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PHYSIOLOGICAL RESPONSES TO STRESS IN THE VIBRIONACEAE

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1. The Vibrionaceae

1.1. A GENERAL DESCRIPTION

The family Vibrionaceae (Domain Bacteria, Phylum Proteobacteria, Class Gammaproteobacteria) is comprised of mostly motile gram-negative chemoorganotrophs, possessing at least one polar flagellum (Farmer III and Janda, 2005; Thompson and Swings, 2006). Vibrios are facultative anaerobes, having both respiratory and fermentative metabolisms, and the mol% G+C of the DNA is 38-51% (Farmer III and Janda, 2005). Cells are usually 1 μm in width and 2-3 μm in length, and most are oxidase positive. The vast majority of vibrios require Na^+ for growth and survival, usually 0.5-3% NaCl for optimum growth. Additionally, most species are susceptible to the vibriostatic agent 0/129 (Thompson and Swings, 2006). In recent years, a two-chromosome configuration, one large and the other small (both circular), has been discovered to be a universal feature for all members of the Vibrionaceae (Iida and Kurokawa, 2006). The Vibrionaceae are ubiquitously distributed throughout aquatic habitats, freshwater and marine waters (Madigan and Martinko, 2006), including rivers, estuaries, lakes, coastal and pelagic oceanic waters, the deep sea, and saltern ponds (Urakawa and Rivera, 2006). Although as many as eight genera have been assigned to the *Vibrionaceae*, the two most specious are *Vibrio* and *Photobacterium* (Thompson and Swings, 2006). A third genus, *Salinivibrio* is worthy of mention due to its unusual ability to grow in a wide range of salinity (0-20% NaCl; Ventosa, 2005) and temperature (5-50°C; Bartlett, 2006) (refer to Table 1.).

Numerous species are pathogenic and cause disease in aquatic animals and humans (Farmer III *et al*., 2005), *Vibrio cholerae* being the most notorious example as the causative agent of cholera (Colwell, 2006). *V. vulnificus* and *V. parahaemolyticus* can also cause severe illness in humans as a result of consuming contaminated seafood (Hulsmann *et al*., 2003; Wong and Wang, 2004). Furthermore, every year *V. harveyi* (Owens and Busico-Salcedo, 2006), *V. anguillarum* (Miyamoto and Eguchi, 1997; Crosa *et al*., 2006), and *V. parahaemolyticus* (Austin, 2006) cause substantial economic losses to the aquaculture industry worldwide.

Study of the Vibrionaceae also has applications in ecoysystem health and conservation biology, especially in light of increasing contemporary concerns about human-induced global climate change. It is already clear that temperature is an abiotic factor that is critical for numerous vibrio symbioses (as discussed below), and it is possible that anthropogenic increases in the prevailing ocean temperature could have profound effects on ecosystems mediated partly through alterations in these symbioses. For example, *V. shiloi* is a pathogen of corals that causes coral bleaching at warmer ocean temperatures such as those expected to prevail in the future (Banin *et al*., 2003). For these reasons, the Vibrionaceae has galvanized tremendous basic and applied research. Increasing interest in recent years in the utilization of the genes responsible for light production from the bioluminescent bacteria *V. fischeri* for developing bioreporter monitoring and biosensor technologies illustrates this (Ripp *et al*., 2006).

1.2. SYMBIOSES WITHIN VIBRIONACEAE

Vibrio species not only occur as free-living members of the bacterioplankton but also regularly form symbioses—relationships between two or more organisms that encompass parasitisms, mutualisms, and commensalisms—with other aquatic organisms, including fish, invertebrates, algae, and other microorganisms (Nishiguchi and Nair, 2003; Meibom *et al.*, 2005). Within marine animals, *Vibrio* species are commonly found in the digestive tract and on their surfaces, including skin and chitinous exoskeletons (Urakawa and Rivera, 2006). Host-associated vibrios are provided with a microenvironment rich in nutrients and organic molecules compared to the surrounding seawater (Urakawa and Rivera, 2006). Hence, the vibrio population within or on the host is often several orders of magnitude higher than in the oceanic water column (10^2 cells/ml). *V. cholerae* reach a population level as high as 10^4-10^6 cells/copepod, while *V. halioticoli* can reach a population size at 10^6-10^9 cells/g of fresh gut in abalones (*Haliotis discus hannai*; Sawabe *et al*., 1995). Although some vibrios are pathogenic towards their hosts, numerous *Vibrio* species are part of the normal microflora of animals living in the ocean, such as oysters (Olafsen *et al*., 1993), blue crabs (Davis and Sizemore, 1982), sharks (Grimes *et al*., 1985), and hydroids (Stabili *et al*., 2006). The metabolic, physiological, and genetic traits permitting the Vibrionaceae to attach, colonize, proliferate in, and circumvent the defense mechanisms of their hosts to cause disease are undoubtedly homologous to those responsible for the establishment of mutualisms. These traits have a common and ancient evolutionary origin, giving rise to many different independent lineages (Nishiguchi and Nair, 2003).

