Joseph Berberena

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Joseph Berberena

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1.1 Introduction: basic bacteriology and etiology of cholera

Cholera is an acute, infectious, severely dehydrating, sometimes fulminating enteritis endemic in India and Southeast Asia contained in marine and fresh water as well as food infected by contaminated feces. There is a relationship between the endemicity of cholera and the immunity or sub-immunity of the population of some geographic areas to Vibrio cholerae infection. The basis of cholera endemnicity has yet to be fully elucidated. Cholera may spread to other regions of the world experiencing warm temperatures achieving epidemic or pandemic status. Epidemic outbreaks of cholera involve vast geographical areas and large populations as observed in the many developing countries affected by this disease. Zuckerman states that there exists no evidence of imported cholera leading to epidemic illness in industrialized countries (World Health Organization. The Immuno- logical Basis for Immunization Series Module 14: Cholera, 2010, p.3). A vibrio of the Vibrionaceae family refers primarily to a Vibrio genus of naturally free-living, aquatic, gram-negative, facultative anaerobic bacteria consisting of straight, curved, or comma shaped rods whose single or multiple polar flagella provide motility in a manner microscopically appearing as rapid movement or vibration.

The taxonomy of cholera in descending order of inclusion consisting of Kingdom, Phylum, Class, Order, Family, Genus, and Species is Bacteria, Proteobacteria, Gamma-proteobacteria, Vibrionales, Vibrionaceae, Vibrio, and Vibrio cholerae respectively. The definition of vibrio is derived from the Latin word vibrare referring to rapid or vibratory movement. The genus Vibrio consists of Gram-negative bacteria that are auto-autochthonous saprophytes found in estuarine, riverine, and marine salt or fresh waters whose facultative anaerobic capability enables them to live or grow in the presence or absence of oxygen. Vibrio cholerae has been isolated in water, sediment, plankton, finfish, and shellfish samples from coastal, estuarine, and riverine environments. Vibrio cholerae have been found to inhabit fresh as well as brackish surface waters and are capable of surviving and multiplying in association with phytoplankton and zooplankton independently of infected human beings according to Huq et al. (Maheshwari et al. 2011, 423). The pathogenic species Vibrio cholerae is a facultative anaerobic Gram negative non-spore forming comma shaped bacillus about 1.04- 1.06 μm in length producing a soluble exotoxin that is a permeability factor for infection of the epithelial cells of the small intestine. Solely humans are the natural host for Vibrio cholerae which enters the body as the result of oral ingestion of contaminated food or water, or ingestion of raw food contaminated by vibrio cholerae laden fecal material or water respectively used as a fertilizer or for irrigation. Raw or undercooked contaminated fruits, vegetables, shellfish, fish, or any food contaminated during preparation or storage are involved in cholera transmission. Foods common to affected areas such as rice, millet, as well as other grains and legumes of neutral pH are efficient growth media for Vibrio cholerae. The freezing of food and liquids does not prevent the transmission of cholera. Ingestion of a substantial 10^8-10^{11} inoculum of infectious Vibrio cholerae organisms in

people exhibiting normal gastric activity and an inoculum of 10^4-10^6 in those exhibiting a deficient amount of hydrochloric acid (HCl) in the gastric juice (achlorhydria or hypochlorhydria) causes illness (Ryan, 2000, 561).

The etiological agent of cholera, Vibrio cholerae, also referred to as Koch's bacillus, is the cause of endemic, epidemic, and pandemic Asiatic cholera in human beings.

The cause of clinical cholera is infection by cholera toxin (CT, Ctx), a potent ADP-ribosylating exotoxin, also referred to as choleragen or cholera holotoxin, elaborated by the bacterium Vibrio cholerae. This intestinal pathogen causes the life-threatening diarrhea characteristic of cholera.

A sudden onset of watery diarrhea and vomiting occurs in about one to five days after the ingestion 2of Vibrio cholerae. The process of secretion involves the elaboration and release of a specific product from a gland or cell. Secretory action may vary from the separation of a specific substance from the blood to the elaboration of a novel chemical substance. The secretory process in bacterial species involves the transport or translocation of effector molecules. In the case of the pathogenic bacterium Vibrio cholerae, cholera toxin is transported from the cytoplasm of the cell interior to the exterior.

This cholera endotoxin is a protein molecule elaborated by Vibrio cholerae strains that colonize the small intestinal mucosa that act on epithelial cells causing hypersecretion of the electrolytes chloride (Cl^-) and bicarbonate (HCO_3^-) as well as the voluminous secretion of isotonic fluid from th e mucosal surface. Severe manifestations of cholera are characterized by a diagnostic painless watery diarrhea referred to as rice-water stools due to its resemblance to the white colored water seen during the washing of uncooked rice. The cholera patient can discharge as much as twenty liters of watery diarrhea daily containing 10^9 Vibrio cholerae in each milliliter of rice-water stool. The adult cholera patient can lose as much as one liter of water each hour. The result is excessive fluid loss, saline depletion, acidosis, and shock that is potentially fatal if not promptly and adequately treated.

Less than 10% of cholera patients exhibit symptoms of moderate or severe dehydration.

More than 90% of cholera cases are of mild or moderate severity and are difficult to clinically identify from other kinds of acute diarrhea (Crucell Sweden AB. Product Monograph Dukoral® 2012, 19).

Around 75% of cholera infected individuals do not develop any symptoms. Pathogenic Vibrio cholerae remain in their feces between 7 and 14 days and are capable of being shed back into the environment to infect other individuals (World Health Organization. Prevention and Control of Cholera Outbreaks:

WHO Policy and Recommendations. 2008, 1). Maier and Pepper state that the fatality rate for untreated cholera patients reaches 50% within a few hours or days after the onset of the disease (Kitaoka, 2011, 397).

The chemical and mechanical stages of digestion from the mouth through the small intestine change nutrients into forms capable of passing through the absorptive epithelial cells lining the intestinal mucosa for passage into the underlying blood and lymphatic vessels. These nutrient forms are glucose, fructose, and galactose monosaccharides, single amino acids, protein dipeptides and tripeptides, fatty acids, glycerol, as well as

monoglycerides from triglycerides. These digestive nutrients pass from the gastrointestinal tract into the blood or lymph in the process of absorption.

