of the cultured bacteria, they appear to be a good candidate for clustering around phytoplankton. *Thalassiosira* is a genera of common coastal diatoms and have served as a longstanding model as a bacterial chemoattractant. Live or dead *T. weissflogii* cells have been shown to increase the number of motile bacteria in seawater samples (Grossart et al. 2001) and dead cells induced clustering in bacteria (Long & Azam 2001).

We first asked if the bacteria were attracted to algal extracellular products before investigating if the bacteria could use these chemotactic signals to localize around individual algae. In order to determine if *Roseobacter* strains were chemotactic to

phytoplankton exudates, a microfluidics device was used to assess bacterial chemotaxis to 0.22 μm filtered phytoplankton spent medium. This microfluidic assay revealed positive chemotaxis to spent medium of both the diatom *T. weissflogii* and cyanobacterium *Synnechococcus elongatus* compared to a blank of fresh media, indicating that the bacteria were responding to material produced by the phytoplankton not the inorganic nutrients or vitamins in F/2 (Figure 7).

Once it was established that these bacteria were attracted to material produced by the phytoplankton, this interaction was investigated further by mixing heat-shocked algae with the *Roseobacter* strains. Motile *Roseobacter* were observed to chemotax towards and attached to the dead algae (Figure 8) Non-motile bacterial mutants lacked the ability to colonize the dead algae, and thus will likely make poor use of spatially localized resources. When this experiment was repeated with algae in exponential phase no clustering was observed, although it is possible that ambient lighting/ growth phase etc. was not appropriate to induce photosynthesis. In may be possible that the bacteria did not sense the algae due to nutrient carry over from the media or that the low concentration of nutrients produced by photosynthesizing algae was not enough to induce chemotaxis in these bacteria. It remains an open question if the benefit received by interaction with spatially localized resources outweighs the energetic cost of chemotaxis and motility.

This paper represents the first evidence of *in situ* clustering of bacteria around phytoplankton. There has been much speculation as to the importance of phytoplankton-induced clustering. Although previous experiments have failed to observe these interactions, potentially due to inactive bacterial populations, poor chemoattractive

phytoplankton or sampling at the wrong scale. Bacteria may have the capability to cluster around algae; however the extent and importance of clustering may vary over time, with peaks coinciding with phytoplankton bloom events (Figure 3). While most modeling focuses on clustering around actively photosynthesizing bacteria, it may actually be dying algal blooms, which induce clustering (Grossart et al. 2001) or attachment (Verity et al. 1988). This is suggested by our model bacteria being chemotactic to the spent media/dead algal cells but not to exponential phase *T. weissflogii*. As nutrient-limited phytoplankton release more carbon, an exponentially growing culture may not be a good proxy for the highly starved phytoplankton of the oceans. A previous study of the motility response of natural bacterial populations to algae found only a dying algal bloom stimulated bacterial motility (Grossart et al. 2001). Bacterial chemotaxis may be the means to locate a nutrient source for attachment. Within a few seconds, motile marine bacteria rapidly attach to even non nutriative surfaces such as glass (Fenchel 2001), and can colonize algae intracellularly (Miller & Belas 2006).

This study suggests that bacteria may cluster around phytoplankton in the oceans and that chemotaxis-driven clustering could dramatically enhance the nutrient uptake of bacteria by enabling them to take advantage of these hotspots. However, the extent of this relationship remains unclear: do bacteria cluster around phytoplankton at all times and or does clustering occur only under certain circumstances like blooms or dying algae. By examining the ocean at the macroscale, do we neglect the important features occur at the bacterial scale and their importance for biogeochemical cycling of the world's oceans?

WORKS CITED:

Azam F, Ammerman JW (1984) Cycling of organic matter by bacterioplankton in pelagic marine ecosystems: microenvironmental considerations. In: Fasham MJR (ed) Flows of energy and materials in marine ecosystems. Plenham Press, New York, p 345-360

Azam F, Cho BC (eds) (1987) Bacterial utilization of organic matter in the sea, Vol. Cambridge University Press, New York, NY

Barbara GM, Mitchell JG (2003) Bacterial tracking of motile algae. FEMS Microbiology Ecology 44:79-87

Bell W, Mitchell R (1972) Chemotactic and growth responses of marine bacteria to algal extracellular products. The Biological Bulletin 143:265-277

Blackburn N, Fenchel T, Mitchell J (1998) Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science 282:2254-2256

Bowen JD, Stolzenbach KD, Chisholm SW (1993) Simulating bacterial clustering around phytoplankton cells in a turbulent ocean. Limnology and Oceanography 38:36-51

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature 438:90-93

Daubin V, Moran NA, Ochman H (2003) Phylogenetics and the cohesion of bacterial genomes. Science 301:829-832

Fenchel T (2001) Eppur si muove: many water column bacteria are motile. Aquatic Microbial Ecology 24:197-201

Gasol JM, Duarte CM (2000) Comparative analyses in aquatic microbial ecology: how far do they go? FEMS Microbiology Ecology 31:99-106

Granum E, Kirkvold S, Myklestad SM (2002) Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion. Marine Ecology-Progress Series 242:83-94

Grossart HP (1999) Interactions between marine bacteria and axenic diatoms (*Cylindrotheca fusiformis, Nitzschia laevis,* and *Thalassiosira weissflogii*) incubated under various conditions in the lab. Aquatic Microbial Ecology 19:1-11

Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. Environmental Microbiology 7:860-873

Grossart HP, Riemann L, Azam F (2001) Bacterial motility in the sea and its ecological implications. Aquatic Microbial Ecology 25:247-258

Guerrini F, Mazzotti A, Boni L, Pistocchi R (1998) Bacterial-algal interactions in polysaccharide production. Aquatic Microbial Ecology 15:247-253

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of Marine Invertebrate Animals. Plenum Press, New York, NY, p 26-60

Jackson GA (1989) Simulation of bacterial attraction and adhesion to falling particles in an aquatic environment. Limnology and Oceanography 34:514-530

Kent AD, Yannarell AC, Rusak JA, Triplett EW, McMahon KD (2007) Synchrony in aquatic microbial community dynamics. The ISME Journal 1:38-47

Krembs C, Juhl AR, Long RA, Azam F (1998) Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. Limnology and Oceanography 43:307-314

Lancelot C (1979) Gross excretion rates of natural marine phytoplankton and heterotrophic uptake of excreted products in the southern North Sea, as determined by short-term kinetics. Marine Ecology-Progress Series 1:179-186

Li WKW, Harrison WG, Head EJH (2006) Coherent sign switching in multiyear trends of microbial plankton. Science 311:1157-1160

Long RA, Azam F (2001) Microscale patchiness of bacterioplankton assemblage richness in seawater. Aquatic Microbial Ecology 26:103-113

Miller TR, Belas R (2006) Motility is involved in *Silicibacter* sp TM1040 interaction with dinoflagellates. Environmental Microbiology 8:1648-1659

Miller TR, Hnilicka K, Dziedzic A, Desplats P, Belas R (2004) Chemotaxis of *Silicibacter* sp strain TM1040 toward dinoflagellate products. Applied and Environmental Microbiology 70:4692-4701

Mindl B, Sonntag B, Pernthaler J, Vrba J, Psenner R, Posch T (2005) Effects of phosphorus loading on interactions of algae and bacteria: reinvestigation of the 'phytoplankton-bacteria paradox' in a continuous cultivation system. Aquatic Microbial Ecology 38:203-213

Mitchell JG, Okubo A, Fuhrman JA (1985) Microzones surrounding phytoplankton form the basis for a stratified marine microbial ecosystem. Nature 316:58-59

Moran MA, Buchan A, Gonzalez JM, Heidelberg JF, Whitman WB, Kiene RP, Henriksen JR, King GM, Belas R, Fuqua C, Brinkac L, Lewis M, Johri S, Weaver B, Pai G, Eisen JA, Rahe E, Sheldon WM, Ye WY, Miller TR, Carlton J, Rasko DA, Paulsen IT, Ren QH, Daugherty SC, Deboy RT, Dodson RJ, Durkin AS, Madupu R, Nelson WC, Sullivan SA, Rosovitz MJ, Haft DH, Selengut J, Ward N (2004) Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. Nature 432:910-913

Moran MA, Gonzalez JM, Kiene RP (2003) Linking a bacterial taxon to sulfur cycling in the sea: Studies of the marine *Roseobacter* group. Geomicrobiology Journal 20:375-388

Müller-Niklas G, Agis M, Herndl GJ (1996) Microscale distribution of bacterioplankton in relation to phytoplankton: Results from 100-nl samples. Limnology and Oceanography 41:1577-1582

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography 25:943-948

Rhee G-Y (1972) Competition between an alga and an aquatic bacterium for phosphate. Limnology and Oceanography 17:505-514

Riemann L, Steward GF, Azam F (2000) Dynamics of bacterial community composition and acitivity during a mesocosm diatom bloom. Applied and Environmental Microbiology 66:578-587

Seymour JR, Mitchell JG, Pearson L, Waters RL (2000) Heterogeneity in bacterioplankton abundance from 4.5 millimetre resolution sampling. Aquatic Microbial Ecology 22:143-153

Seymour JR, Mitchell JG, Seuront L (2004) Microscale heterogeneity in the activity of coastal bacterioplankton communities. Aquatic Microbial Ecology 35:1-16

Verity PG, Villareal TA, Smayda TJ (1988) Ecological investigations of blooms of colonial *Phaeocystis pouchetti* . II The role of life-cycle phenomena in bloom termination. Journal of Plankton Research 10:749-766

FIGURES AND TABLE

Table 1. Comparison of cell counts for 10 ml and 1 µl samples

Sample Date	10ml (*10^5/ml)	1µl (*10^5/ml)
April 2003	21.8	21.6
June 2003	13.6	14.4
July 2003	23.2	23.2
August 2003	27.2	22.2
September2003	51.9	44.9
October 2003	19.8	22.8
November2003	18	19.5
January 2004	3.9	5.5
March 2004	5.8	12.7
April 2004	12.9	17.8
May 2004	31.5	22.2

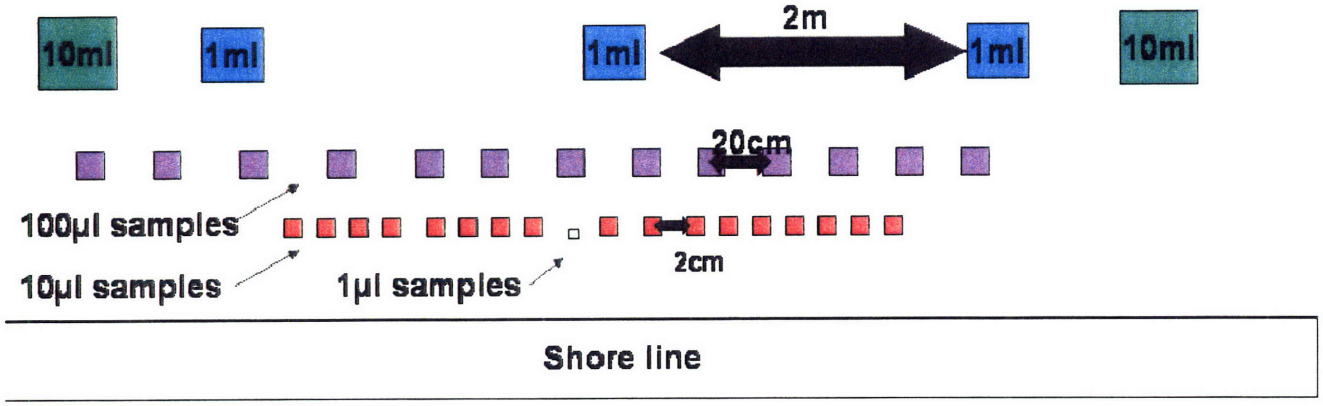


Figure 1. Seawater sampling scheme to capture environmental variability at multiple spatial scales. Seawater samples ranged from 1µl to 10ml with sample size and distance between samples concomitantly increasing by an order of magnitude. Samples at the same size range were taken parallel to shore moving against the water motion to avoid re-sampling the same piece of water.