In some instances, vibrios are intimate symbionts providing such an essential role that their hosts would be unable to survive in nature without them (Douglas, 2002). These roles include protection from pathogens, enhanced metabolic function, elevated environmental tolerance, or nutrient acquisition. The symbiosis between *V. halioticoli* and abalones (*Haliotis*) is one such example. In this case, the bacterial partner serves in alginate degradation, a brown algal polysaccharide the abalone consumes while grazing, and provides the gastropod host with an important energy source (Sawabe, 2006). Another example is *V. fischeri*, which is a bioluminescent symbiont of sepiolid squids and monocentrid fishes, and benefits these animals through a behavior termed counterillumination, allowing the hosts to conceal themselves from potential predators or prey (Jones and Nishiguchi, 2004). Considering host interactions partaken by vibrio

bacteria encompass the entire symbiosis continuum, from pathogen to indispensable microbial mutualist (Nishiguchi, 2001; Nishiguchi and Jones, 2004), a paradigm shift is emerging where some *Vibrio* species are considered beneficial and may have potential in the development of probiotics for commercially important aquaculture animals (Verschuere *et al*., 2000).

2. Stress regulation

2.1. GENERAL DESCRIPTION

Despite the fact that biologists uniformly recognize some environments as stressful, attempts to unequivocally define or quantify stress are difficult (Lenski and Bennett, 1993). The *Oxford Dictionary of Ecology* defines stress as, "A physiological condition produced by excessive pressures that are detrimental to an organism" (Allaby 2005), while the *Dictionary of Ecology, Evolution, and Systematics* states a stress is "…Any environmental factor that restricts growth and reproduction of an organism or population or causes a potentially adverse change in an organism or biological system; any factor acting to disturb the equilibrium of a system" (Lincoln *et al*., 1998). For many evolutionary biologists and ecologists, a more satisfying definition is one treating stress as any environmental factor (biotic or abiotic) reducing fitness (Lenski and Bennett, 1993).

"Stress," broadly considered, must also include any biotic or abiotic factors that fluctuate, and thus require organisms to adapt to them physiologically in order to survive. Most bacteria encounter such stressful changes in the environment, including the Vibrionaceae. They grow and survive in a multitude of habitats while possessing various lifestyles: aquatic sediments, fresh and brackish waters, oceans, symbionts of host organisms, saprophytes on detritus, and as free-living cells (Nishiguchi and Jones, 2004; Urakawa and Rivera, 2006; Dunlap *et al*., 2007). These different environments and lifestyles should not be viewed as static and permanent but rather as transient and cyclical (Urakawa and Rivera, 2006; Dunlap *et al*., 2007), where microbes migrate between each habitat while encountering stressful conditions (McDougald and Kjelleberg, 2006). These different habitats vary in a myriad of abiotic and biotic factors; consequently, the Vibrionaceae have evolved diverse physiological responses to stress and variable environments.

Previous research has shown fluctuating environments and stressors (e.g., oxygen and reactive forms, extreme salinities/temperatures) have important influences in symbiosis (Xu *et al*., 2004). For instance, a temperature downshift from 26°C to 18°C caused dramatic changes in the microbiota of the gastrointestinal tract in red hybrid tilapia, with tremendous proliferation of *Vibrio* spp. and a concomitant decrease in *Flavobacterium* (LeaMaster *et al*., 1997). Vibrio bacteria have also been shown to be distributed differentially both within host species located in different habitats, as well as in various seasons throughout the water column (Jones *et al*, 2007; Jones *et al*., 2006). The effect of fluctuating environments on the growth of non-host associated vibrios has been investigated less, but there are still some intriguing recent findings. For example, saline stress has been shown to affect the quality of organic carbon produced by vibrios

living in simple, microbial loop foodwebs. This phenomenon affects the quality of carbon available to other trophic levels (Odic *et al*., 2007).