This process occurs by means of diffusion, facilitated diffusion, osmosis, and active transport. Ninety percent of absorption occurs in the small intestine. The remaining ten percent of absorption occurs in the stomach and large intestine. The large intestine receives and absorbs any unprocessed material passed from the small intestine. The processes of digestion and absorption of nutrients and most of the water present occur in the duodenum, jejunum, and ileum of the small intestine. Water absorption in the gastrointestinal tract occurs by means of osmosis from the intestinal lumen through absorptive cells into blood capillaries. Water absorption from the small intestine involves the absorption of electrolytes and nutrients for the maintenance of osmotic balance with the blood since water moves across the intestinal mucosa in both directions. The large intestine consists of the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anal canal. Although the colon is the portion of the large intestine extending from the cecum to the rectum, it is often used inaccurately to refer to the entirety of the large intestine. The large intestine contains bacteria that convert proteins to amino acids, break down amino acids, and manufacture some B vitamins as well as vitamin K. It also functions in the absorption of some water, ions and vitamins, feces formation, and defecation. The churning and peristaltic action of the large intestine drive the contents of the colon into the rectum for defecation of unabsorbed elements. In 1881, German physician and 1905 Nobel Prize in Physiology or Medicine laureate Robert Koch discovered the comma shaped Vibrio cholerae bacillus in culture from specimens taken from the intestinal tissue of Egyptian patients who died after 3 manifesting the typical clinical symptoms of cholera. He is credited with the discovery of Vibrio cholerae (Nair & Narain, 2010, 237). Entry of this bacterium into the human body is via the mouth.

An antigen (Ag) is a substance that has the ability to provoke a response by the immune system and react with the antibodies or cells resulting from the immune response. Antibodies are proteins produced by plasma cells in response to a specific antigen. The antibody also referred to as an immunoglobulin (Ig) combines with an antigen to effectuate its inhibition, neutralization,or destruction. Vibrio cholerae resides in aquatic environments and their associated zooplanktonand fauna as well as in the infected human and animal intestine. The cholera exotoxin is an antigenic extracellular toxin that when released into the environment can rapidly cause disease in small amounts. The primary site of action of Vibrio cholerae and its symptom producing cholera toxin is the intestinal mucous membrane. Only five micrograms ($5 \times 10^{-6}\mu g$) of cholera toxin cause the disease and the characteristic diarrhea for which the CT is responsible (Lopez-Gigosos, 2011, 57).

Sambu Nath De and D.N. Chatterjee reported their discovery of the cholera endotoxin in 1959.

They demonstrated that Vibrio cholerae alters the permeability of the intestinal mucosa causing aberrant fluid secretion, watery diarrhea, severe rapid dehydration, prostration, and the loss of sodium (Na^+), Chloride, Potassium (K^+) and Bicarbonate electrolytes (Nair & Narain, 2010, 237).

The origins of many of the electrolytes absorbed by the small intestine are ingested food and liquids, as well as gastrointestinal secretions. An isotonic solution has the identical

concentration of impermeable solutes as the semi-fluid portion of the cytoplasm which is also referred to as cytosol or intracellular fluid in which cellular organelles and inclusions except the nucleus are suspended and solutes are dissolved. The dehydration observed in clinical cholera is an isotonic occurrence since the measured cation concentration in stool water is equal to the cation concentration in plasma water. The anion concentrations of stool and plasma water are nearly equal. Although cholera toxin impairs intestinal permeability, it does not disrupt the intestinal mucosa or alter intestinal motility.

Although Vibrio cholerae was initially considered to cause non-inflammatory diarrhea, an intestinal inflammatory response has recently been reported according to Silva et al., Harrison et al., as well as Qadri et al.(World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera, 2010, p.8). Vibrio cholerae and its products do not cause sloughing of the cells of the intestinal mucosa unless an affected individual has been in a state of severe and prolonged shock. Thus, nutritional solids and oral rehydration fluid therapy can be beneficially ingested by the cholera patient to replace the electrolytes lost and sustain metabolic function. In 1959, N.K. Dutta demonstrated that the symptoms of the disease are caused by cholera toxin (Nair & Narain, 2010, 237). The incubation period may be only several hours. It is usually two to three days or as long as five days. This period varies inversely with the size of the Vibrio cholerae challenge, being the shortest when a large number of organisms are ingested at a given time. The symptomatic infection is characterized by the sudden onset of a voluminous watery diarrhea, vomiting, dehydration, and prostration. The major pathologies in cholera are severe dehydration and electrolyte loss that can be fatal if proper oral rehydration therapy is not promptly initiated. Despite the possible life-saving effect of oral rehydration, this therapy exerts no effect on the course of the illness or the dissemination of infection,

1.2 Vibrio cholerae serology, biotyping, and diagnosis

The Vibrio cholerae species of bacterium consists of serotypes classified on the basis of their respective somatic O surface antigen oligosaccharide. Serotype, also referred to as serovar or serogroup, refers to a taxonomic subdivision of bacteria based on the types and combinations of constituent antigens in a cell as well as a formula expressing the antigenic analysis that is the basis of the subdivision. It can also refer to a viral species group having a close antigenic 4 relationship. Vibrio cholerae subspecies classification is characterized by serogroups whose antigenic lipopolysaccharide composition differs and no known cross-rectivity between different serogroups occurs. The O blood group antigen is an innate host factor that determines the susceptibility of the host to cholera. Barua and Paguio state that the O blood group antigen confers serotype-specific protection against infection with Vibrio cholerae O1; with O blood group individuals demonstrating higher antibody responses compared to other blood groups as a response to the live attenuated CVD103-HgR cholera vaccine according to Lagos et al. (World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera, 2010, p.6). However, patients of the O blood group are more likely to suffer more severe symptoms and fatal outcomes upon infection with either the Vibrio cholerae O1 or O139 serogroup according to Faruque et al. and Harris et al. (Ibid.). People having the O blood group run a much higher risk of illness from Vibrio

cholerae than individuals of other blood groups. People living in the endemic Ganges River Delta region of India have the lowest prevalence of O blood group genes. This may be due to long-term genetic selection of O group individuals from the population where cholera has been endemic. However, the lifetime incidence of cholera infection is high for this population.

Strains belonging to the O1 serogroup are divided into the Classical and El Tor biotypes. Synonyms for a Vibrio cholerae strain may denote the geographical region in which it was first isolated.

Each O1 biotype can be subdivided into the toxigenic O1 Inaba, Ogawa, and Hikojima serotypes. Ogawa strains produce A, B and a small amount of C antigens. Inaba strains only produce the A and C antigens. A third toxigenic Hikojima serotype producing antigens A, B and C is rare, unstable, and not fully defined. The toxigenic O1 serotype consisting of the Inaba, Ogawa and Hikojima subtypes is characterized by variations in the composition of the O1 antigen. These antigenic variations are factors A, B and C, which are quantitative but not qualitative. The Ogawa phenotype is dominant over the Inaba form. The Vibrio cholerae O1 Hikojima serotype agglutinates with both anti-Inaba and anti-Ogawa antisera. The respective Inaba, Ogawa, and Hikojima serotypes comprise the antigenic formulas AB, AC, and ABC in which the 'A' antigen is common according to Sakazaki and Tamura (Ibid., 1). Inactivating genetic mutations of factor B, which may occur during cholera epidemics when Ogawa is the etiologic agent, may result in an irreversible change into the Inaba form.