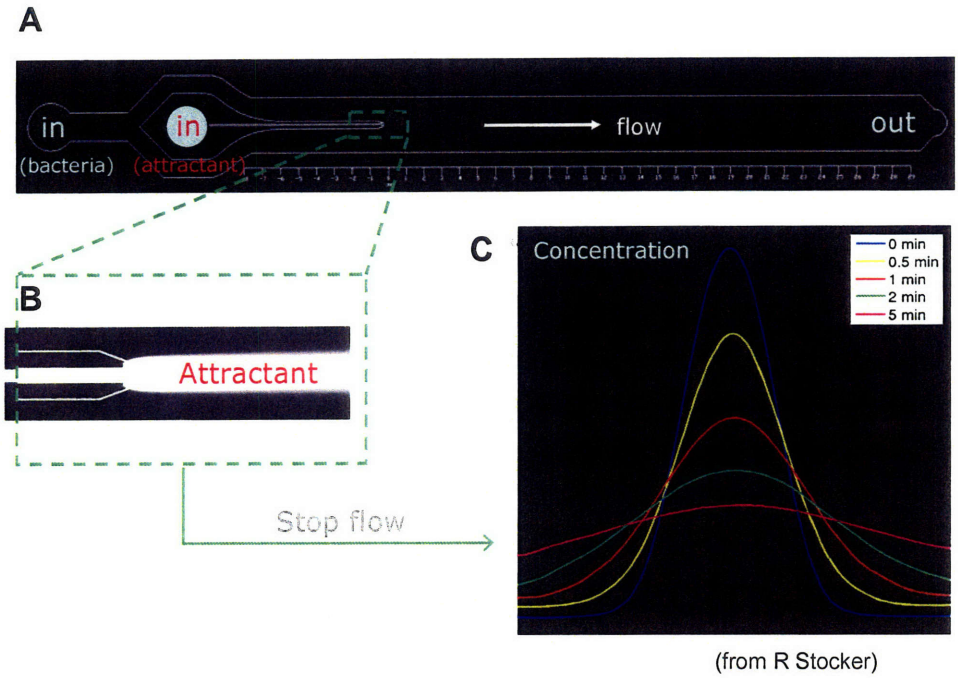


Figure 2. Microfluidics channel design. A. Outline of the channel showing inlets for the bacteria and attractant (center), which are both controlled by an external syringe pump, with flow traveling to the right. B. Inlet for the attractant visualized using fluorescene dye. C. Diffusion of substrate across the width of the channel after stoppage of flow allowing bacterial chemotaxis to occur. (Provided by R. Stocker)

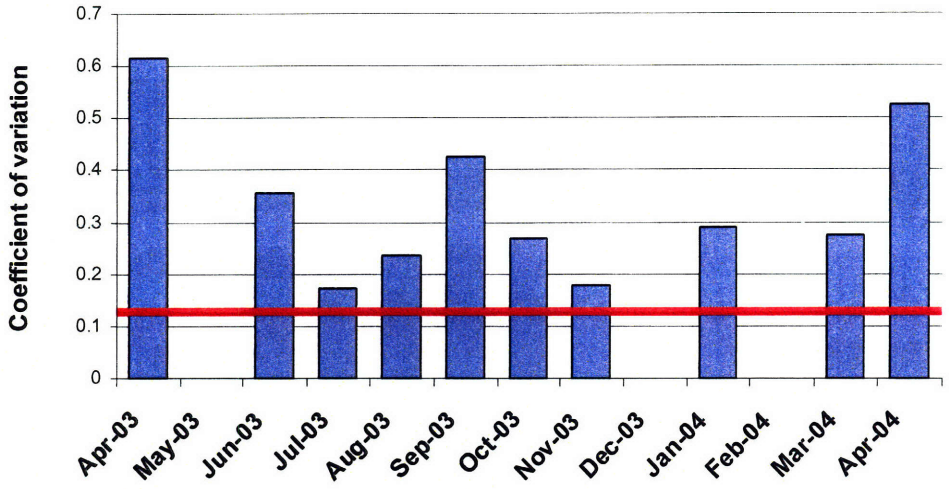
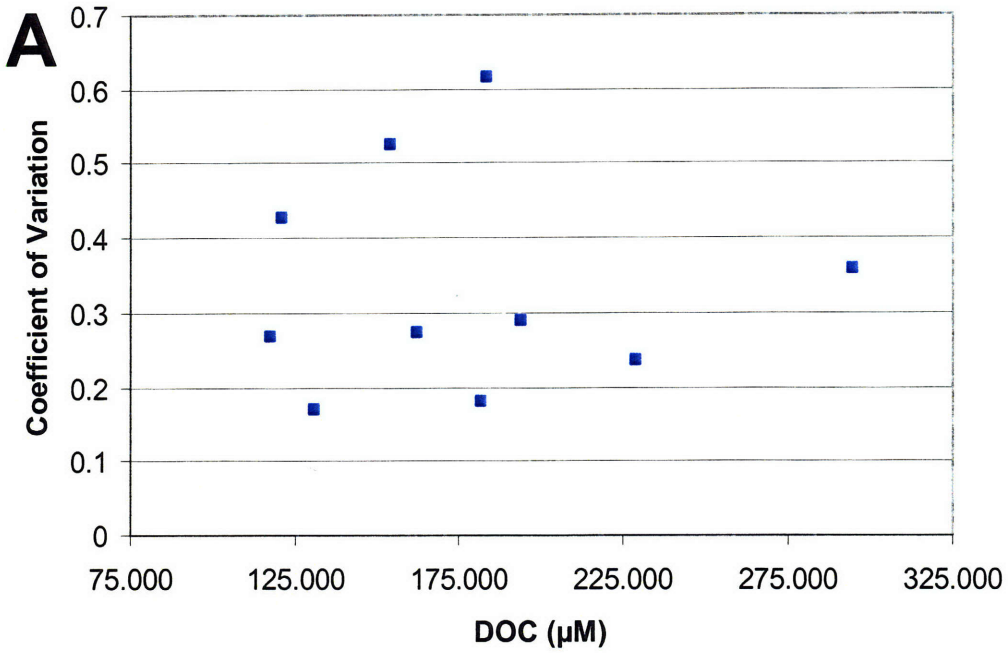
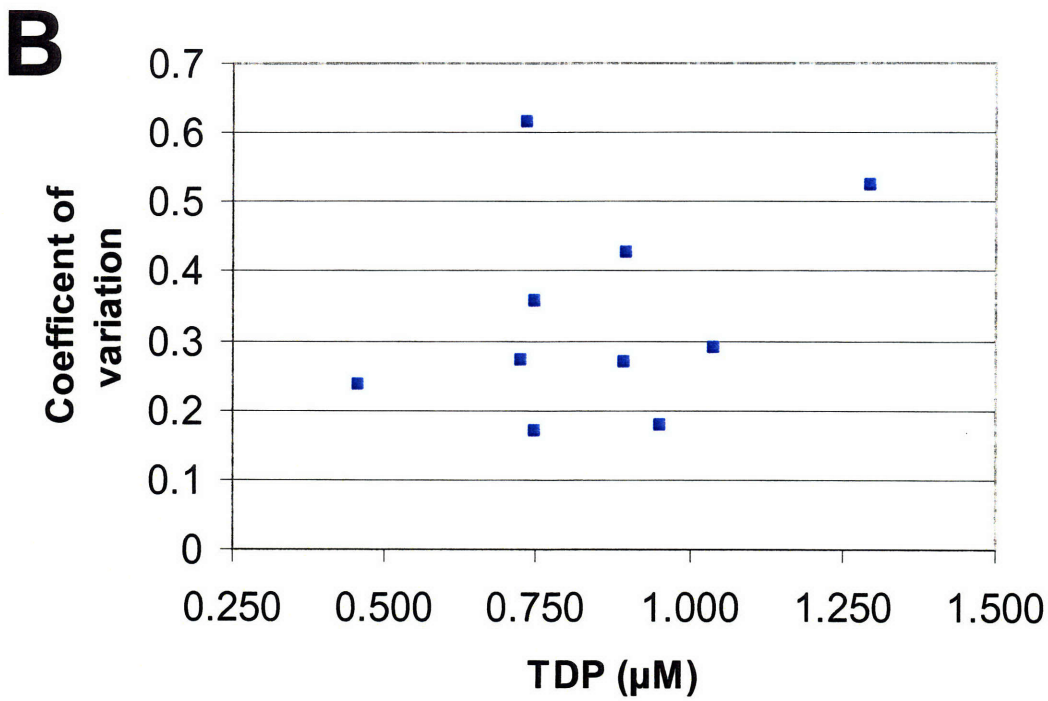
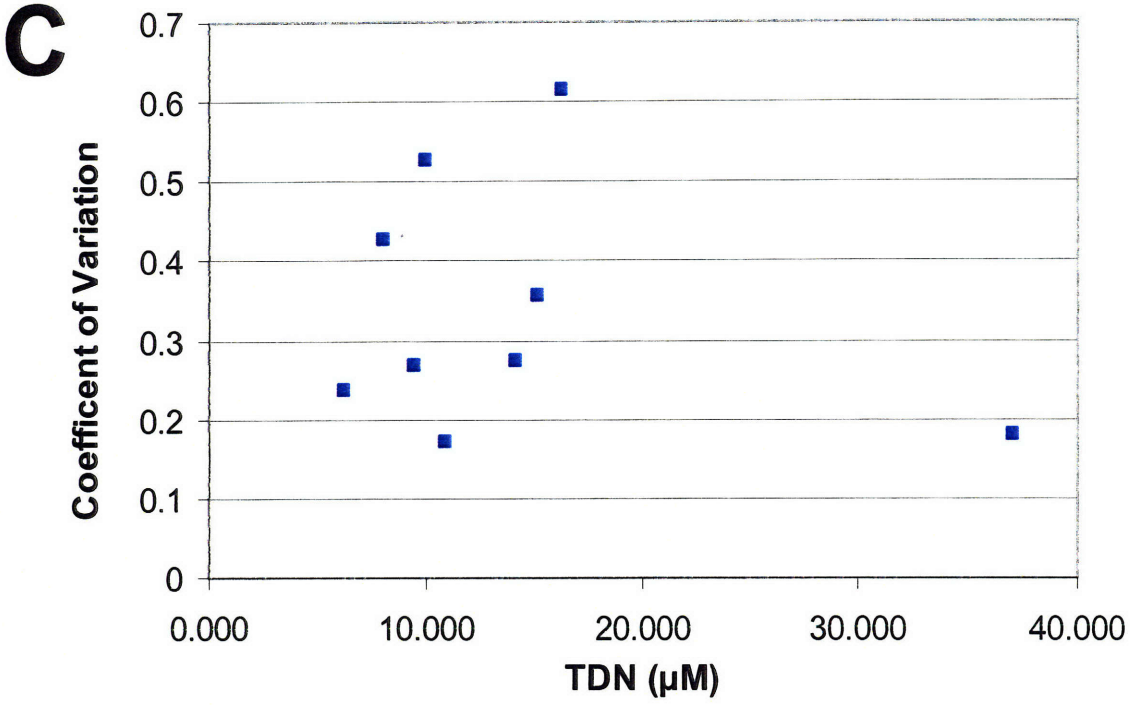