The purpose of this review is to discuss the physiological responses of non-cholera vibrios to stress, especially to stressors likely encountered during symbiosis or during transitions from one host or lifestyle to another. We will also draw connections, wherever possible, among work that addresses vibrios from evolutionary, ecological, and molecular physiological points of view. We refer readers interested in *V. cholerae* to another recent review (Prouty and Klose, 2006).

2.2. TEMPERATURE

Vibrios encounter a broad range of temperatures, from those prevailing in marine habitats, to the higher temperatures tolerated by vibrios that can infect humans. Temperature is a significant determinant in shaping ecological associations of vibrios with countless host organisms, including eels (Amaro *et al*., 1995; Marco-Noales *et al*., 1999), squid (Jones *et al*., 2006; Nishiguchi, 2000), sea bream (Bordas *et al*., 1996), oysters (Kaspar and Tamplin, 1993), and coral (Rosenberg *et al*., 2007). For example, *V. shiloi* and *V. coralliilyticus*, both pathogens of coral, produce virulence factors implicated in bleaching and killing their hosts. In both cases, the production of these virulence factors is strongly regulated by temperature. At winter temperatures (16-20°C), virulence factors are not produced, while summer temperatures (25-30°C) induce virulence factor production (Rosenberg *et al*., 2007).

Temperature is a critical abiotic factor affecting other pathogenic symbioses, too. For example, chemotaxis is important for virulence of the fish pathogen *V. anguillarum*, and it is strongly affected by temperature. *V. anguillarum* is most robustly chemotactic at 25°C, and the chemotactic response diminishes in both cooler (5°C, 15°C) and warmer (37°C) conditions (Larsen *et al*., 2004). The stationary-phase associated sigma factor encoded by *rpoS* is required for *V. vulnificus* to survive heat shock (Hulsmann *et al*., 2003). An important virulence factor in *V. vulnificus* is capsular polysaccharide (CPS); CPS production appears to be controlled by a phase variation mechanism that can be detected by examining colony phenotype. Encapsulated cells make opaque colonies, while *cps*- cells make translucent colonies. Conversion from CPS+ to *cps*- (from opaque to translucent) is affected by temperature, as increasing the temperature from 23°C to 37°C increased switching for several different isolates (Hilton *et al*., 2006).

Since Vibrionaceae are aquatic microorganisms residing mostly within oceans, which are the largest cold environment on earth (Urakawa and Rivera, 2006) making up 71% of the earth's surface (Atlas and Bartha, 1998), some members of this group have been extensively selected to thrive in cold temperatures (Bartlett, 2006). Thus, although clinical and human pathogenic Vibrionaceae are mesophilic and capable of growth at ≥37°C, some members of this bacterial family's ancient lineage have adapted to low temperatures. Examples include *Photobacterium profundum*, *V. logei*, *V. wodanis*, and *V. salmoncida*. *Photobacterium* spp. have been more frequently observed to be the more prevalent member of the Vibrionaceae in the cold deep-sea, whereas the genus *Vibrio* is more common in cold ocean surfaces. These species are capable of growth at ≤5°C. Vibrios such as *V. diabolicus*, isolated from a deep-sea hydrothermal vent annelid

Alvinella pompejana, are heat tolerant, but no evidence exists that any member of the Vibrionaceae are thermophilic (Urakawa and Rivera, 2006).

Cold shock responses have been studied in *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* (McGovern and Oliver, 1995; Bryan *et al*., 1999; Datta and Bhadra, 2003; Huels *et al*., 2003; Lin *et al*., 2004;). Within the later two species, cold shock response increases survival at lower temperatures by a translation-dependent process. Research in this area is particularly important when considering applications of low temperature usage for food storage of shellfish (Bryan *et al*., 1999). As is generally true of stress responses in microorganisms, cold shock response involves changes in gene expression. Expression of cold shock proteins (CSPs) reach maximal levels during acclimation and includes the up-regulation of several proteins, including small homologous peptides 65-70 residues long in the CspA family (Ermolenko and Makhatadze, 2002). Most studies of the CspA family have been studied in greater detail in bacteria such as *E. coli* and *B. subtilis*. Proteins in this family have five antiparallel β strands that form a β barrel, creating a characteristic cold-shock protein domain well conserved throughout all three domains of life. CSPs often bind single-stranded mRNA and DNA, and are believed to assist bacteria in coping with unstable secondary structures at lower temperatures during ribosomal translation, mRNA degradation, termination of transcription, and perhaps nucleoid condensation, thereupon giving CSPs the function of nucleic acid chaperones. Additionally, there may also be a suppression of protein synthesis to prevent miscoding of polypeptides until the cold shock response is initiated (Ermolenko and Makhatadze, 2002).