The relationship between Vibrio cholerae serotype and memory consists of the capability of Inaba serotype induction of cross-reactive antibodies to the Ogawa serotype. However, the Ogawa serotype is not capable of inducing cross-reactive antibodies to the Inaba serotype. Mosley et al. believe that the difference in the specific immune response may be attributable to the difference in the immuno- dominant lipopolysaccharide epitope (Ibid., 12). The clinical effects of these two forms of cholera are similar. Vibrio cholerae strains of the VC O1 serogroup are subdivided into the three Inaba, Ogawa, and Hikojima serotypes as well as the two Classical and El Tor biotypes. O139 is the O antigen determinant for Vibrio cholerae O139 Bengal. Of the two hundred six serogroups discovered to date, cholera toxin producing Vibrio cholerae of the O1 and O139 serogroups cause cholera. There is also a toxigenic non-O1 serotype. Vibrio cholerae O139 was discovered in 1992 (Sack and Sack 2004, 223). It is the one hundred thirty-ninth serotype in the typing scheme for VC. The O1 and O139 serotypes of Vibrio cholerae cause epidemic disease. The present cholera pandemic is caused by the El Tor biotype of Vibrio cholerae O1. This biotype replaces the Vibrio cholerae O1 classic biotype as the dominiant Vibrio cholerae biotype. O141 represents a subsequent serotype isolated in the stool and occasionally the blood of patients in the United States. Toxigenic VC O141 has been associated with sporadic cholera diarrhea and bloodstream infection. Bacteremia is the presence of viable bacteria in the circulating blood. Vibrio cholerae O2-O140 are serotypes not associated with epidemics or pandemics categorized as non-O1 and non-O139 Vibrio cholerae. The Non-O1 and non-O139 serogroups do not produce cholera toxin. The sequencing of the entire genome of Vibrio cholerae has revealed the existence of 24 isolates.

Cholera diagnosis from a stool specimen involves microbiological identification of Vibrio cholerae and its characterization into the O1 or O139 serogroups. Microscopic examination for the presence of Vibrio cholerae motility in stool samples by direct bright or dark field microscopy is a favored but involved process requiring cold-chain specimen transportation as well as a suitably equipped and expertly staffed clinical laboratory. The gold standard for the identification of bacterial cultures requires 2~3 days and biochemical tests performed in an appropriately equipped laboratory by trained personnel (Sinha 2012, 524). Chilled Cary-Blair agar gel preservative transport medium self-contained in a sealable tube containing a rectal swab for fecal sample collection is used to collect the sample and is then cooled and transported to the diagnostic laboratory maintaining a cold chain for sample preservation. Enrichment of the suspected Vibrio cholerae organisms occurs in Alkaline Peptone Water (APW) and is subsequently plated onto selective Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar medium and incubated for the isolation and detection of the presence of diagnostic yellow colored colonies of VC. Further identification for serogroup is performed by means of slide agglutination kit testing using Vibrio cholerae antisera. A Polymerase Chain Reaction (PCR) consists of the laboratory production of many copies of a gene from the separation of both strands of DNA having the gene segment whose location is marked with a primer along with the DNA polymerase mediated assembly of a copy next to each segment and continuous copying of the gene copies.

The Real-time Polymerase Chain Reaction (RT-PCR) assay involves the extraction of total stool sample DNA resulting in a stabilized sample. Separate specific RT-PCR assays are performed using VC O1 *wbe* and VC O139 *wbf* gene specific primers. Processing and measurement is by means of RT-PCR instrumentation and a corresponding biochemical solution kit. Quantification of specimen Vibrio cholerae serotype O1 and/or O139 loads occurs based on the comparison of DNA data is obtained using reference sets from known cell numbers of the Vibrio cholerae serotypes being tested for. Diagnostic RT-PCR testing is more sensitive than other assay methods. Immunochromatography involves the separating of components of a mixture by chromatographic and immunoassay methods. Such chromatographic-immunoassays (CIAs) achieve low detection thresholds and are highly sensitive and specific. The Monoclonal Antibody (Mab) for the bacteria is conjugated on colloidal immunogold particles functioning as a mobile phase immunosensor. Another antibody is immobilized as a stationary phage immunosensor in line onto nitrocellulose membrane coated with Vibrio cholerae O1 and O139 'O' antigen specific monoclonal antibodies coupled to colloidal gold. The dipping of this membrane onto appropriately diluted or buffered Vibrio cholerae specimens results in discrete bands besides that of the control. The resulting antigen-antibody complex or Sandwich Assay is visualized as a colored capture line. A second antigen system consisting of murine Immunoglobulin G (IgG) and anti-mouse IgG functions as an internal control. Depending on the characteristics of the particular brand of test, a negative dipstick assay sample may appear as one colored line and a positive result as two colored lines. Involved testing procedures do not provide for the rapid cholera diagnosis necessary in the clinical or field setting when timely and appropriate implementation of rehydration and supportive treatment is essential for the minimization of morbidity and mortality. There are several brands of rapid diagnostic Vibrio cholerae test

kits available. The Sensitive Membrane Antigen Rapid Test (SMART) Kit is a fifteen minute colorimetric dipstick immunoassay for the direct presumptive detection of the Vibrio cholerae O1 content in human feces. Monoclonal antibody specific for the Vibrio cholerae O1 antigen circumvents the inherent problems involving polyclonal anti-O1 antibody use for the identification of VC O1 from clinical and environmental specimens. The homogeneity of the reagents is attributable to the use of monoclonal antibodies. It consists of a monoclonal antibody-polyclonal antibody sandwich assay format. Its monoclonal antibody is specific for the A antigen of the VC O1 lipopolysaccharide. The Crystal VC Dipstick Kit is another rapid highly sensitive and 6specific fifteen minute immunochromatographic test for the detection of Vibrio cholerae O1 and O139 in the clinical or field setting. Results are visualized as separate bands on a single test strip for the simultaneous and differential detection of Vibrio cholerae O1 and O139 in the presence of an inbuilt control band. Early detection as well as timely rehydration and antimicrobial therapy are essential to minimize the morbidity and mortality of cholera. The natural untreated course of cholera results in death in twenty to fifty percent of symptomatic cases (Institut Pasteur. Rapid Diagnosis of Cholera Based on Immunochromatography). Cholera survivors recover in about four to six days acquiring protective immunity after one week that can last for several months.

O antigens are saccharides comprising part of the cell wall lipopolysaccharide. A lipopoly- saccharide (LPS) is a compound or complex of lipid and carbohydrate. The carbohydrate components of the lipopolysaccharide of the cell wall of Vibrio cholerae are designated as O antigens. Chattterjee and Chaudhury describe three lipopolysaccharide domains comprising an oligosaccharide part (O-SP) consisting of glycosidically-linked sugars, the branched heteropoly- saccharide core sugars linking O-SP to lipid A, and the phosphoglycolipid part of lipid A anchoring to the bacterial membrane (World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera, 2010, 1). Cox and Perry as well as Hisatune et al. state that the O-SP and core region distinguish serogroups O1 and O139 and the O-SP terminal sugar differentiates Ogawa and Inaba O1 serogroups (Ibid.). The production of monoclonal antibodies in a laboratory setting involve the cloning of a specific antibody producing B-cell of the immune system capable of binding to a specific antigen. Antiserum is immune serum containing one or more antibodies demonstrably specific for a given antigen. Serotyping involves the use of specific absorbed antisera or monoclonal antibodies against the O antigen of the bacterial lipopolysaccharide of the particular Vibrio cholerae strain being tested. Individual Vibrio cholerae strains are differentiated by the structure of the cell wall lipopolysaccharide detectable by the use of specific antisera prepared to the somatic O antigen. All Vibrio cholerae O antigen groups share a common flagellar H antigen. Since flagellar or H antigens seem to be identical for all Vibrio cholerae strains, they are of little value for strain identification.