Figure 3. Coefficient of variation (CV) for 1µl samples measured over a seasonal cycle. Peaks in CV correspond roughly to peaks in eukaryotic cell abundance. Red line indicates the CV obtained for filtered and homogenized seawater subsampled in the same manner as monthly samples.

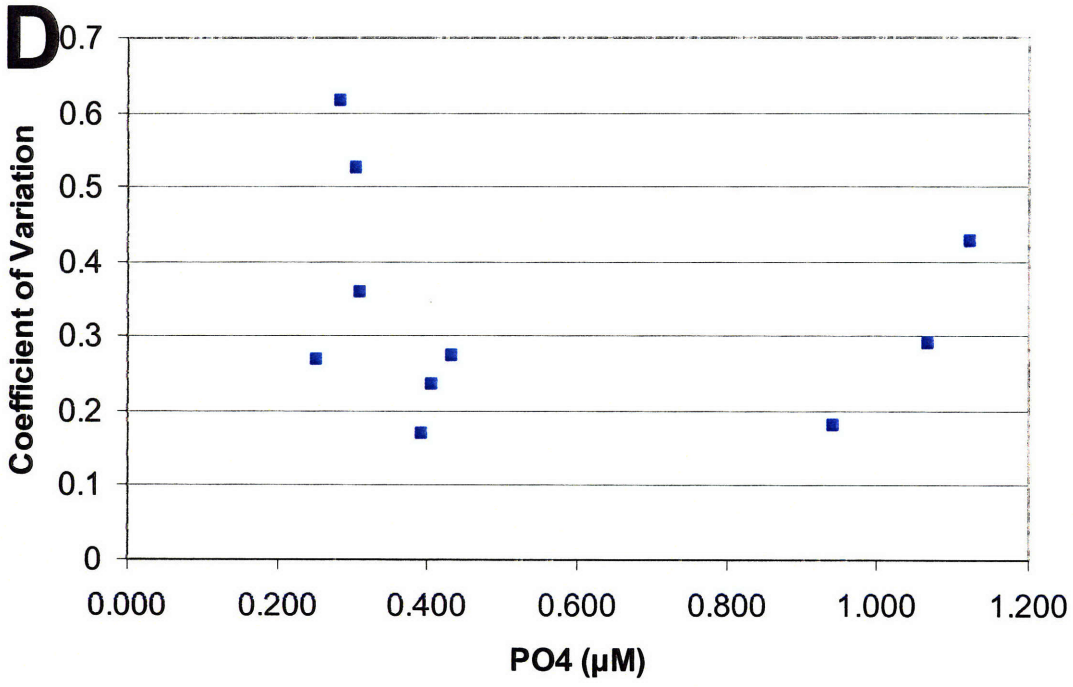

A
Coefficient of Variation
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
75.000
125.000
175.000
225.000
275.000
325.000
DOC (µM)


B
Coefficent of variation
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.250
0.500
0.750
1.000
1.250
1.500
TDP (µM)

C


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Coefficent of Variation
0.000
10.000
20.000
30.000
40.000
TDN (µM)

D


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Coefficient of Variation
0.000
0.200
0.400
0.600
0.800
1.000
1.200
PO4 (µM)

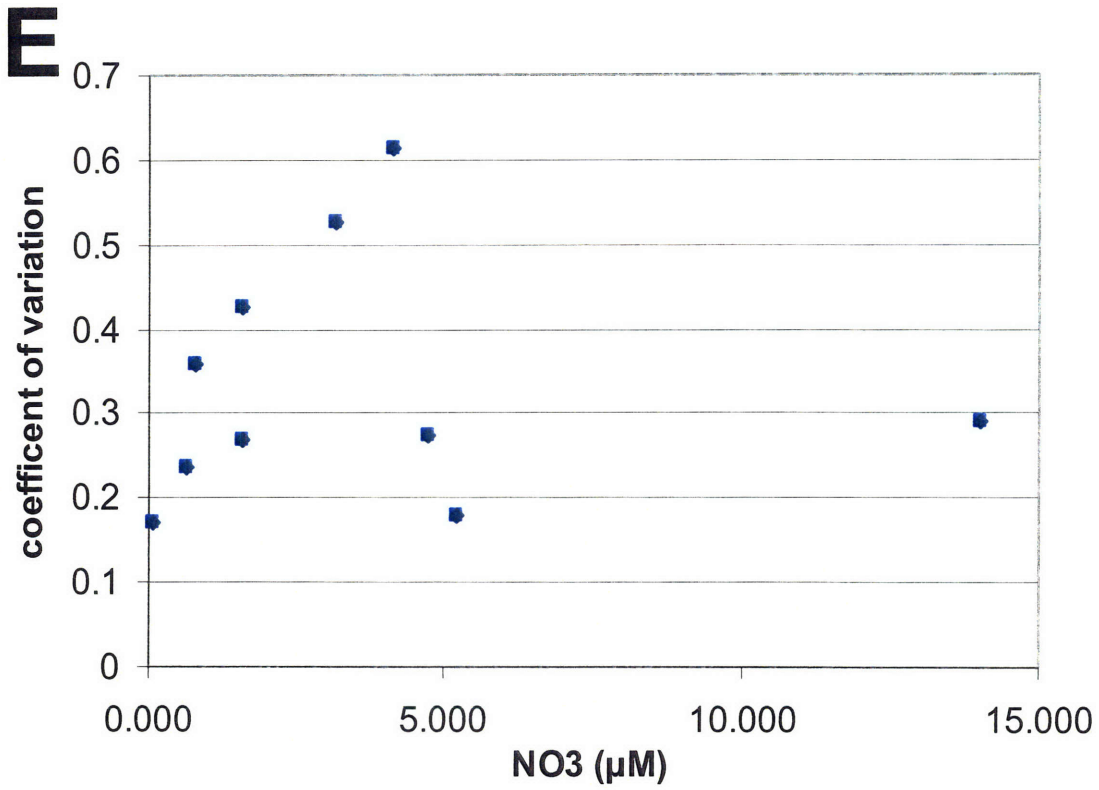


Figure 4 Comparison of different organic and inorganic nutrient concentrations with the coefficient of variation of prokaryotic cell abundance (1 μl samples). A. Dissolved Organic Carbon (DOC), B. Total Dissolved Phosphorus (TDP) C. Total Dissolved Nitrogen (TDN), D. Phosphate (PO_4), E. Nitrate (NO_3).

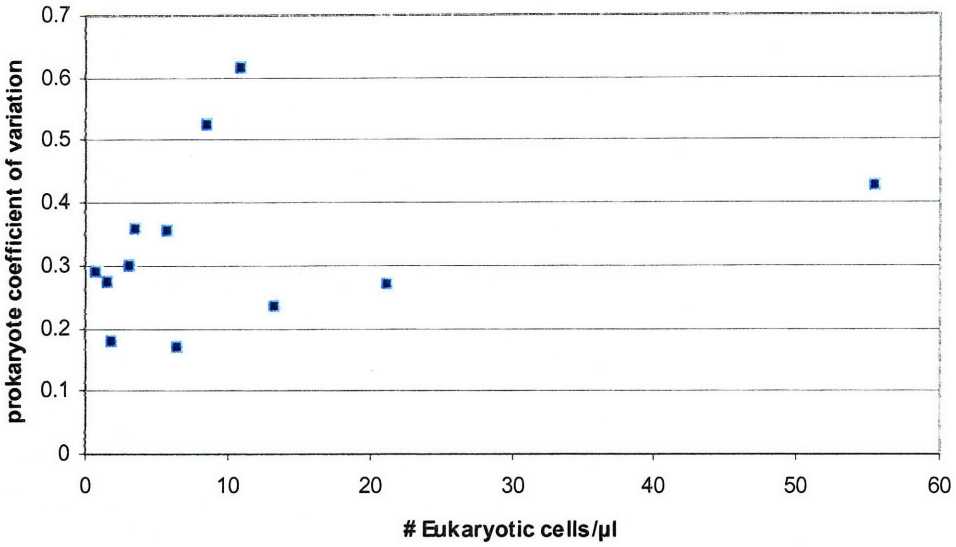


Figure 5. Coefficient of variation of prokaryotic cell abundance in 1 μl samples versus the average number of eukaryotic cells for the same sampling event.