To maintain functional membrane fluidity with decreasing temperature, vibrios are known to increase the unsaturation of fatty acids comprising their cell membranes. Adaptation to a fully psychrophilic lifestyle regularly, but not always, involves a decrease in enthalpy-driven interactions for the catalytic activity of enzymes, increasing the number of functional conformations permitted for enzyme-substrate complexes (Bartlett, 2006). For instance, the amino acid residues of psychrophilic enzymes within the cytosol display additional hydrophilic associations with the solvent, while simultaneously lessening internal hydrophobic interactions. This yields enzymes that are less condensed relative to mesophilic counterparts, which can result by increasing the α-helix and decreasing the β-sheet character of the secondary structure.

2.3. pH STRESS

pH stress is ecologically and evolutionarily significant because symbiotic vibrios include some gastrointestinal pathogens that must somehow survive the acidic challenge encountered in the digestive environment. Recently, molecular mechanisms of survival in the face of pH stress have been studied most intensively in the species *V. vulnificus*. This microorganism is an opportunistic pathogen of humans, acquired by ingesting contaminated seafood. There appears to be multiple overlapping signal transduction networks that together sense and respond to acid challenge. For example, the alternative sigma factor encoded by the *rpoS* gene is required for *V. vulnificus* to survive acid stress (pH <5) in both stationary and exponential phases of microbial growth (Hulsmann *et al*., 2003; Park *et al*., 2004). Other regulatory proteins such as CadC (accessory protein for the Cd^{2+} efflux ATPase CadA), SoxR (superoxide response regulator), and Fur (ferric

uptake regulator protein) are also needed for survival of acid stress. The CadC regulator in *V. vulnificus* induces *cadAB* expression, leading to the production of CadA (lysine decarboxylase) and CadB (lysine-cadaverine antiporter). Lysine decarboxylation is one step toward the production of cadaverine, which accumulates in the extracellular space during an acid stress response. The AphB transcription factor also enhances expression of *cadAB* under stressful acidic conditions, by directly activating a promoter that drives *cadC* production (Rhee *et al*., 2002; 2006).

The physiological response that leads to survival of acid stress is connected to the response that protects *V. vulnificus* from superoxide stress. The SoxR regulator, already known to regulate genes important for surviving oxidative damaging agents, also induces the *cadAB* operon (Kim *et al*., 2006). This induction does not require the CadC regulator, and cells lacking CadA are more sensitive to oxidizing agents than wild-type cells. Furthermore, simply increasing the amount of *cadAB* expression by supplying these genes on a multicopy plasmid is sufficient to reduce the induction of a superoxide dismutase that the cells would normally produce upon challenge with the oxidizing agent methyl viologen. Together, these results suggest that extracellular cadaverine not only neutralizes the local environment surrounding cells in acidic medium but also scavenges superoxide radicals (Kim *et al*., 2006).

Yet another connection between survival of acid and oxidative stresses in *V. vulnificus* was revealed with the discovery that cells grown to exponential phase and then subsequently exposed to acidic (pH=5.0) conditions induce the expression of *sodA*, a locus encoding a manganese-containing superoxide dismutase (MnSOD; Kim *et al*., 2005). MnSOD is positively regulated by SoxR and negatively regulated by Fur, but is not transcriptionally regulated by *rpoS*. Regulation by SoxR is likely indirect, while regulation by Fur is direct via binding to the *sodA* promoter. An explanation for the induction of MnSOD upon low pH is that shocked cells accumulate superoxides. In fact, use of a scavenger to prevent intracellular superoxide accumulation eliminated pH-dependent induction of MnSOD. Thus, it appears that MnSOD is not induced directly by acidic conditions per se, but rather by oxidizing agents that are themselves produced in response to acid shock. Deletion of any SOD in *V. vulnificus* (FeSOD, encoded by *sodB*; CuZnSOD, encoded by *sodC*, or MnSOD) led to decreased survival of exponential cells exposed to acid stress (pH=5.0). Therefore, acid resistance in *V. vulnificus* involves not only stress responses to acidity itself but also counteractions to superoxides that accumulate intracellularly upon acid stress (Kim *et al*., 2005). Additional investigations of pH stress in vibrios other than *V. vulnificus* are beginning to expand this area of research (Wong and Wang, 2004).