Chapter two:the genetics of cholera

2.1 Vibrio cholerae nucleic acid genetics, crisper/cas adaptive Immunity, plasmids, and virology

Vibrio cholerae O1 consists of the classical and El Tor biotypes. Differentiation of biotypes is possible by analysis of phenotypic traits as well as specific genetic markers. The O139 Bengal strain of Vibrio cholerae is capsulated exhibiting dissimilarities in the "O" antigen component of the bacterial polysaccharide as compared with O1 strains. Analysis of the *whf* gene cluster encoding the biosynthesis of the "O" antigen in the O1 and O139 strains showed replacement of a region encoding for O1 antigen specificity by a genetic element of larger size encoding O139 specificity.

Potentially pathogenic strains of Vibrio cholerae can emerge from the exchange of O-antigen biosynthesis genes. Non-O1 and non-O139 serotypes possess genetic sequences similar to those of O1 strains in their *whf* gene cluster; although other virulence encoding genes exhibit diversity. Transposition refers to the movement of a gene or a set of genes from one site in the genome to another. Genetic sequences similar to known insertion genes is indicative of transposition in the exchange of O1 for O139-specific gene clusters. Horizontal Gene Transfer (HGT, also referred to as lateral gene transfer) processes occur when a bacterium incorporates genes from another bacterium without being its offspring. Vibrio species exchange genetic material by means of Horizontal Gene Transfer. The strain that has emerged in this process is generally more virulent.

Deoxyribonucleic Acid (DNA) comprises the primary genetic material of cellular organisms and DNA viruses mainly occurring in the cell nucleus. DNA is a linear or circular polymer whose sugar is deoxyribose. Ribonucleic Acid (RNA) comprises the genetic material in RNA viruses functioning in the movement of genetic information in cells. Its sugar is ribose.

A virus is one of a group of infectious microscopic agents characteristically lacking an independent metabolism possessing the capability to replicate solely within living host cells.

Viruses reproduce with genetic continuity and their mutation is possible. The individual viral particle or virion is comprised of nucleic acid as a nucleoid, DNA or RNA (not both in a given virion), and a protein shell referred to as a capsid containing and protecting the nucleic acid which may be in a multilayered configuration. The three subgroups of viruses based upon their host specificity are bacterial viruses, animal viruses, and plant viruses; classified according to their origin, mode of transmission, or their manifestations occasionally named for the geographic location where they were initially isolated. A bacterial virus can effectuate transmissible lysis of bacteria. A virus particle attaches to the bacterial cell wall and its nucleoprotein enters the cell whereby viral synthesis occurs. The virus leaves the cell upon its physical destruction. A bacteriophage (also referred to as a phage or bacterial virus) is a virus that lyses bacteria having a specific affinity for bacteria containing either RNA or DNA found in association with all bacterial groups. A given phage is parasitically mated to a specific bacterial type. The word ending –

phage denotes an entity that eats or destroys. The bacteriophage is a virus particle that attaches to the bacterial cell wall.

Bacteriophages can infect virtually all types of bacteria. Approximately half of identified bacteria possess the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPER-Associated Proteins (CRISPER/Cas) adaptive immune system which functions primarily to provide immunity against bacteriophages. The CRISPER/Cas system confers sequence-specific protection from invading nucleic acids including phage. A bacteriophage-encoded CRISPER/

Cas system was observed to target and counteract a phage inhibitory chromosomal island of a bacterial host. The bacteriophage utilized the stolen immune system to disable and overcome the host Vibrio cholerae bacteria's defense system against phages. Thus, the bacteriophage is capable of infecting, replicating, and proliferating within the host cholera bacteria, then killing the host and proliferating producing additional phage offspring capable of killing more Vibrio cholerae bacteria. The bacteriophage encodes its particular CRISPR/Cas adaptive and functional response capable of utilizing the innate immune system of its Vibrio cholerae bacterial host to disable it to evade the innate immunity of the host enabling phage survival and proliferation.

This system demonstrates a flow of genes between the bacteriophage and the bacteria suggesting the possibility of developing phage therapies to treat bacterial infections as its origins and characteristics become more fully understood. Cholera related bacteriophages (also referred to as vibriophages) are from the genus Inovirus of the Inoviridae family. Their morphology is that of long slender proteinaceous tubes whose individual particles contain a single-stranded circular DNA genome that is converted to a double-stranded replicative form (RF) in infected cells recoverable as a plasmid. A plasmid is an extrachromosomal self-replicating structure of bacterial cells carrying genes for varied functions not essential for cellular growth; consistingof cyclic double-stranded DNA molecules replicating independently from chromosomestransmitting by means of successive cell divisions genes specifying functions such as antibiotic resistance, conjugation, production of enzymes, toxins, and antigens as well as the metabolism of sugars and other organic compounds.

Plasmids are transferable from one cell to another by conjugation and transduction. These processes will be further defined. Plasmids play a m ajor role in genetic exchange in numerous species of bacteria. Vibrio cholerae is the host for diverse vibriophages including virulent phages and temperate phages consisting of the kappa-type phages produced by many strains of the El Tor biotype. A temperate bacteriophage possesses genetic material in the form of a prophage that becomes part of the affected bacterial cell (that as a result is referred to as a lysogenic bacterium) remaining present through numerous cycles of cell division. The temperate bacteriophage infects but does not lyse its host bacterial cell.

The other group of vibriophages includes the filamentous phages possessing a single-stranded DNA (ssDNA) genome. A nucleoprotein consists of a simple basic protein generally in the form of a histone or protamine in combination with a nucleic acid.

Protamines are basic proteins that occur combined with nucleic acids. Histones are simple proteins comprised of many basic groups that are soluble in water. Deoxyribose and ribose are nucleoproteins consisting of deoxyribonucleic acid-protein and ribonucleic acid-protein complexes respectively.

Viral nucleoprotein enters the cell resulting in viral synthesis and its liberation upon physical disruption of the cell. Whether referred to as bacterial viruses or bacteriophages, theses entities are generally bacterial species specific, but may be strain-specific or capable of infecting more than one species of bacteria.