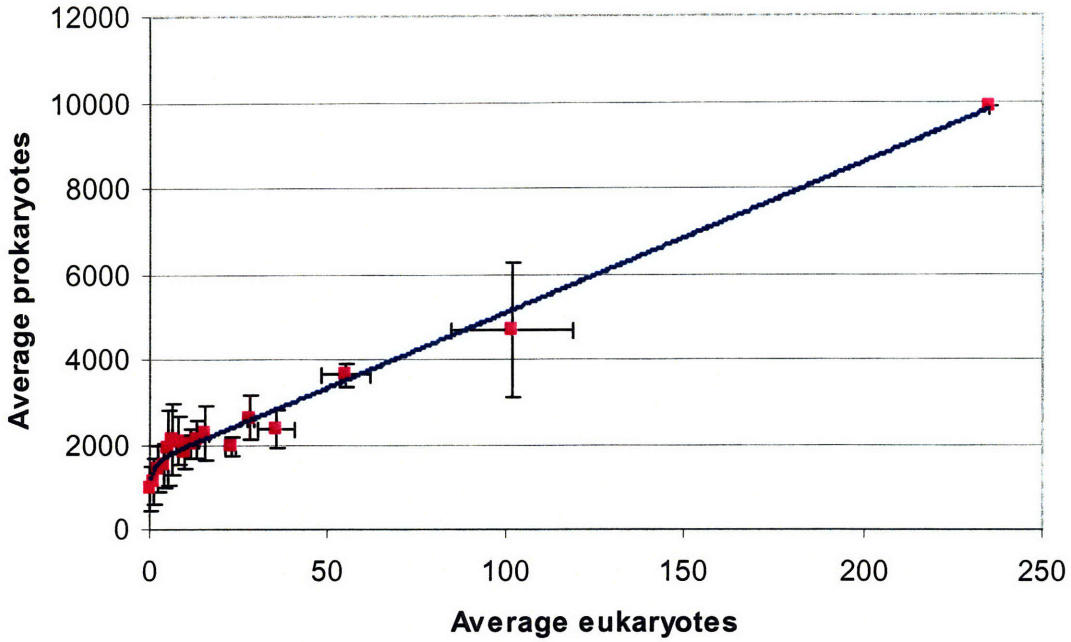


Figure 6. Comparison of average cell concentration of prokaryotes and eukaryotes in the same samples. 1µl samples from all months were binned by eukaryotic cell numbers; the dots on the graph depict averages with error bars indicating one standard deviation of the binned values. Binned averages were manually fitted with the equation: Prokaryotic cells= 1550 +35(Eukaryotes) –exp(-Eukaryotes*0.5) as shown by blue line.

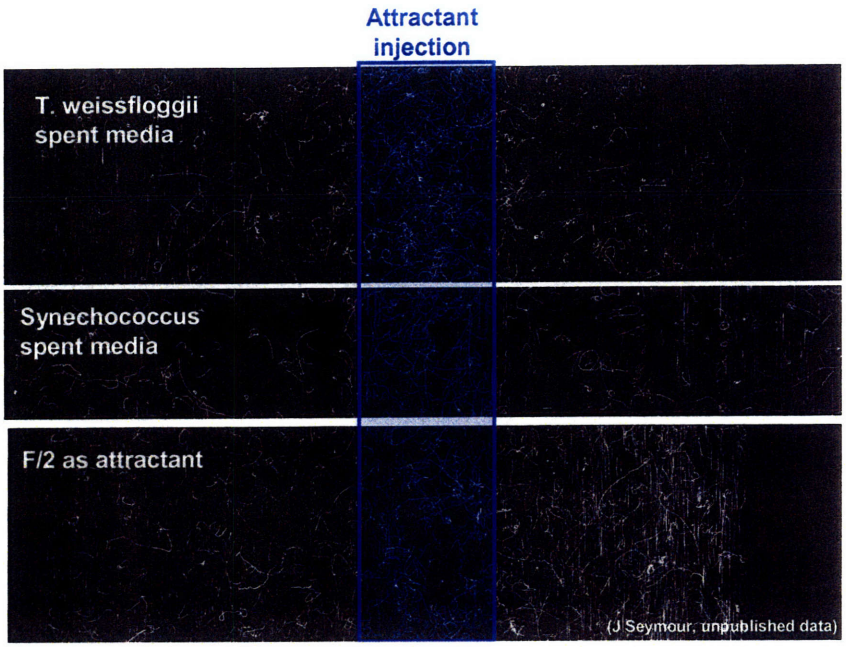


Figure 7. Chemotaxis of bacteria to phytoplankton extracellular products. Chemotaxis of *Silicibacter* TM1040 is show, with white tracks indicating the path of the bacteria. The shaded blue region indicated the area where the attractant was injected in the microfluidics device. The accumulation in the area of spent media indicates that the bacteria are chemotactic toward this substrate.

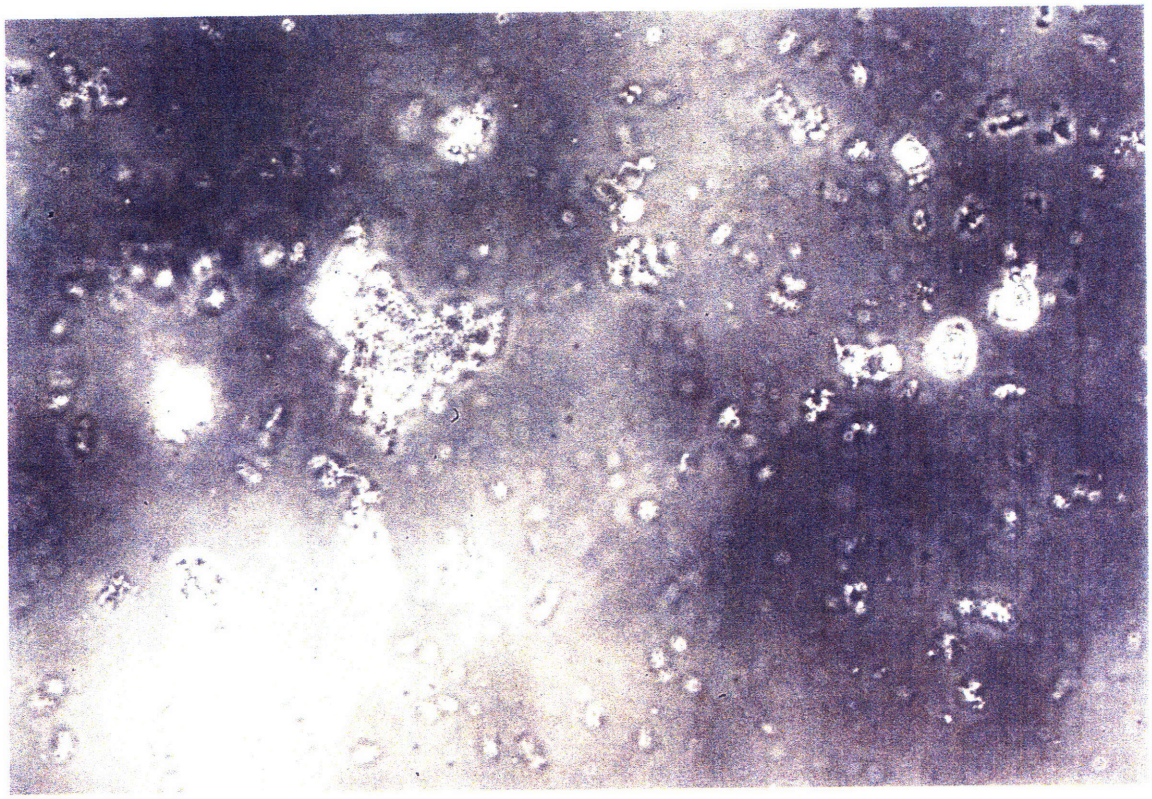


Figure 8. Motile *Roseobacter* Y4I attaching to heat-shocked cells of the diatom *T. weissflogii*.

Chapter Five

Conclusions and Future Work

CONCLUSION

Scientists have long been puzzled by "the Paradox of the Plankton": how highly diverse plankton with similar nutrient requirements coexist in unstructured aquatic environments (Hutchinson 1961). It has since been proposed that the oceans are in fact structured both by basin-wide gradients of nutrients and temperature and at a fine scale where zooplankton, particles and algae create microhabitats for bacteria. This microhabitat structuring promotes bacterial diversity as groups of bacteria with similar metabolic capabilities do not compete for resources if they are adapted to distinct water column lifestyles. Not all bacteria may be adapted to make use of these microhabitats, marine bacteria can be divided into two groups: passive bacteria which efficiently use low bulk nutrient levels in the oceans and opportunistic bacteria which exploit patchy high nutrient environments (Buchan et al. 2005, Giovannoni & Stingl 2005, Polz et al. 2006). My thesis focuses on microenvironments which can be exploited by opportunistic bacteria, including organic particles, chitinous zooplankton, and phytoplankton. This study examines the role of aquatic microenvironments in bacterial diversity: closely related organisms may co-exist in the same environment without competing for resources by a combination of habitat, metabolic, and behavioral differentiation. This topic was approached by asking two basic questions: (i) Within the bacterial family *Vibrionaceae* do phylogenetic groups engage in microenvironmental specialization or functional differentiation? (ii) Is there small scale clustering of bacteria around point sources in the coastal ocean?

Such questions delve into unresolved problems in microbial ecology such as what constitutes an ecological population and how can we identify ecological adaptation of "wild" bacterial populations. This investigation was motivated by previous research into bacterial community structure; a comprehensive sampling of 16S rRNA sequence diversity from the coastal ocean revealed that the majority of sequences were very closely related, or "microdiverse" (Acinas et al. 2004). These 16S rRNA gene sequences resolve into clusters, such clusters have previously been suggested to constitute ecologically coherent populations (Cohan & Perry 2007). According to this model, ecologically adapted clusters become genetically isolated by an adaptive mutation which then "selectively sweeps", purging the diversity in all organisms occupying the same niche (Cohan & Perry 2007). If a sweep occurs rapidly relative to recombination, diversity is purged at nearly all alleles in niche-specific clusters; once established, clusters may be relatively stable because a rapid drop in homologous recombination rates with sequence distance may isolate them genetically (Dykhuizen & Green 1991, Allen et al. 2007, Cohan & Perry 2007).

There have, however, been alternative explanations for the formation of these clusters not linked to ecological differentiation. First, if homologous recombination is very rapid among closely related genomes within a population, adaptive alleles could sweep through the populations, purging diversity at only this single allele (Doolittle & Papke 2006, Polz et al. 2006). Similarly, horizontal gene transfer by illegitimate recombination may move adaptive genes among dissimilar genomes, thus eroding ecological cohesion of closely related genomes (Doolittle & Papke 2006). Further, it has recently been shown that clusters may arise under allopatric differentiation (Whitaker et

al. 2003) and even under sympatry by neutral drift (Fraser et al. 2007). Finally, there is only sparse evidence for ecological differentiation between microdiverse clades, namely *Procholorcoccus* sequence clusters in the ocean partitioned along gradients of temperature, nutrients and light (Johnson et al. 2006).

Bacteria adapted to oligotrophic conditions like *Procholorcoccus* partition nutrients along continuous, large scale environmental gradients (Johnson et al. 2006). More metabolically versatile bacteria such as vibrios and *Roseobacter*, are adapted to these large scale features but additionally to microenvironments such as particles, algae and zooplankton which can be spatially and temporally variable (Polz et al. 2006). This thesis explores the importance of marine microenvironments in the productivity and diversity of opportunistic bacterial populations.