2.4. NUTRITIONAL STRESS

Ninety-five percent of the open ocean is oligotrophic, averaging a scant 50 g of carbon fixed per square meter per year by primary productivity (Atlas and Bartha, 1998). Host organisms, however, are nutrient rich. As vibrios experience transient free-living and host-associated life cycles, these microbes thus encounter feast or famine conditions in which they are either host-associated (feast) or living in the water column or sand (famine). They must therefore undergo long intervals with little or no growth and metabolic dormancy in their free-living state, followed by brief periods of rapid growth during symbiosis (McDougald and Kjelleberg, 2006). Given this natural history, it is no

surprise that many *Vibrio* species possess extraordinarily quick generation times during periods of high nutrient availability, enabling them to out-compete and outgrow other microbial species (Eilers *et al*., 2000; Giovannoni and Rappe, 2000).

They also appear to have been selected for effective starvation response mechanisms, and the molecular basis of these responses has been studied in some detail (Urakawa and Rivera, 2006). Researchers found that incubation of *V. vulnificus* in chambers suspended in natural estuarine waters led to *in situ* expression of both *rpoS* and *katG* (catalase peroxidase), regardless of different prevailing temperature and salinity conditions found in the summer and winter (Smith and Oliver, 2006). Perhaps these genes were expressed specifically to adapt to nutritional stress during the *in situ* incubation; regardless of the time of year, the dissolved organic carbon was only 2.83 mg/L. This *in situ* work is consistent with previous findings, which demonstrated that *rpoS* mutants were less able to survive starvation conditions initially, compared with wild-type, but after 14 days exhibited survival identical to wild-type (Hulsmann *et al*., 2003).

A study of *V. anguillarum*, which are fish pathogens, demonstrated a linkage between nutritional stress and virulence. Chemotaxis is an essential activity during infection, and starving (through incubation in phosphate-buffered saline) and *V. anguillarum* cells remained virulent as exponential-phase cells after 2 days, and were still chemotactic post 8 days starvation using LD_{50} (Larsen *et al*., 2004).

2.5. DNA DAMAGE

Like all cells, *Vibrios* must have mechanisms for repairing DNA damage caused by common environmental assaults such as exposure to UV irradiation. In *V. vulnificus*, *rpoS* mutants are much more sensitive to UV irradiation than their wild-type counterparts in exponential phase (Park *et al*., 2004). In *V. harveyi*, the small GTP-binding protein CgtA is required for survival upon exposure to ultraviolet light. Its role in the repair of damaged DNA is likely indirect, as CgtA stimulates *recA* gene expression (Zielke *et al*., 2003). The coevolution of DNA-interacting proteins and genome dialects, intergenome differences as a result of horizontal gene transfer, has recently been attributed to stress (Paz *et al*., 2005). Evolution of bioluminescence as a mechanism to aid DNA repair via the activation of light-dependent photolyase has also been proposed (Czyz *et al.*, 2003).

2.6. OXIDATIVE STRESS

Vibrio species encounter oxidative stress during colonization of animal hosts, even during mutualistic associations such as the symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (Ruby and McFall-Ngai, 1999). Thus, the question of the mechanisms by which certain *Vibrio* species survive oxidative stress has been under intense investigation. Several groups have investigated the role of the *V. vulnificus* sigma factor encoded by *rpoS* in survival following a challenge with the oxidizing agent H_2O_2. In one circumstance (strain C7184o), an *rpoS* mutant was much more sensitive to H_2O_2 than its wild-type counterpart during stationary phase (Hulsmann *et al*., 2003). Another case using a different pathogenic isolate of *V. vulnificus* (ATCC 29307), the *rpoS* mutant was more sensitive than wild-type to H_2O_2 challenge during exponential phase, but not during stationary phase (Park *et al*., 2004). However, the

ATCC 29307) *rpoS* mutant had reduced catalase activity in both exponential and stationary phases, despite the fact that differential survival upon challenge with H_2O_2 was only observed in exponential phase. Therefore, different roles of *rpoS* during oxidative challenge between these two *V. vulnificus* isolates might indicate that adaptation to oxidative stress has taken different pathways (i.e., convergent evolution) in distinct *V. vulnificus* isolates.