2.2 Vibrio cholerae transformation, transduction, and conjugation

Transformation, transduction, and conjugation are forms of horizontal gene transfer. Transformation is the exchange of genetic material involving the uptake of short DNA fragments mediating the exchange of any portion of a chromosome. Transduction involves the transfer of DNA by a bacteriophage the length of which is limited to the size of the phage head. The recipient and donor must possess specific cell surface receptors for phage binding. Eukaryotes are organisms whose cells contain nuclear material surrounded by a limiting membrane capable of mitosis; a process of cell reproduction involving modification of the nucleus resulting in two daughter cells having the same chromosome and DNA content as that of the parent cell. Plasmids are circular double stranded molecules of DNA replicating independently from the host chromosome that transmit by means of successive cell divisions genes specifying the antibiotic resistance of R plasmids, conjugation in F plasmids, production of antigens, enzymes, toxins, as well as the metabolism of sugars and various organic compounds. Conjugation and transduction transfer plasmids from one cell to another.

Transduction involves the transfer of genetic information by means of a bacteriophage vector from one bacterium to another. Transformation involves introduction of exogenous DNA into a cell changing its phenotype. It is a process by which bacterial plasmids can be introduced into bacterial cells. A transposon also referred to as a transposable element is a DNA segment possessing a repeat of an insertion sequence of genes at each end, capable of migrating from one to another plasmid within the same bacterium, to the bacterial chromosome, to another bacterial chromosome, or to a bacteriophage. The DNA segment moves from one position in the genome to another. Conjugation consists of the transfer of long DNA fragments, mediated by conjugal plasmids or conjugal transposons, occurring between distantly related bacteria, bacteria and eukaryotic cells, or from cell to cell contact. A conjugative plasmid is an extra-chromosomal element effecting its own extracellular transfer of various functional genes not essential for cellular growth from one bacterial cell to another by means of conjugation. Conjugal transposons are also transferred by means of conjugation. Bacteria reproduce asexually through the process of binary fission involving bacterial division resulting in a genetically exact copy of the bacteria. Bacterial reproduction can result in the generation of clones. Horizontal gene transfer processes can introduce DNA sequences displaying little homology with the remaining DNA of the recipient cell. Donor gene sequences can be stably incorporated into the recipient chromosome by genetic recombination should homologous sequences be shared between the donor DNA and the recipient chromosome. When homologous sequences flank sequences absent in the recipient,a small or large insertion from another strain of unrelated bacteria may be acquired by the recipient. Large insertions acquired from another bacterium, frequently resulting from differences in GC content or codon usage, are referred to as “islands” being

absent from related bacterial strains. Varying sequences of the nucleotides Guanine (G), Adenine (A), Thymine (T), and Cytosine (C) encode genetic information. DNA is a polynucleotide whose monomers are G,A,T, and C that individually possess a different nitrogenous base. Adenine and guanine consist of five carbon and four nitrogen atoms numbered 1 through 9 arranged in a ring structure that lie in the same plane. Cytosine and thymine consist of four carbon and two nitrogen atoms numbered 1 through 6 also arranged in a ring structure that lie in the same plane. Adenine and guanine are purines that are larger than the cytosine and thymine pyrimidines. The structural backbone of DNA possesses five carbons numbered 1', 2', 3', 4', and 5' distinguishing them from the numbering of the purine and pyrimidine rings. Purine and pyrimidine are bases that are nucleic acid constituents. The hydroxyl (OH) groups located on the 5'- and 3'- carbons are linked to a phosphate group forming the backbone of DNA polymer which has an alternating sugar-phosphate sequence. The deoxyribose sugars unite at both the 3'- and 5'-hydroxyl groups to phosphate groups in an ester link configuration also referred to as phosphodiester bonds. Ribose is the sugar component of ribonucleic acid that unlike deoxyribose possesses a hydroxyl group at the 2'-position. A nucleoside is one of four DNA bases covalently attached to the C1' sugar position. 2'-deoxyribose is the sugar in deoxynucleosides. Ribonucleosides possess ribose as sugar.

Unlike nucleotides, nucleosides do not possess phosphate groups. Deoxyadenosine (dA), deoxyguanosine (dG), deoxycytosine (dC), and deoxythymidine (dT or T) are different DNA nucleosides. A nucleotide is a nucleoside possessing one or more phosphate groups covalently attached to hydroxyl groups 3' and/or 5'. The structure of DNA is that of a double stranded helixmacromolecule whose sugar-phosophate backbones wind in a right-handed spiral around a helical axis consisting of two polynucleotide chains running in opposite directions held together by means of weak ther modynamic forces. The individual nucleotide bases located on the inside of the helix are stacked in a step-like fashion. Adenine forms two hydrogen bonds with thymine on the opposite strand.

Guanine forms three hydrogen bonds with cytosine on the opposing strand. The sugar phosphate backbone exists outside of the helix for polar phosphate group interaction with the polar environment; the nitrogenous bases occur inside the helix stacked perpendicular to the helical axis.

2.3 Vibrio cholerae a-b toxins, the toxin co-regulated pilus, lytic bacteriophages, the vibrio pathogenicity island, and cystic fibrosis transmembrane conductance regulator mediated fluid efflux

Vibrio cholerae produces a diarrheagenic cholera toxin that is a protein enterotoxin binding to enteric cell membranes stimulating the adenyl cyclase (also called adenylate cyclase or adenylyl cyclase) system causing Cl^- and HCO_3^- ion hypersecretion. Adenyl cyclase is the 3', 5'-cyclic adenosine monophosphate synthesase enzyme acting on ATP to form 3', 5'-cyclic AMP.

It is a lyase class enzyme occurring in plasma cell membranes catalyzing from ATP. A kinase is a phosphotransferase catalyzing high-energy phosphate group transfer from a

donor such as ATP to an acceptor compound such as an alcohol, carboxyl, nitrogenous or another phosphate group.

Cyclic adenosine monophosphate (cAMP, 3' 5'-AMP, 3', 5'-cyclic adenosine monophospate) is a cyclic nucleotide serving as an intracellular and sometimes extracellular second messenger that mediates the action of many peptide or amine hormones binding to cAMP-dependent kinases releasing free catalytically active subunits. The cAMP produced is an important metabolic regulator. Adenyl cyclases change cellular cAMP levels in response to limitations in nutrient availability.

Ctx is a highly evolved multifunctional bacterial protein toxin that engages the trafficking and signaling machinery of the host cell. There are two copies of cholera enterotoxin genes in classical biotype strains and one copy of the Ctx gene in most El Tor strains. Cholera toxin is a member of a family of A-B enterotoxins of bacterial origin comprising structurally and functionally distinct enzymatically active A polypeptide (*ctxA*) and receptor-binding B (*ctxB*) subunits.