In Chapter 2, I show that subclusters within the family *Vibrionaceae* display distinct environmental distributions, suggesting that these groups are adapted to different microhabitats. Approximately 1000 co-occurring vibrio isolates were obtained from sequentially filtered seawater, corresponding to zooplankton-enriched, particulate, and planktonic water column size fractions at two time points. Sequencing a single housekeeping gene (*hsp60*) for all isolates revealed numerous microdiverse clusters, corresponding generally to named vibrio species, with approximately half of all isolates at both time points belonging to *V. splendidus*. A visual inspection of the tree reveals that few clusters were present in both spring and fall samples. This finding is in agreement with quantitative PCR studies showing seasonal trends in abundance, most likely related to water temperature (Thompson et al. 2004, Thompson et al. 2005). However, there was also striking partitioning of *Vibrionaceae* clades based on the size fraction of isolation,

suggesting that spatial resource partitioning in microenvironments also occurs in the water column. The majority of phylogenetically defined clusters were non-randomly distributed between size fractions. Although no groups were exclusively present in the single size fraction, associations with microenvironments appeared to be driven by the free-living or zooplankton-associated rather than particulate size fractions.

While the majority of ecologically distinct clusters corresponded to named species, the *V. splendidus*-like cluster appears to contain 10 separate subclusters with distinct spatial and temporal preferences, suggesting that changes in habitat preference can also occur within a named species. Two groups within *V. splendidus*, displayed rapid size-fraction preference switches, these groups have distinct seasonal and size fraction signatures and are separated by as little as a single base pair in the *hsp60* gene. Sequencing additional housekeeping genes (*pgi*, *adk*, *mdh*), revealed that for one group the extremely close relationship in *hsp60* was probably the result of lateral gene transfer as the sequences of these additional housekeeping genes separate the clades with different habitat preferences. However, the other clusters exhibits almost identical sequences in all genes, suggesting rapid resource diversification is on-going in this group. In fact *V. splendidus*, which is the most abundant group during warm water conditions (Thompson et al. 2004, Thompson et al. 2005), may be so successful because resource diversification allows it to occupy multiple environmental niches. This finding is consistent with high levels of genomic diversity in isolates (Thompson et al. 2005).

The observed strength of resource partitioning is unexpected as the vibrios are in low abundance in the environment. Even the "free-living" lifestyle may be subdivided by further metabolic differentiation; this partitioning of resources could indicate that

competition is quite strong in aquatic environments. Future work should address the true extent of resource subdivision to determine if it occurs on a finer scale, for example examining the populations on particles of different origins and on zooplankton by location in body (exoskeleton, gut, etc.). Very fine scale phylogenetic relationships could be established by sequencing multiple housekeeping loci and comparing allelic patterns, similar to pathogen typing schemes (Hanage et al. 2006). To compliment this approach and begin to assess what genes allow bacteria to colonize new niches, genome sequencing of closely related strains inhabiting different environmental niches could establish the genetic basis of lifestyle differentiation. A further question would be if adaptation to microscale habitats is a phenomenon unique to the *Vibrionaceae*, a preliminary study of clades within the *Roseobacter* observes that microdiverse clusters appear to correlate with distinct water-column lifestyles (Buchan et al. 2005), suggesting that microhabitat adaptation may be a common feature among the bacterioplankton.

In chapter 3, the conservation of both chitinoclastic ability and the chitin metabolic pathway is investigated among members of the *Vibrionaceae*. Since marine sources of chitin are particulate: crabs, diatom fibrils, zooplankton, it was initially postulated that the vibrios which live attached to zooplankton or particles (as documented for some groups in Chapter 2) are more likely to metabolize chitin. However, chitin metabolism is a near ubiquitous feature among the vibrios; although β- chitin was metabolized more often than the more tightly bound α-chitin. The chitin degradation pathway appears ancestral in the vibrios and the conservation of this pathway was examined further for additional vibrio isolates using the extracellular chitinase (*chiA*). This study examined *chiA* gene diversity using genomic sequences and PCR-amplified

genes from environmental and clinical isolates. Genomes within the photobacteria contained either additional or deeply divergent homologs of *chiA,* suggesting lateral gene transfer is responsible for this phylogenetic signature. Moreover, this research confirmed an earlier observation that the *chiA* gene in alpha Proteobacteria appears to be laterally acquired (Cottrell et al. 2000), and suggested the source of this gene was a vibrio. Although *chiA* appears to be laterally transferred, it is unknown if these alternate chitinase sequences confer a selective advantage under certain conditions or different specificity on chitin substrates.

Chitin-degradation is apparently well conserved in the *Vibrionaceae*, including the genera *Vibrio*, *Photobacteria*, and *Enterovibrio*, although it has apparently been lost in the *V. supersteus*-like isolates. Interestingly *V. supersteus*-clade are also one of the few non-motile vibrios (B. Kirkup, unpublished data), suggesting that these organisms have adopted an alternate lifestyle. While the presence of chitin-degrading genes did not appear to correlate with the water-column habitat of isolates, this data does suggest that either chitin-degradation is an important lifestyle for vibrios at certain times or that vibrio genomes carry unused metabolic ability, which may allow organisms to rapidly switch between niches (as observed in Chapter 2).

Further research into the vibrio chitin metabolic pathway should be undertaken to test the bioinformatic and expression-based pathway predictions in Chapter 3 concerning the incorporation of deacetylated residues in the chitin catabolic cascade. While the majority of the chitin degradation pathway is highly conserved, extracellular chitinases appear patchily distributed in the genomes and may allow organisms to fine tune regulation and growth on chitin under different circumstances. Multiple and highly

divergent *chiA* genes within the photobacteria also warrant biochemical investigation to determine if these genes display different specificity or regulation. Chitin degradation appears to be an important and ancestral characteristic among the *Vibrionaceae*. Although lifestyle differentiation means that these organisms do not all live attached to chitinous surfaces or particles (e.g. *V. ordalii*, Chapter 2), they retain chitin degradation capacity.

Finally, Chapter 4 examines bacterial clustering in the presence of algae in the coastal ocean. Microscale seawater samples were counted at roughly monthly time intervals over a seasonal cycle; concurrently, data was obtained for bulk nutrient levels as well as counts of eukaryotic cells and particle-attached prokaryotes. The highest level of bacterial patchiness was observed in 1 μl seawater samples collected in April 2003, when the number of prokaryotic cells varied by up to 9-fold. Overall, patchiness exhibited seasonal variation with peaks corresponding to predicted phytoplankton blooms and a positive correlation with eukaryotic cell number. The presence of high eukaryote concentrations when patchiness peaks does not automatically imply that bacteria are clustering around phytoplankton; algae could indirectly stimulate motility by organic matter production (Grossart et al. 2001). In order to clarify the microscale relationship of bacteria and phytoplankton, the number of prokaryotic and eukaryotic cells in each 1 μl sample were plotted. An empirically fitted line revealed a roughly linear relationship suggesting the presence of an additional 35 prokaryotic cells for each eukaryotic cell. This trend line was visible both between and within a single month. Although patchiness peaks occurred at high concentrations of eukaryotic cells, this trend line encompassing all

of the data, may indicate that bacteria also cluster at low algal cell number when patchiness cannot be detected by microscopic observation.

Using a system of cultured algae and bacteria, it was confirmed experimentally that bacteria chemotactic to algal extracellular products could cluster in the nutrient plume of a single algal cell, suggesting that motility in the presence of phytoplankton may indeed be favorable. A model system of *Roseobacter* and the diatom *Thalassiosira weissflogii* was used to investigate algal-bacterial interactions in greater detail, as alpha Proteobacteria and *Roseobacter* in particular are thought to be stimulated by phytoplankton (Grossart et al. 2005). A microfluidic assay confirmed that these bacteria chemotax towards the exudates released during algal growth. Further, motile cells could also aggregate around and attach to heat-shocked diatom cells, demonstrating that nutrient plumes released by a single cell were sufficient to induce chemotaxis and allow these bacteria to utilize patchy resources. An isogenic *Roseobacter* motility mutant did not colonize particle and thus was likely only exposed to spatially-averaged nutrient concentrations. On-going work is investigating the cost-efficiency of motility. At what level of resource patchiness does the energy gained from chemotactic clustering make up for the cost of motility?

Further work in this area should investigate the phylogeny of bacteria which chemotax towards phytoplankton exudates and how abundant these organisms are in the coastal ocean. If this group includes all *Roseobacter*, a large component of coastal bacterial populations (Buchan et al. 2005), spatial interactions with phytoplankton may be an important bacterial lifestyle. Such studies will help to clarify the importance of chemotaxis to phytoplankton extracellular products in coastal ocean environments and

which bacteria benefit from such clustering. How marine bacteria populations respond to phytoplankton products and detritus will inform modeling of deep sea carbon export, especially ocean iron fertilization schemes. Moreover, it will establish that the relationship of bacteria with phytoplankton includes a spatial component of interaction rather than just bulk coupling.

Overall, this work advances our understanding of microscale features in microbial ecology and suggests that adaptation to these microenvironments allows fine-scale resource partitioning and increased bacterial productivity. Differential association with microenvironments, such as particles and zooplankton, could allow co-existence of closely related strains with overlapping metabolic capabilities through resource subdivision. However such partitioning may not be evident in vibrio genomes as ancestral traits may be maintained in populations adapted to different lifestyles, providing these organisms with a rich repository of alternate metabolic capabilities and lifestyles. Although metagenomic inventories reveal environment-specific distributions of functional genes (DeLong et al. 2006), microhabitat specialization and levels of *in situ* gene expression remain poorly explored for closely-related bacteria. While microenvironmental and metabolic differentiation was only investigated in a single bacterial family such resource subdivision may be a common feature of the bacterioplankton and suggests that competition is intense and the resource space is finely subdivided. The relevant water column compartments are likely dependent on the type of bacteria; for example, alpha Proteobacteria appear adapted to cluster around phytoplankton and metabolize algal, extracellular products where Bacteroidetes are thought to be particle adapted. Understanding the tremendous diversity and productivity

of aquatic bacteria requires interrogating the environment in which they live at the bacterial scale.