The SoxRS regulon and superoxide dismutases (SODs) have also been implicated in physiological responses needed to survive oxidative stress. In many cases, the production of SODs is linked to survival of multiple stressors. For example, in *V. vulnificus*, extracellular acid stress provokes the accumulation of intracellular superoxides and so further provokes a superoxide response (Kim *et al*., 2005; 2006). An extracellular superoxide dismutase is an important virulence factor in the coral pathogen *V. shiloi*; its production is induced by high temperature, indicating a connection between survival of oxidative stress and temperature stress (Banin *et al*., 2003). In *V. harveyi*, a pathogen of the farmed black tiger prawn, exposure to the superoxide generating drug menadione induces expression of both the OxyR and SoxRS regulons. *V. harveyi* also seem to exhibit physiological adaptation to oxidative stress, as exposure to sub-lethal doses of menadione protect *V. harveyi* cells from subsequent exposure to otherwise lethal concentrations of H_2O_2. Growing *V. harveyi* cells in high-salinity medium prior to exposure to menadione also led to increased protection to this oxidizing agent, suggesting a coupling between osmotic stress physiology and oxidative stress responses in this organism (Vattanaviboon and Mongkolsuk, 2001).

V. harveyi, like most vibrios, are bioluminescent. The enzyme luciferase directly catalyzes the production of photons and is encoded by the *luxAB* genes. The LuxD protein, encoded in the same operon, is an acetyltransferase that produces fatty acid substrates for the luminescence reaction. Mutants with null mutations in *luxA* or *luxB*, but not mutants with a null mutation in *luxD*, are hypersensitive to several oxidative stressors such as H_2O_2, cumene hydroperoxide, t-butyl hydroperoxide, and ferrous ions. Curiously, this hypersensitivity was found over a narrow range of concentrations of these agents, occurring neither above nor below this range. Nevertheless, *luxA* and *luxB* mutants were rescued by supplied antioxidants in the growth medium. This suggests that bioluminescence may have evolved as a response to oxidative stress (Barros and Bechara, 1998; Szpilewska *et al*., 2003).

As in *V. harveyi*, bioluminescence in *V. fischeri* consumes reducing power. The physiology suggests a possible relationship between redox homeostasis, responses to oxidative stress, and bioluminescence. Recent evidence demonstrates that the ArcAB system in *V. fischeri* represses expression of the *luxICDABEG* operon. Possible inactivation of the ArcA repressor by oxidative stress, experienced during the early stages of host colonization, likely derepresses *luxICDABEG* expression when *V. fischeri* colonize *E. scolopes*. This hypothesis, may explain why some strains of symbiotically competent *V. fischeri* such as ES114 are not visibly luminescent *in vitro* yet are visibly luminous during symbiosis (Bose *et al*., 2007).

Additional connections between oxidative stress physiology and host colonization may be present due to the need to survive stressors produced by the host. For example, in halophilic *V. fluvialis*, an opportunistic pathogen that causes gastroenteritis in humans, requires the *hupO* gene for surviving exposure to H_2O_2 during exponential phase (Ahn *et al*., 2005). HupO is a virulence factor that binds to hemin and likely affects intracellular

accumulation of hemin-associated iron during infection. The mechanism of *hupO*-associated H_2O_2 resistance is independent of catalase activity; therefore, the molecular details of how iron deficiency is connected to oxidative stress through HupO remain to be determined.

A relatively unexplored but related topic is the question of survival in the face of nitrosative stress, which symbionts also encounter when colonizing a host. It is increasingly clear that *V. fischeri* encounter nitric oxide during host colonization, as the host tissues lining the spaces where *V. fischeri* must traverse to colonize juvenile squids contain cells that produce NO and nitric oxide synthase (NOS; Davidson *et al*., 2004). NO production is normally considered to be a defensive strategy to prevent harmful bacterial infections, so it is striking that, in this case NO seems to function as part of the normal process of host-symbiont colonization that leads to a highly specialized and mutually beneficial symbiosis.