CtxA and ctxB respectively refer to the gene code of each cholera toxin. B subunit heterogeneity has been observed on the basis of DNA and protein sequencing. Thus, *ctxB* typing can differentiate between strains of Vibrio cholerae. The two cholera toxin subunits are encoded by the *ctxA* and *ctxB* genes which are not part of the innate bacterial genome. *CtxAB* is one of the principal virulence factors of Vibrio cholerae. Lysis involves the destruction of bacteria, red blood cells, and other antigens by a lysin. Any substance that causes lysis, including specific complement-fixing antibodies that destroy cells and tissues is a lysin. Complement consists of a group of serum proteins controlling inflammation, activation of phagocytes, as well as the lytic attack on cell membranes activated by interaction with the classical complement pathway which consists of host cell antibodies that uptake the antigen using complement C1, C2, and C4 and the C3 convertase generating the immune system response. Host cell antibodies uptake antigens using complement proteins C1, C2, and C4 as well as C3 convertase that generates this pathway in an adaptive immune response. The alternative complement pathway coats the antigenic microorganism for uptake by phagocytes in an innate immune response. Complement receptors C1-C4 are cell surface receptors for C3 complement fragments. CR1, CR2, and CR3 are complement receptors for activated C3 fragments. CR1 and CR2 possess many complement control protein domains of the classical and alternative pathways. Integrins are a family of cell surface adhesion molecules (CAMs). Some interact with CAMs, others with components of the extracellular matrix or complement fragments. Integrase is the enzyme which which acts upon the integrin. CR3 and CR4 are integrins which function as cell surface adhesion molecules involved in intercellular adhesion. Some integrins interact with (CAMs), others with complement fragments, and others with extracellular matrix components. Certain chemical substances as well as antibodies are lysogens that induce lysis. These genes are borne on the genome of the CTX phi (CTXΦ) filamentous temperate lysogenic baceriophage whose genes encode the *ctxAB* cholera toxins through which these important genes of Vibrio cholerae are acquired.

CTXΦ is a lysogenic filamentous bacteriophage carrying structural genes encoding cholera toxin, as well as the Vibrio Pathogenicity Island (VPI) carrying genes for the pilus colonization factor toxin coregulated pilus. CTXΦ infection can convert nonpathogenic

Vibrio cholerae strains into toxigenic strains. The CTXΦ prophage forms varying arrays in the bacterial genome together with the related RS1 satellite phage. CTXΦ is a prophage in toxigenic Vibrio cholerae encoding cholera toxin. This prophage is active in the El Tor and O139 VC strains and is capable of generating extrachromosomal CTXΦ in a replicative process producing infective CTXΦ particles. The CTXΦ prophage is defective and inactive in classical VC strains and does not give rise to CTXΦ particles capable of producing infection. Filamentous bacteriophages are unusual in that the infected host remains viable and the phages are continually assembled and extruded from the cell. This is unlike most bacterial viruses assembled in the cellular cytoplasm and released by cell lysis. The CTX element is transmissible and can give rise to viral particles under appropriate conditions. An endotoxin from Vibrio cholerae consists of two protomers.

A protomer is one of the identical subunits comprising an oligomeric protein. The two protomers are the heavy (H) or A subunit and the light (L) or B subunits. The A subunit is an ADP-ribosilating enzyme functioning in CT toxicity by means of the stimulation of target cell adenylate cyclase and the corresponding fluid hypersecretion and electrolyte loss characteristic of cholera. The A catalytic subunit is cleaved by proteolysis into the A1 and A2 fragments. Fragment A1 is an ADP-Ribose monotransferase. The B subunit binds cholera toxin to the Monosyalosyl Ganglioside GM1 ganglioside receptors on the surface of intestinal epithelial cells and enables the uptake of the A1 fragment. This A1 catalyzed transfer of ADP-Ribose to the alpha subunits of heterotrimeric G proteins results in production of cyclic AMP which is believed to modulate the release of fluid and electrolytes from intestinal crypt cells. G proteins will be discussed further in subsequent sections of this text. CTX intoxication by cells consists of B pentamer-mediated receptor binding and entry into a vesicular pathway, translocation of the enzymatic A1 domain of the A subunit into the target cell cytoplasm, where covalent modification of intracellular targets leads to adenylate cyclase activation resulting in clinical cholera. The lysogenic filamentous bacteriophage genetically encodes a virulence factor. Phage conversion is the process by which bacteriophages convert their bacterial host from a non-pathogenic to a pathogenic strain through the provision of bacteriophage-encoded virulence genes to the host. A pilus is a minute filamentous appendage of certain bacteria associated with antigenic properties as well as cellular sex function. Pili (also referred to as fimbria) are filamentous adhesions binding prokaryotes together for genetic material transfer. The pathogenicity of Vibrio cholerae is dependent upon two important virulence factors; the Toxin-Coregulated Pilus (TCP) functioning as a colonization factor as well as the cholera exotoxin. The toxin coregulated pilus is a type IV pilus encoded in VPI I that allows Vibrio cholerae to aggregate in a mechanism that protects individual cells from the shearing forces in the small intestine. Vibrio cholerae cells secrete cholera toxin which activates the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in the small intestinal epithelia functioning in massive fluid efflux into the intestinal lumen resulting in the rapid fluid loss characteristic of cholera diarrhea. The toxin coregulated pilus is produced under the same conditions as cholera toxin and was named accordingly.

2.4 The genetics and mechanisms of bacterial gene recombination

The infecting bacteriophage attaches itself to a toxin coregulated pilus, a fine filamentous appendage on the surface of the cholera bacterium, serving as a bacteriophage receptor.

This receptor is an intestinal colonization factor. Pilin is the protein composing bacterial pili.

Vibrio cholerae produces several kinds of pili and carries genes for numerous pilin-like proteins.

The bacteriophage deposits its genes that code for CTX into the bacterium. CTXΦ employs the toxin-coregulated pilus as the primary receptor as well as TolQ, TolR, and TolA operon proteins as co-receptors to inject its DNA into the host cell. An operon is a chromosomal segment functioning in transcription and genetic regulation. *Att* refers to the functional attachment site.

CTXΦ utilizes the host XerC and XerD recombinases to integrate into the VC bacterial genome, in an integration process involving site-specific recombination between the att (phage-like attatchment site) attP of CTXΦ as well as the *attB* sequence within the *dif* site of chromosome I of Vibrio cholerae which is a sequence at which the XerCD recombinases resolve chromosomal dimmers in this process of DNA replication. XerC and XerD are tyrosine recombinases targeting the nucleotide deletion-induced filamentation (*dif*) sequence associated with the chromosome's replication terminus which is located in non-coding sequences containing a highly conserved XerD binding site. Bacterial DNA replication of circular chromosomes by homologous recombination is capable of generating concatenated chromosomes affecting cell viability.

Chromosomal deconcatenation takes place specifically at the *dif* chromosome site.

The *dif* locus is also believed to be involved in the integration and excision of exogenous DNA in addition to its role of chromosomal dimer resolution. The Vibrio cholerae filamentous phages CTXΦ and VGJΦ integrate into the host chromosome at the same VC chromosome I *dif* site.

It has been demonstrated that upon appropriate folding, the single stranded phage DNA of Vibrio cholerae forms a *dif*-like structure irreversibly combining with the bacterial *dif* through the use of host XerC and XerD recombinases (Carnoy, 2009). Thus *dif* is a preferential integration site for single-stranded filamentous phages whose genome has *dif*-like genetic motifs. Bacteriophage attachment sites are DNA regions required for site-specific recombination. The structure of the attachment sites is believed to be important for the recognition of recombination partners at these *att* sites by the recombinases mediating the site-specific recombination process. These sequences, sites, and recombinases will be further elucidated.