WORKS CITED:

Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL, Polz MF (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430:551-554

Allen EE, Tyson GW, Whitaker RJ, Detter JC, Richardson PM, Banfield JF (2007) Genome dynamics in a natural archaeal population. Proceedings of the National Academy of Sciences of the United States of America 104:1883-1888

Buchan A, Gonzalez JM, Moran MA (2005) Overview of the marine *Roseobacter* lineage. Applied and Environmental Microbiology 71:5665-5677

Cohan FM, Perry EB (2007) A systematics for discovering the fundamental units of bacterial diversity. Current Biology 17:R373-R386

Cottrell MT, Wood DN, Yu LY, Kirchman DL (2000) Selected chitinase genes in cultured and uncultured marine bacteria in the alpha- and gamma-subclasses of the proteobacteria. Applied and Environmental Microbiology 66:1195-1201

DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, Frigaard NU, Martinez A, Sullivan MB, Edwards R, Brito BR, Chisholm SW, Karl DM (2006) Community genomics among stratified microbial assemblages in the ocean's interior. Science 311:496-503

Doolittle WF, Papke RT (2006) Genomics and the bacterial species problem. Genome Biology 7

Dykhuizen DE, Green L (1991) Recombination in *Escherichia coli* and the definition of biological species. Journal of Bacteriology 173:7257-7268

Fraser C, Hanage WP, Spratt BG (2007) Recombination and the nature of bacterial speciation. Science 315:476-480

Giovannoni SJ, Stingl U (2005) Molecular diversity and ecology of microbial plankton. Nature 437:343-348

Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. Environmental Microbiology 7:860-873

Grossart HP, Riemann L, Azam F (2001) Bacterial motility in the sea and its ecological implications. Aquatic Microbial Ecology 25:247-258

Hanage WP, Fraser C, Spratt BG (2006) Sequences, sequence clusters and bacterial species. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1917-1927

Hutchinson GE (1961) The paradox of the plankton. The American Naturalist XCV:137-145

Johnson ZI, Zinser ER, Coe A, McNulty NP, Woodward EMS, Chisholm SW (2006) Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science 311:1737-1740

Polz MF, Hunt DE, Preheim SP, Weinreich DM (2006) Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. Philosophical Transactions of the Royal Society B-Biological Sciences 361:2009-2021

Thompson JR, Pacocha S, Pharino C, Klepac-Ceraj V, Hunt DE, Benoit J, Sarma-Rupavtarm R, Distel DL, Polz MF (2005) Genotypic diversity within a natural coastal bacterioplankton population. Science 307:1311-1313

Thompson JR, Randa MA, Marcelino L, Tomita-Mitchell A, Lim E, Polz MF (2004) Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Applied and Environmental Microbiology 70:4103-4110

Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301:976-978

Appendix One

Evaluation of 23S rRNA PCR primers for use in phylogenetic studies of bacterial diversity

Dana E Hunt, Vanja Klepac-Ceraj, Silvia G. Acinas, Clement Gautier,
Stefan Bertilsson, Martin F Polz

Dana E Hunt, Vanja Klepac-Ceraj, Silvia G. Acinas, Clement Gautier, Stefan Bertilsson, Martin F Polz (2006) Evaluation of 23S rRNA PCR Primers for Use in Phylogenetic Studies of Bacterial Diversity *Applied and Environmental Microbiology* **72(3)** 2221-2225.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2006, p. 2221–2225 Vol. 72, No. 3
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.3.2221–2225.2006

Evaluation of 23S rRNA PCR Primers for Use in Phylogenetic Studies of Bacterial Diversity†

Dana E. Hunt, Vanja Klepac-Ceraj,‡ Silvia G. Acinas, Clement Gautier,§ Stefan Bertilsson,¶ and Martin F. Polz*

Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received 8 September 2005/Accepted 19 December 2005

The availability of a diverse set of 23S rRNA gene sequences enabled evaluation of the specificity of 39 previously published and 4 newly designed primers specific for bacteria. An extensive clone library constructed using an optimized primer pair resulted in similar gene richness but slightly differing coverage of some phylogenetic groups, compared to a 16S rRNA gene library from the same environmental sample.

There has been renewed interest in the use of the 23S rRNA gene with the decrease in sequencing costs and the growing popularity of techniques such as microarrays (3, 13), analysis of the 16S-23S intergenetic region (7, 9), fluorescence in situ hybridization, and quantitative PCR. The 23S rRNA gene offers the same advantages as the 16S rRNA gene (e.g., universal distribution, conserved function, and invariant and variable regions), yet it includes additional diagnostic sequence stretches due to a greater length, characteristic insertions and/or deletions (12), and possibly better phylogenetic resolution because of greater sequence variation (4, 10–12, 20). However, use of the 23S rRNA gene for bacterial community analysis is hampered by the lack of established broad-range bacterial PCR amplification and sequencing primers.

This study incorporates data from large-scale sequencing efforts to develop new and evaluate existing bacterium-specific 23S rRNA PCR amplification primers. Additionally, this study includes the first well-sampled environmental clone library of 23S rRNA sequences, greatly increasing the number of 23S rRNA gene sequences.

Evaluation of primers. To check the specificity of PCR primers, an alignment of 23S rRNA gene sequences was developed using the ARB software package (http://www.arb-home.de). Bacterial 23S rRNA sequences were obtained from published sources: the European rRNA database (22), National Center for Biotechnology Information complete bacterial genomes (as of 6 February 2005), the ARB LSU database, and environmental bacterial artificial chromosome clones (16, 18). To ensure broad environmental representation of these primers, sequences were also retrieved using BLAST from the Sargasso Sea assembled database (21) with full-length query 23S rRNA sequences from the genomes of representative organisms (*Shigella flexneri* 2a strain 301, *Pirellula* strain 1, *Prochlorococcus marinus* CCMP 1986, *Streptomyces coelicolor* A3, *Bradyrhozobium japonicum* USDA110, and *Bacteriodes fragilis* YCH46). Using this method, 1,415 nonredundant 23S rRNA sequences of >400 bp each were retrieved from the Sargasso Sea data set. Initial alignments of a total of 2,176 sequences were constructed using the ARB Fast Aligner with manual editing based on secondary structure and the existing ARB alignment. This data set was not corrected for skewing, due to overrepresentation of common laboratory organisms, pathogens, and organisms abundant in the Sargasso Sea.

The primers developed in this study (129f, 189r, 457r, and 2490r, with numbering based on *Escherichia coli* position) (6) show excellent correspondence to sequences in the aligned database (Table 1); additionally, some mismatches may be the result of PCR or sequencing error. Although some previously published "universal" bacterial primers display broad range, this extensive database indicates that other suggested target regions are not sufficiently conserved to serve as bacterial PCR primers (Table 2). Primers for ITS amplification show various degrees of specificity: the region corresponding to the position of primer 129f is highly conserved (8, 15), but other primers are less conserved and exclude a large fraction of bacterial diversity (7, 19).

On the basis of their broad specificity, length of amplified sequence, and good amplification properties, we propose using the primers 129f (modified in this study) and 2241r for studies of bacterial 23S rRNA diversity. These primers amplify a large portion of the 23S rRNA, consistently produce only a single band of PCR product, and are highly conserved across the bacterial sequences currently available (Tables 1 and 2). Positive amplification was achieved with a diverse set of isolates under the following conditions: 3 min at 94°C; then 30 cycles, each consisting of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C; and a final 5-min extension at 72°C. All isolates used to test the primers produced PCR product of the correct size; the phyla of bacteria are listed and the number of isolates tested is given in parentheses: α-*Proteobacteria* (7), β-*Proteobacteria* (2), δ-*Proteobacteria* (1), ε-*Proteobacteria* (1), γ-*Proteobacteria* (22), *Firmicutes* (7), *Bacteroidetes* (8), and *Cyanobacteria* (2).

* Corresponding author. Mailing address: Massachusetts Institute of Technology, 48-421, 77 Massachusetts Ave., Cambridge, MA 02139. Phone: (617) 253-7128. Fax: (617) 258-8850. E-mail: mpolz@mit.edu.

† Supplemental material for this article may be found at http://aem.asm.org/.

‡ Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

§ Present address: Laboratory for Optics and Biosciences, Institut National de la Santé et de la Recherche Médicale, Ecole Polytechnique, 91128 Palaiseau Cedex, France.

¶ Present address: Limnology/Department of Ecology and Evolution, Norbyvägen, Uppsala University, SE-75236 Uppsala, Sweden.

TABLE 1. Percentage mismatches to the 23S rRNA gene dataset for primers designed in this study[a]

Primer[b]	Nucleotide and % mismatch																		
129f	C	Y	G	A	A	T	G	G	G	G	V	A	A	C	C				
	0.3	0.1	2.4	0.2	0.5	0.6	2.4	2.1	1.9	**9.0**	4.0	0.5	1.1	2.2	2.4				
189r	T	A	C	T	D	A	G	A	T	G	T	T	T	C	A	S	T	T	C
	0.1	0.2	2.3	0.1	0.0	0.0	0.3	0.1	0.3	4.7	1.6	0.6	0.0	0.3	0.3	4.8	0.0	0.0	0.2
457r	C	C	T	T	T	C	C	C	-	T	C	A	C	G	G	T	A	C	T
	3.0	0.1	4.4	0.1	0.0	1.3	0.7	**5.5**	0.5	0.2	1.3	3.7	0.1	**5.2**	0.6	0.0	0.0	0.2	0.1
2490r	C	G	A	C	A	T	C	G	A	G	G	T	G	C	C	A	A	A	C
	0.3	0.3	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.3	0.2	0.2	0.9	0.9	0.1	0.9	0.1	0.7	0.2

[a] Degenerate positions in the sequences were assumed to equally contribute to all possible nucleotides. Boldface type indicates that >5% of database sequences do not match the primer. A hyphen indicates insertions in more than two sequences.
[b] Primer 129f is modified from 130f (9b), 189r is modified from 11A (20a), and 457r is modified from 473r (10).

Analysis of 23S rRNA clone library. The 129f-2241r primer set was subsequently used to construct a clone library to evaluate coverage and relative distribution of phyla in comparison with a 16S rRNA clone library constructed from a parallel sample (1). A surface seawater sample from the marine end of Plum Island Sound estuary (northeastern Massachusetts) was collected as previously described (1). Cells were lysed using bead beading (5), and DNA was purified using phenol:chloroform:isoamyl alcohol extraction, sodium acetate and ethanol precipitation, and RNase I treatment (17). DNA was amplified in 10 replicate 20-μl PCRs, each reaction mixture containing 50 ng of purified DNA template. PCR conditions were as follows: 3 min at 94°C; then 15 cycles, each consisting of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C; and a final 5-min extension at 72°C. PCR products were pooled, precipitated with ethanol, and gel extracted (QIAGEN gel extraction kit). Amplicons were cloned using the TOPO-TA kit (Invitrogen).

A total of 535 operational taxonomic units (OTUs) were identified, based on sequential digests with restriction enzymes HhaI and MspI of cloned inserts amplified using internal plasmid primers (M13). Inserts with restriction patterns adding up to >2,500 nucleotides were excluded, as they were assumed to originate from more than one cloned 23S rRNA gene insert. To determine the phylogenetic coverage, at least one member of each OTU was sequenced and grouped into higher taxonomic groups (subphylum or phylum). Both 129f and 457r were used as sequencing primers on plasmids extracted using a QIAprep Spin Miniprep kit (QIAGEN) and M13-amplified PCR products, respectively. A total of 614 clone library sequences were edited using Sequencher, and phylum-level identification of the OTUs was made using discontinuous megaBLAST with a scoring metric (match = 4; mismatch = −5) to allow identification of sequences highly divergent from those present in the database. The cutoff for categorization of a sequence was a sequence length of 300 bp of at least 85% similarity to an organism of known phylogeny.