2.7. OSMOTIC STRESS

Vibrios live in environments that vary in salinity, and therefore experience high (hyperosmolar) and low (hypoosmolar) osmotic stress. During hypoosmolarity, the obstacles to cellular homeostasis are maintaining appropriate cytoplasmic concentrations of metabolites and ions, preventing cell lysis, and preserving ionic strength and pH (Bartlett, 2006). During hypoosmotic shock, some vibrios may increase putrescine content to compensate for decreased K^+ that are necessary to stabilize the phosphate backbones of nucleic acids. Hyperosmolarity, however, promotes dehydration and shriveling of cells. Microorganisms must be able to import or synthesize counterbalancing solutes that are compatible with metabolic and physiological functions. K^+ uptake is frequently stimulated to compensate for the increased external osmolarity. However, negative counter-ions (e.g., glutamate) must also be concurrently imported into the cell or synthesized de novo to sustain the same intracellular net charge (Sleator and Hill, 2001). Alternatively, cells can forgo K^+ uptake and import or synthesize neutral compatible solutes, as they carry no charge. Ectoine is such an example and its biosynthesis may be unique to the genus *Vibrio* (Bartlett, 2006). *V. fischeri* is also known to possess the ability to synthesize the disaccharide trehalose, which is also a neutral compatible solute for high osmolar stress. Incorporating polyunsaturated fatty acids in the cell membrane may also alleviate vibrios of excess toxic Na^+ by allowing their departure through the more fluid membrane (Valentine and Valentine, 2004).

As mentioned previously, *V. vulnificus* is an opportunistic human pathogen that can survive a range of osmolar conditions, from high-salt (or sugar) environments used to curb colonization of shellfish intended for human consumption, to those typical in marine environments and lower osmolarites as encountered in some compartments of the human body. For example, *rpoS* mutant *V. vulnificus* (C7184o) in stationary phase were much more sensitive to hyperosmolarity stress than their wild-type counterparts (Hulsmann *et al*., 2003). In contrast, *rpoS* mutant *V. vulnificus* (ATCC 29307) was no more sensitive to hyperosmolarity stress than its wild-type counterpart (Park *et al*., 2004). Perhaps these isolate-specific observations indicate that the *rpoS* regulon is not identical across all *V. vulnificus* isolates, evidence that a strain-specific genomic context is present for gene expression. Also in *V. vulnificus*, loss-of-function mutations in the *putAP* operon cause hypersensitivity to high osmolarity (Kim *et al*., 2006). The operon

encodes a proline dehydrogenase and a proline permease; proline dehydrogenase is part of a pathway that converts proline into glutamate, a well-known osmoprotectant. Two promoters, separated by 6 base pairs, control production of two transcripts from this operon. One is monocistronic and encodes only *putA*, while the second encodes both *putA* and *putP*. Expression of mRNA that hybridizes to a *putA* probe declines in stationary phase, while both *putA* and *putAP* transcripts are induced by proline and negatively regulated by glutamate (Kim *et al*., 2006). In contrast, high osmolarity induced higher *putA* mRNA levels but did not affect levels of bicistronic *putAP* mRNA, suggesting that only one of the two promoters is responsive to osmotic conditions. Additionally, both *putA* and *putAB* transcripts were dramatically reduced in a *crp*-mutant, suggesting a possible connection between survival of acid stress and nutritional status (as surveyed by intracellular cAMP levels; Lee and Choi, 2006). *Crp* mutants in other bacteria, such as *E. coli*, can have pleiotropic effects, so this relationship between nutritional status and survival of saline stress remains tentative in vibrio bacteria.

Osmotic stress has also been observed to have effects in other vibrios. For example, in the fish pathogen *V. anguillarum*, chemotactic responses to serine are decreased by high osmolarity (≥1.8% NaCl) relative to optimal osmolarity conditions (0.8% NaCl; Larsen *et al*., 2004). Proteomic analyses have been completed of *V. alginolyticus* and *V. parahaemolyticus* at different NaCl concentrations to examine resultant changes in gene expression through these physiological shifts (Xu *et al*., 2004; Xu *et al*., 2005). Since marine pathogens constantly face changes in osmolarity as they shift between marine waters and their native hosts, proteins such as outer membranes are selected to accommodate such changes. Outer membrane proteins OmpW, OmpV, and OmpTolC were discovered to be responsive osmotic stress proteins in *V. alginolyticus* (Xu *et al*., 2005). OmpV was expressed at low NaCl concentrations, but not at higher concentrations. Conversely, OmpW and OmpTolC displayed reverse changes, being expressed at high NaCl concentrations and down-regulated at low NaCl levels. Interestingly, differential expression of outer membrane proteins has been suggested by several researchers to play significant roles in symbiosis, including immunogenicity and virulence (Jones and Nishiguchi 2006; Xu *et al*., 2005). Not only were OmpW and OmpV identified in *V. parahaemolyticus* osmoregulation, but elongation factor TU and polar flagellin were implicated as well (Xu *et al*., 2004). Elongation factor TU and polar flagellin were respectively downregulated and upregulated at higher salinities, while OmpW and OmpV showed analogous patterns of expression, as in *V. alginolyticus*.