2.5 The vibrio cholerae chri-ii genome, bacteriophage replication, and the ctxφ filamentous phage

Vibrio cholerae bacteria possesss a multipartite genome consisting of two circular chromosomes; the primary Vibrio cholerae chromosome I (ChrI) and the secondary Vibrio cholerae chromosome II (ChrII). ChrI possesses numerous genes that code for essential biosynthetic pathways such as DNA replication, transcription, translation, cell wall biosynthesis, and pathogenicity. ChrII possesses fewer essential genes, additional species-specific genes, and more unknown Open Reading Frames (ORFs) encoding functional viral genes. ORFs will be described in subsequent sections of this text. Vibrio cholerae chromosomal replication occurs by means of a specific initiator molecule. Replication of ChrI is initiated at the Origin of Replication Locus I (*OriI)* by the DnaA protein which is the common chromosomal DNA replication initiator and global transcription regulator functioning in chromosomal replication in a majority of bacteria in a process involving ATP. Replication of ChrII is regulated at the plasmid-like *oriII* by the ChrII specific initiator RctB Vibrio specific factor. Replication of the ChrI and ChrII chromosomes is synchronous. ChrII is shorter and completes replication before ChrI.

It is believed that the genetic cross-talk between both systems prevents wide fluctuations in protein and chromosome abundance and facilitates coordination of chromosome replication and segregation. These processes are yet to be completely defined.

Bacteriophage transmission occurs in the gastrointestinal tract. Vibrio cholerae infects the mucosa of the small intestine. A bacterium devoid of these genes would not be pathological.Thus, some cholera bacteria cause disease while others of the same strain do not cause illness.

The bacteriophage is capable of colonizing different strains of cholera. Bacteriophage relationships to the host bacteria are specific. Phages are named after the bacterial species group, or strain for which they are specific. The genome of the CTX element is that of a filamentous bacteriophage. Choleragenic Vibrio cholerae cells are capable of prolific genomic rearrangement involving its main virulence locus coding for cholera toxin genes carried by the CTXΦ filamentous phage according to Waldor and Mekalanos (World Health Organization.

The Immunological Basis for Immunization Series Module 14: Cholera, p.22). Thus, release of a live CTX locus attenuated vaccine strain into the environment is capable of reverting to a virulent strain due to acquisition of wild-type cholera toxin genes borne by the phage. Cholera vaccination will be further discussed in a subsequent section of this text. Some non-O1 and non-O139 serogroups of Vibrio cholerae may carry virulence genes coding for CTX. They may be transferable to novel serogroups by a filamentous bacteriophage that binds to the toxin-coregulated pilus by a VC generalized transducing bacteriophage. CTX producing strains possess toxin-coregulated pili and are diarrheagenic in humans. Solely pathogenic Vibrio cholerae strains carrying the A-B type cholera enterotoxin encoded by *ctxAB* genes residing in the CTXΦ lysogenic filamentous bacteriophage genome cause epidemic and pandemic cholera. Stable lysogen formation by CTXΦ in Vibrio cholerae occurs. Bacteriophage replication does not result in Vibrio cholerae cell lysis. Thus, CTXΦ

can be horizontally and vertically transferred. These strains theoretically have epidemic potential.

2.6 Filamentous bacteriophages, the mannose sensitive Hemagglutinin, the type iv pilus, biofilm, chitin, competence for natural transformation, vibrio cholerae lipopolysaccharides lipopolysaccharides, cytokines, andimmune responses

Lytic bacteriophages, unlike phages that enhance bacterial virulence, can cause the death of their bacterial hosts by lysis. In the mid-nineteenth century it was recognized that certain elements, presumed to be bacteriophages, were protective against waterborne Vibrio cholerae.

According to Faruque as well as Jensen, it has been found that blooms of certain bacteriophages coincided with a decreased titer of Vibrio cholerae in environmental waters subjected to analysis (Boyd, 2008, 49). Faruque et al. state that environmental and human host Vibrio cholerae populations are subject to natural control by serogroup-specific bacteriophages (Kitaoka, 2011, 404). Research breakthroughs may eventually result in therapeutic modalities for the treatment of cholera patients and/or a use of these bacteriophages for the control of Vibrio cholerae proliferation in environmental waters. A number of filamentous bacteriophages other than CTXΦ and RS1Φ have been characterized from the Vibrio cholerae O1 and O139 serogroup isolates identified as phages 493, VSK, VSKK,VEJΦ, VGJΦ, KSF-1Φ, fs1, and fs2. Thus, there exists a variety of bacteriophages involved in the horizontal transfer of cholera toxin genes. Phage 493 is believed to have played a role in the emergence of the epidemic serotype of Vibrio cholerae O139 although its genetic structure and sequence have yet to be fully described. VGJΦ can infect Vibrio cholerae O1 classical and El Tor as well as VC O139strains. Bacteriophages VGJΦ as well as VEJΦ are capable of transmitting the genome of phage CTXΦ and the genes encoding cholera toxin horizontally among Vibrio cholerae strains expressing the Mannose Sensitive Hemagglutinin Type IV receptor pilus. Integration of VGJΦ as well as other filamentous phages is dependent upon the XerCD recombinases of their host.

VGJΦ elaborates functions required for the production and transmission of CTXΦ and RS1Φ. Mannose is an aldohexose epimer having glucose at carbon number two in its structure occurring in the oligosaccharides of many glycoproteins and glycolipids. An agglutinin is an antibody capable of aggregating a particulate antigen such as a bacterium following its combination with its homologous antigen. A substance other than an antibody capable of agglutinating particles is also defined as an agglutinin. Lectin is an example of such an agglutinin. Lectin is a highly specific carbohydrate-binding protein. A lectin is any of a group of hemagglutinating proteins mainly found in plant seeds specifically binding to the branching sugar molecules of glycoproteins and glycolipids on cell surfaces. Particular lectins specifically agglutinate erythrocytes of certain blood groups and malignant cells while sparing their normal counterparts. Other lectins stimulate lymphocyte proliferation. Lectins will be discussed further in subsequent sections of this text. A hemagglutinin (HA) is an antibody or lectin that agglutinates erythrocytes. The Mannose Sensitive Hemagglutinin (MSHA) is a subunit protein occurring as a Type IV surface pilus (or fimbria) on Vibrio

cholerae O1 of the El Tor biotype and VC O139. Since convalescing patients and vaccinees develop anti-MSHA antibody responses, MSHA may be immunogenic. Filamentous phages utilize pili as their receptors. The MSHA pilus, like the toxin-coregulated pilus of CTXΦ, is used as a receptor. MSHA pili can function as receptors of genetically diverse filamentous phages that can mediate horizontal gene transfer. Its MshA structural subunit gene is essential for the production of the MSHA Type IV surface pilus present on Vibrio cholerae O1 El Tor and VC O139 strains associated with human cholera. Although Vibrio cholerae O1 classical biotype strains express the *mshA* pilin subunit, they do not assemble MSHA pili on the surface of the bacterial cell and do not display an MSHA–dependent hemagglutination phenotype. Vibrio cholerae O1 of the classical biotype does not elaborate MSHA; the gene occurs and is expressed at levels comparable to those of other strains.