A comparison of 23S and 16S rRNA (1) gene clone libraries constructed from replicate water samples yielded gross similarities but also some important differences (Fig. 1). The observed levels of richness in the two libraries were comparable when the digestion-defined OTUs in the 23S library were approximated by 99% sequence identity clusters (1, 14) in the 16S rRNA library (535 versus 520 for the 23S and 16S rRNA gene libraries, respectively). In both libraries, *Bacteroidetes* and α-*Proteobacteria* were the most abundant groups (2). However, the 23S library displayed a higher percentage of *Bacteriodetes* (42.8% versus 32.5%) and lower percentages of γ-*Proteobacteria* (3.9% versus 22.8%), *Actinobacteria*, and minor groups. This comparison is of interest because it may reflect the primer bias of either 23S or 16S rRNA primers, a shallower depth of sequence coverage in the 23S library masking rare variants, or a limited 23S rRNA database preventing identification of certain groups. *Planctomycetales* were probably excluded by these 23S rRNA primers because the forward primer targets a region not present in their 23S rRNA gene. Additionally, >5% mismatches were observed at position 10 of primer 129f to the set of aligned sequences (Table 1); these mismatches occurred primarily in environmental sequences rather than in cultured isolates, confirming the value of incorporating environmental shotgun sequences in the alignment. This mismatch may explain the low level of abundance of γ-*Proteobacteria* in the clone library, as this alternate sequence is present in the SAR-86 group (16, 18) and other γ-*Proteobacteria* in the database, as well as members of other phyla. This problem can be remedied by adding an additional degeneracy to primer 129f with the final sequence as CYGAATGGGRVAACC; this modified primer paired with 2241r positively amplified a subset

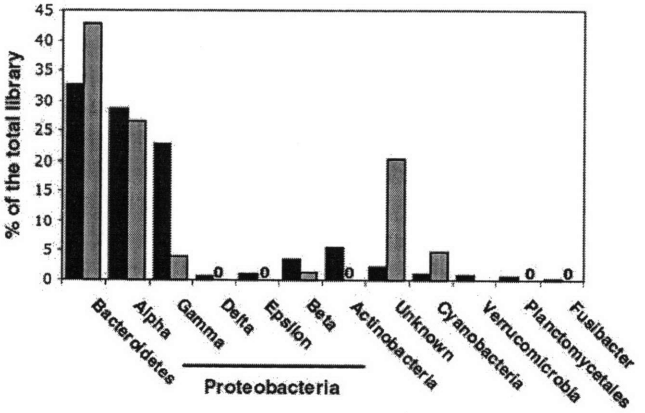


FIG. 1. Relative frequency distribution of major phylogenetic groups detected among the environmental sequences from a 16S rRNA library (black) and a 23S rRNA library (gray).

TABLE 2. Percentage mismatches to the 23S rRNA gene dataset at each position for previously described universal primers[a]

Primer (reference)	Nucleotide and % mismatch																							
ITSReub (7)	G	C	C	A	A	-	G	G	C	A	T	C	C	A	C	C								
	7.9	**6.2**	2.9	**16.4**	**10.8**	0.4	2.9	4.2	**5.8**	2.1	0.5	**10.6**	2.8	**12.8**	**5.3**	4.0								
66r (19)	C	A	C	G	T	C	T	T	T	C	A	T	C	G	S	C	T							
	24.4	**17.6**	3.1	0.9	**7.7**	0.1	**94.4**	0.0	2.6	0.4	**17.0**	0.4	2.9	4.2	**6.2**	**6.0**	1.3							
fprimer6 (3a)	G	C	G	A	T	T	T	C	Y	G	A	A	Y	G	G	G	R	A	A	A	C	C	C	
	50.6	**80.3**	**7.0**	**11.7**	0.1	**46.9**	**5.1**	0.2	0.1	2.4	0.2	0.5	0.3	2.4	2.1	2.0	0.9	**39.3**	0.5	1.1	2.2	2.4	**7.5**	
130f (9b)	C	C	G	A	A	T	G	G	G	G	V	A	A	G*	G*	G*								
	0.3	**8.4**	2.4	0.2	0.5	0.6	2.4	2.1	2.0	**9.0**	4.0	0.5	1.1	**100**	**99.9**	**100**								
130r (9b)	C*	C*	T	T	G	C	C	C	C	A	T	T	C	G	G									
	99.9	**100**	1.1	0.5	**84.5**	**9.0**	2.0	2.1	2.4	0.6	0.5	0.2	2.4	**8.4**	0.3									
11A (20a)	G	G	A	A	C	T	G	A	A	A	C	A	T	C	T	A	A	G	T	A				
	47.0	0.2	0.0	0.0	**17.1**	0.3	0.3	0.0	0.6	1.6	4.7	0.3	0.1	0.3	0.0	**34.0**	0.1	2.3	0.2	0.1				
242r (9b)	K	T	T	C	G	C	-	T	C	G	C	C	R	C	T	A	C							
	2.9	0.2	0.6	0.8	0.5	1.7	0.6	0.0	0.0	2.2	**5.5**	0.0	0.3	0.2	0.3	0.0	0.1							
256f (9b)	A	G	T	A	G	Y	G	G	C	G	A	-	G	C	G	A	A							
	3.1	0.1	0.0	0.3	0.2	0.3	0.0	**5.5**	2.2	0.0	0.0	0.6	1.7	0.5	0.8	0.6	0.2							
23ar (20a)	C	G	G	T	A	C	T	-	G	G	T	T	C	A	C	T	A	T	C	G	G			
	0.1	**5.2**	0.6	0.0	0.0	0.2	0.1	0.4	**38.4**	**10.6**	0.2	**13.0**	0.2	**17.2**	0.1	0.2	0.0	0.1	1.3	0.0	2.4			
rprimer10 (3)	T	T	C	G	C	C	T	T	T	C	C	C	-	T	C	A	C	G	G	T	A	C	T	
	0.5	0.3	0.9	**66.5**	3.1	0.1	4.4	0.1	0.0	1.3	0.7	**5.5**	0.5	0.2	1.3	3.7	0.1	**5.2**	0.6	0.0	0.0	0.2	0.1	
473f (10)	A	G	T	A	C	C	G	Y	G	A	-	G	G	G	A	A	A	G						
	0.1	0.2	0.0	0.0	0.6	**5.2**	0.1	0.1	1.3	0.2	0.5	**5.5**	0.7	1.3	0.0	0.1	4.4	0.1						
559r (9b)	C	A	T	T	M	T	A	C	A	A	A	A	G	G	Y	A	C	G	C					
	0.3	2.5	0.1	**6.2**	0.3	0.7	**25.6**	0.2	0.3	0.1	4.9	0.3	4.3	0.1	0.3	0.1	**6.7**	**6.6**	4.9					
559r (21a)	C	A	T	T	M	T	R	C	A	A	A	A	G	G	Y	A	C	G	C					
	0.3	2.5	0.1	**6.2**	0.3	0.7	**11.4**	0.2	0.3	0.1	4.9	0.3	4.3	0.1	0.3	0.1	**6.7**	**6.6**	4.9					
803r (21a)	T	T	C	G	G	R	G	A	G	A	A	C	S	A	G	M	T	A						
	0.3	0.5	0.5	0.2	**45.0**	2.8	0.8	0.5	**15.8**	3.7	0.3	0.2	4.9	0.3	0.2	0.4	0.1	0.5						
820f (20a)	T	A	G	C	T	G	G	T	T	C	T	C	Y	Y	C	G	A	A						
	0.5	0.1	**10.5**	0.2	0.3	**9.5**	0.2	0.3	3.7	**15.8**	0.5	0.8	2.8	**26.7**	0.2	0.5	0.5	0.3						
975r (10)	T	C	T	-	G	G	G	Y	T	G	T	T	Y	C	C	C	-	T						
	0.2	1.6	**6.3**	0.3	0.8	0.2	2.2	0.1	0.4	**21.0**	0.5	0.5	1.1	2.3	0.2	0.4	0.4	0.7						
43a (20a)	G	G	A	T	G	T	T	G	G	C	T	T	A	G	A	A	G	C	A	G				
	2.0	4.5	0.4	**55.2**	0.2	0.0	2.4	**5.2**	0.0	0.1	1.6	2.2	2.7	0.2	0.0	2.6	0.0	0.0	0.0	0.1				
1075f (10)	G	T	T	G	G	C	T	T	R	G	A	R	G	C	A	G	C							
	0.2	0.0	2.4	**5.2**	0.0	0.1	1.6	2.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.3							
1091r (9b)	R	G	T	G	A	G	C	T	R	T	T	A	C	G	C									
	0.0	1.9	0.0	1.1	0.3	0.2	0.1	0.3	0.0	0.1	0.0	0.1	0.2	0.2	0.1									
1104f (9b)	W	G	C	G	T	A	A	Y	A	G	C	T	C	A	C									
	0.0	0.0	0.2	0.2	0.1	0.0	0.1	0.0	0.3	0.1	0.2	0.3	1.1	0.0	1.9									
1200f (10)	G	G	T	A	G	R	R	G	A	-	G	C	G	T	T	-	C							
	0.6	2.3	0.6	0.6	0.3	**25.8**	2.6	1.0	0.8	0.4	**16.7**	1.5	4.6	0.8	**10.2**	0.3	**15.0**							
1363f (10)	G	A	G	G	C	C	G	A	N	-	-	A	R	G	C	G	-	T	A					
	6.3	2.4	4.5	**8.6**	**22.4**	**24.3**	2.9	1.9	0.0	1.1	0.4	**5.4**	**11.4**	**22.9**	**11.4**	0.5	0.4	2.9	1.8					
53a (20a)	G	G	A	C	-	A	A	C	A	G	G	T	T	A	A	-	T	A	T	T	C	C		
	0.0	0.6	**53.3**	**80.0**	3.8	0.2	**29.9**	**23.9**	**28.6**	3.0	**7.7**	0.9	**11.2**	**26.4**	0.1	2.8	**8.6**	0.5	1.3	0.1	**8.4**	3.3		
1623f (9b)	A	A	A	C	C	G	W	C	A	C	A	G	G	T	R	G								
	3.1	0.0	1.7	0.1	**5.5**	3.4	0.1	0.1	3.0	0.1	2.5	4.9	0.0	0.4	0.6	**5.7**								
62ar (20a)	G	G	G	G	C	C	A	T	T	T	T	G	C	C	G	A	G	T	T	C				
	10.8	**8.4**	**54.9**	**42.1**	**55.9**	**27.8**	0.2	**26.6**	2.9	0.1	0.0	0.3	0.2	**8.7**	**34.4**	0.0	0.2	0.1	0.0	0.2				