3. Experimental evolution and the viable but non-culturable state

3.1. EXPERIMENTAL EVOLUTION WITH VIBRIOS

In recent years, experimental evolution with microorganisms has emerged as an exciting new sub-discipline of evolutionary biology addressing diverse issues (Lenski *et al*., 1991; Bennet, 2002; Lenski, 2002), including microbial adaptation to variable environments and stress (Bennett and Lenski, 1999; Bennett and Lenski, 1997; Lenski and Bennett, 1993). The elegance of this scientific approach is the ability investigators have to control the selective regimen of the experimental conditions, to observe evolution and adaptation on a human time scale due to short generation times of

microorganisms, and the ability to compare evolving lineages from different evolutionary time points directly to a specifically known ancestor through the usage of a -80°C "frozen fossil record." This cryogenically preserved "fossil record" allows identification of changes in gene expression responsible for adaptation and loci subject to selection through subsequent genetic analysis (Riehle, *et al*., 2003). Although experimental evolution studies in the past were largely initiated with *Escherichia coli* as the major study microorganism, the list of other microbial species used in parallel studies has expanded in recent years. To date, the usage of Vibrionaceae in experimental evolution has principally been absent; however, such work is currently underway in our laboratory, as we are in the process of conducting serial passage experiments with *V. fischeri* derived from the sepiolid squid *Euprymna scolopes* (Hawaiian species) and evolving them through the novel host congener *E. tasmanica* (Australian species; Nishiguchi *et al*., 1998; Nishiguchi, 2002). Moreover, we are expanding such experimental evolution projects to address the ability of *V. fischeri* to adapt to abiotic factors at extreme limits of permissible growth based on previous studies that have shown introgression of various *V. fischeri* haplotypes to different habitats (Jones *et al*., 2006), as well as seasonal changes that cause changes in the viable *V. fischeri* baceterioplankton community (Jones *et al*., 2007).

3.2. THE VIABLE BUT NON-CULTURABLE STATE

A substantial literature now exists and continues to develop surrounding the viable but nonculturable (VBNC) state, whereby microorganisms normally culturable do not grow in liquid or agar media because of their entry into a dormancy where cells are still metabolically active and presumed to enhance resistance and survival to stress, a phenomenon first widely reported in the Vibrionaceae but is now believed to exist in other prokaryotes (Roszak and Colwell, 1987; Colwell, 2000). Nevertheless, the existence of VBNC cells has been contested and continues to be challenged (Bogosian and Bourneuf, 2001; Wong and Wang, 2004; McDougald and Kjelleberg, 2006). Past research on cells ostensibly in the VBNC state have included the identification of molecules and mechanisms (e.g., temperature upshift) that apparently resuscitate VBNC cells, enabling them to re-grow in microbiological culture media once again. Nonetheless, skepticism persists because of the possibility that any observed re-growth is the result of injured cells having recovered their healthy state, and not the result of resuscitating cells from a genuine VBNC condition (Bogosian and Bourneuf, 2001). Skeptics point out, as of yet, that no genes have been identified through null mutations or knockouts that may be responsible for vibrios entering a developmental program or pathway leading to a physiologically differentiated VBNC state. Convincing evidence would perhaps require loss-of-function experiments followed by complementation or over-expression gain-of-function studies (Bogosian and Bourneuf, 2001; McDougald and Kjelleberg, 2006). Proponents of the VBNC state remain convinced of its validity, perhaps not least because of the state's power to explain some ecological observations related to isolation of vibrio colony-forming units during different times of the year. Continued work into this area will surely lead to intriguing research, along with lively debate, for years to come.

4. Conclusions

Finally, extended examinations into the genetic traits and physiological responses characteristic of Vibrionaceae—quorum sensing, biofilm formation, two-chromosome architecture, induction of recombination machinery in the utilization of integrons and horizontal gene transfer—is essential to more completely understand their roles in regulating cellular homeostasis against stress (Boucher and Stokes, 2006; Iida and Kurokawa, 2006; Rowe-Magnus *et al*., 2006). There has not been much recent work specifically on stress and its effects on any of these phenomena, providing fertile ground for additional physiological investigations. Future work in areas such as experimental evolution, community ecology and population structure of vibrios in the environment, as well as specific trade-offs between symbiotic and free-living lifestyles should provide key insights into the adaptive radiation and speciation of this extensive group of bacteria.

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