Continued on following page

TABLE 2—*Continued*

Primer (reference)	Nucleotide and % mismatch																			
1685r (21a)	C	C	T	T	M	T	C	S	C	-	G	A	A	S	T	T	A	C	G	G
	8.5	**6.6**	0.0	0.0	0.9	0.1	**21.6**	0.1	2.7	0.9	4.9	0.0	0.4	0.1	**5.6**	0.1	0.1	0.0	**10.8**	**8.4**
69ar (20a)	C	T	T	A	G	G	A	C	C	G	T	T	A	T	A	G	T	T	A	C
	0.3	0.3	0.3	1.5	0.8	2.0	0.3	**5.8**	**5.9**	0.7	0.1	3.0	0.0	0.8	0.6	0.5	0.3	0.1	0.0	0.2
1930r (9b)	C	G	A	C	A	A	G	G	A	A	-	T	T	T	C	G	C	T	A	C
	3.7	0.0	0.7	0.5	0.9	0.1	0.4	0.3	1.1	0.0	0.4	1.1	0.4	0.2	0.3	0.3	0.1	0.1	0.1	0.1
2069f (9b)	G	A	C	G	Y*	A	A	A	G	A	C	C	C	C	R	T	G			
	0.1	0.0	0.2	3.1	**99.8**	0.5	0.5	0.3	0.1	0.1	0.3	0.0	0.0	3.5	0.0	**5.8**	1.6			
2241r (9b)	A	C	C	G	C	C	C	C	A	G	T	H	A	A	A	C	T			
	0.4	0.4	0.7	2.6	0.4	0.4	0.1	0.0	0.0	0.3	0.9	1.0	0.1	0.1	0.2	0.2	0.6			
2436f (10)	T	C	-	G	C	T	C	A	A	C	G	G	A	T	A	A	A	A	G	
	0.9	2.7	0.3	3.0	1.5	0.3	4.1	0.3	3.0	**6.1**	0.7	3.5	1.9	0.8	0.4	0.2	0.0	0.3	0.2	
2498r (9b)	G	A	G	Y	C	G	A	C	A	T	C	G	A	G	G					
	0.3	0.1	0.3	0.0	0.3	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.3	0.2					
93ar (20a)	C	G	A	C	G	-	T	T	C	T	G	A	A	C	C	C	A	G	C	T	C
	0.2	0.2	0.1	0.2	0.2	0.2	2.0	0.1	**10.7**	0.2	**38.6**	0.2	0.2	0.2	0.2	0.1	0.1	2.9	0.2	0.2	0.2
2603f (21a)	A	R	A	M	-	C	G	T	C	G	T	G	A	G	A	C	A	G		
	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.2	2.0	0.0	0.0	0.1	0.1	0.2	0.2	0.2		
2669f (9b)	A	G	T	A	C	G	A	G	-	A	G	G	A	C	C	G	G			
	0.5	0.2	0.1	0.2	0.3	0.2	0.2	0.6	0.3	0.1	0.1	0.1	0.1	0.6	0.3	3.5	**8.0**			
2744r (10)	C	T	T	-	A	G	A	T	G	C	Y	T	T	C	A	G	C			
	2.2	0.1	0.2	0.4	0.1	**5.7**	0.1	0.2	0.2	0.4	1.5	0.1	0.0	0.2	0.2	2.3	3.2			
2747r (9b)	G	Y	T	T	-	A	G	A	T	G	C	Y	T	T	C					
	17.4	0.0	0.1	0.2	0.4	0.1	**5.7**	0.1	0.2	0.2	0.4	1.5	0.1	0.0	0.2					
97ar (20a)	C	C	C	G	C	T	T	-	A	G	A	T	G	C	T	T	T	C	A	G	C
	2.3	**35.9**	**19.0**	**17.4**	2.2	0.1	0.2	0.4	0.1	**5.7**	0.1	0.2	0.2	0.4	**5.6**	0.1	0.0	0.2	0.2	2.3	3.2
2758f (9b)	Y	T	G	A	A	R	G	C	A	T	C	T	-	A	A					
	0.0	0.2	0.2	0.0	0.1	1.5	0.4	0.2	0.2	0.1	**5.7**	0.1	0.4	0.2	0.1					

[a] Primers follow the naming convention of the original publication but are ordered according to their position along the 23S rRNA sequence. Positions shown in boldface indicate that >5% of sequences do not match the primer at that position. Degenerate positions in the sequences were assumed to equally contribute to all possible nucleotides. Hyphens indicate insertions in more than two sequences. *, probable typographic error in the published primer sequence.

of 14 isolates from diverse phyla. The large number of unknowns is due to the difficulty of 23S rRNA sequence identification because of the poor depth of sequence coverage, especially for less-well-studied phyla (i.e., β-, δ-, and ε-*Proteobacteria*).

Applications using 23S primers, especially techniques such as automated rRNA intergenic spacer analysis that are highly sensitive to the primers chosen (7), should be reevaluated and perhaps modified in light of this data. Nonetheless, this comparison of 16S and 23S rRNA gene sequences shows that reasonable coverage and agreement between broad-range primer pairs can be achieved.

The alignment used to check the primers is available online (see the supplemental material and the ARB database, available for download at http://web.mit.edu/polz/seq=align.html).

Nucleotide sequence accession numbers. Sequences were submitted to GenBank with accession numbers DQ312516 to DQ313129.

This research was supported by grants from NSF, NIEHS, and DOE (Genomes to Life).

Thanks to Ivan Ceraj for programming assistance and Patricia McAndrew for help with initial clone library screening.

REFERENCES

1. **Acinas, S. G., V. Klepac-Ceraj, D. E. Hunt, C. Pharino, I. Ceraj, D. L. Distel, and M. F. Polz.** 2004. Fine-scale phylogenetic architecture of a complex bacterial community. Nature **430**:551–554.
2. **Acinas, S. G., R. Sarma-Rupavtarm, V. Klepac-Ceraj, and M. F. Polz.** 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl. Environ. Microbiol. **71**:8966–8969.
3. **Anthony, R. M., J. T. Brown, and G. L. French.** 2000. Rapid diagnosis of bacteremia by universal amplification of 23S ribosomal DNA followed by hybridization to an oligonucleotide array. J. Clin. Microbiol. **38**:781–788.

3a. **Anthony, R. M., J. T. Brown, and G. L. French.** 2000. Rapid diagnosis of bacteremia by universal amplification of 23S ribosomal DNA followed by hybridization to an oligonucleotide array. J. Clin. Microbiol. **38**:781–788.

4. **Anton, A. I., A. J. Martinez-Murcia, and F. Rodriguez-Valera.** 1999. Intraspecific diversity of the 23S rRNA gene and the spacer region downstream in *Escherichia coli*. J. Bacteriol. **181**:2703–2709.
5. **Bertilsson, S., C. M. Cavanaugh, and M. F. Polz.** 2002. Sequencing-independent method to generate oligonucleotide probes targeting a variable region in bacterial 16S rRNA by PCR with detachable primers. Appl. Environ. Microbiol. **68**:6077–6086.
6. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148**:107–127.
7. **Cardinale, M., L. Brusetti, P. Quatrini, S. Borin, A. M. Puglia, A. Rizzi, E. Zanardini, C. Sorlini, C. Corselli, and D. Daffonchio.** 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl. Environ. Microbiol. **70**:6147–6156.

8. **Fisher, M. M., and E. W. Triplett.** 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl. Environ. Microbiol. **65**:4630–4636.
9. **Gurtler, V., and V. A. Stanisich.** 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology **142**:3–16.
9a. **Ibrahim, A., J. Hofman-Bang, and B. K. Ahring.** 2001. Amplification and direct sequence analysis of the 23S rRNA gene from thermophilic bacteria. BioTechniques **30**:414–416.
9b. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–147. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., New York, N.Y.
10. **Ludwig, W., G. Kirchhof, N. Klugbauer, M. Weizenegger, D. Betzl, M. Ehrmann, C. Hertel, S. Jilg, R. Tatzel, H. Zitzelsberger, S. Liebl, M. Hochberger, J. Shah, D. Lane, and P. R. Wallnoef.** 1992. Complete 23S ribosomal RNA sequences of gram-positive bacteria with a low DNA G+C content. Syst. Appl. Microbiol. **15**:487–501.
11. **Ludwig, W., R. Rossellomora, R. Aznar, S. Klugbauer, S. Spring, K. Reetz, C. Beimfohr, E. Brockmann, G. Kirchhof, S. Dorn, M. Bachleitner, N. Klugbauer, N. Springer, D. Lane, R. Nietupsky, M. Weizenegger, and K. H. Schleifer.** 1995. Comparative sequence analysis of 23S rRNA from *Proteobacteria*. Syst. Appl. Microbiol. **18**:164–188.
12. **Ludwig, W., and K. H. Schleifer.** 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. FEMS Microbiol. Rev **15**:155–173.
13. **Mitterer, G., M. Huber, E. Leidinger, C. Kirisits, W. Lubitz, M. W. Mueller, and W. M. Schmidt.** 2004. Microarray-based identification of bacteria in clinical samples by solid-phase PCR amplification of 23S ribosomal DNA sequences. J. Clin. Microbiol. **42**:1048–1057.
14. **Moyer, C. L., J. M. Tiedje, F. C. Dobbs, and D. M. Karl.** 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature? Appl. Environ. Microbiol. **62**:2501–2507.
15. **Ranjard, L., F. Poly, J. C. Lata, C. Mougel, J. Thioulouse, and S. Nazaret.** 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl. Environ. Microbiol. **67**:4479–4487.
16. **Sabehi, G., O. Beja, M. T. Suzuki, C. M. Preston, and E. F. DeLong.** 2004. Different SAR86 subgroups harbour divergent proteorhodopsins. Environ. Microbiol. **6**:903–910.
17. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Suzuki, M. T., O. Beja, L. T. Taylor, and E. F. DeLong.** 2001. Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environ. Microbiol. **3**:323–331.
19. **Suzuki, M. T., C. M. Preston, O. Beja, J. R. de la Torre, G. F. Steward, and E. F. DeLong.** 2004. Phylogenetic screening of ribosomal RNA gene-containing clones in bacterial artificial chromosome (BAC) libraries from different depths in Monterey Bay. Microb. Ecol. **48**:473–488.
20. **Trebesius, K., D. Harmsen, A. Rakin, J. Schmelz, and J. Heesemann.** 1998. Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. J. Clin. Microbiol. **36**:2557–2564.
20a. **Van Camp, G., S. Chapelle, and R. De Wachter.** 1993. Amplification and sequencing of variable regions in bacterial 23S ribosomal-RNA genes with conserved primer sequences. Curr. Microbiol. **27**:147–151.
21. **Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Y. Wu, I. Paulsen, K. E. Nelson, W. Nelson, D. E. Fouts, S. Levy, A. H. Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y. H. Rogers, and H. O. Smith.** 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science **304**:66–74.
21a. **Ward, N. L., F. A. Rainey, B. P. Hedlund, J. T. Staley, W. Ludwig, and F. Stackebrandt.** 2000. Comparative phylogenetic analyses of members of the order *Planctomycetales* and the division *Verrucomicrobia*: 23S rRNA gene sequence analysis supports the 16S rRNA gene sequence-derived phylogeny. Int. J. Syst. Evol. Microbiol. **50**:1965–1972.
22. **Wuyts, J., G. Perriere, and Y. V. de Peer.** 2004. The European ribosomal RNA database. Nucleic Acids Res. **32**:D101–D103.