Vibrio cholerae occurs naturally in environmental aquatic habitats as a free-living organism in the water as well as a surface attached biofilm including abiotic surfaces, chitinous surfaces of zooplankton, and mucilagenous surfaces of phytoplankton. Chitin is a white insoluble linear homopolymer existing as the main constituent of arthropod exoskeletons, also occurring in plants and fungi. Chitin is a source of carbon, nitrogen, and energy in the aquatic environment for numerous microorganisms. Vibrio cholerae attaches to the chitinous shells of small crustaceans in the aquatic environment. The chitin- and the toxin-coregulated pili participate in attachment to chitinous surfaces. Chitin induces expression of a regulon functioning in its colonization and metabolism inducing genes believed to encode a type IV assembly complex.

The Vibrio cholerae bacterium exists both as a free-living organism in the water and attached to mucilaginous surfaces of phytoplankton, chitinous surfaces of zooplankton such as the exoskeletons of copepod molts in its riverine, estuarine, and coastal aquatic habitats.

Vibrio cholerae growth on a chitinous surface induces Competence For Natural Transformation in a mechanism of intra-species exchange of genes. Chitin is a nutrient for Vibrio choleraeinducing natural transformation for the acquisition of novel genes from other microbes in its habitat. Three positive regulators of the Vibrio cholerae Competence For Natural Transformation phenotype are the HapT, RpoS, TfoX genes, which will be further discussed in a subsequent section of this text. Chitin induction and activation of the competence mechanism is influenced by an increase in cell density, declining nutrient availability, deceleration of growth, or stress. Genetic and ecological factors influence the emergence of novel pathogenic bacteria in the environmental aquatic reservoir. Chitin-induced natural transformation is believed to be a mechanism essential for O-serogroup conversion occurring in aquatic habitats whereby Vibrio cholerae variants emerge better adapted for environmental niche survival or possessing enhanced pathogenicity for human beings.

The lipopolysaccharides of Vibrio cholerae O1 and O139 are antigens involved in the immune response against cholera. Serologically, three Vibrio cholerae O1 antigens are associated with the A, B, and C B-cell epitopes according to Villeneuve et al. and Wang et al (World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera, 2010, 7). Each of the over two hundred Vibrio cholerae serogroups consists of a highly conserved lipopolysaccharide lipid as well as a core region linked to the serogroup-specific O antigen side chain projecting from the outer membrane whose antigenic character

is determined by its monosaccharide composition, length, and structure. Jacobs states that the Vibrio cholerae lipopolysaccharide is a T-cell independent type I (TI-1) antigen not requiring T-cell involvement for antigen-specific antibody production (World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera, 2010, 7).

B-cells are activated by binding to more than one receptor. The eleven Toll-Like Receptors (TLRs) exhibit recognized pathogen-associated molecular patterns of innate immune responses as a first line of defense against invasive pathogens functioning in the regulation of immune cells, inflammation and cellular survival and proliferation. They are found on the cell's surface or endosomal//lysosomal compartment. The ubiquitous rapid response Nuclear Factor Kappa-light-chain-enhancer of activated B-cells (NF-κB) protein complex controls eukaryotic DNA transcription factors regulating immune and inflammatory responses to infection. Kappa light chains are found in immunoglobulins.

TLRs activate the NF-κB cytokine expression pathway linking adaptive and innate immune responses by the production of inflammatory cytokines such as certain interleukins and tumor necrosis factors, chemokines, cell adhesion molecules, growth factors, immunoreceptors, as well as the induction of co-stimulatory molecules of the complement variety affecting various cells types including epithelial and endothelial cells. The NF-κB factor is involved in various cellular and organismal processes involving immunity, inflammation, cellular development and growth, as well as apoptosis in response to ligation of various immune response receptors including T-cell, B-cell, and toll-like/Il-1 receptors. NF-κB responds to Vibrio cholerae bacterial antigens. Nagai et al. and Peng state that the lipid A portion of lipopolysacharide binds the Toll-like receptor 4 (TLR4) as well as other similar molecules; the TLR4 antibody inhibits the effects of LPS (World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera, 2010, 7). Complement refers to serum proteins produced by the liver found in circulating blood plasma and within body tissues involved in controlling inflammation, activating phagocytes, the prevention of excessive damage to body tissues, and cytolysis (the destruction of cell membranes). Complement receptors on B-cells are capable of binding C3d that may be covalently bound to lipopolysaccharide or the outer membrane structures of bacteria. Complement-antigen binding to the surface receptors of B-cells lowers the required threshold of crosslinking enhancing activation according to Lyubchenko et al. In addition, the cytokines released from macrophages subsequent to lipopolysaccharide binding to the surface TLR4 are capable of modulating B-cell activation according to Corbel and Melchers (World Health Organization.

The Immunological Basis for Immunization Series Module 14: Cholera, 2010, 7).

The role of complement in the immune response will be discussed further in a subsequent section of this text. Vibrio cholerae infection elicits substantial serum and intestinal anti-lipolysaccharide and anti-cholera toxin immune responses.

2.7 Vibrio cholerae o139 evolution from vc o1 involving Horizontal gene transfer and chitin-induced competence For natural transformation

Virtually every case of endemic and epidemic cholera was attributable to the Vibrio cholerae O1 serogroup until 1992. Infection stimulates the antibody response to the O1 side chain that corresponds to the development of a degree of protective immunity. The previously unknown

Vibrio cholera serogroup O139 Bengal, named according to where it was first discovered in the vicinity of the Bay of Bengal, was identified as the causative agent of cholera outbreaks in India and Bangladesh in 1992 which spread to other Asian countries. Analysis of VC O139 Bengal revealed that a DNA segment encoding the the VC O1 antigen *wbe* region had become deleted from this ancestral Vibrio cholerae O1 El Tor biotype and had been replaced by a *wbf* region DNA segment specifying the novel O139 serogroup antigen. It differed from the O1-encoding region in VC El Tor strains in that the O139-antigen gene cluster specifies the novel O139 side chain as well as a capsular polysaccharide (CPS) containing the identical O139-antigenic determinant. The closely related genetic relationship between the ancestral VC O1 El Tor lineage with the novel O139 serogroup, the presence of varying O-antigen encoding gene cassettes at the identical chromosomal locus, as well as regions of homology on either side of this site suggest that horizontal gene transfer occurred replacing the original VC O1 gene cluster with the O139 gene cluster of a Vibrio cholerae environmental strain by means of a mechanism yet to be fully elucidated. This VC O1-to-O139 gene cluster exchange may be mediated by chitin-induced Competence For Natural Transformation occurring between two varying Vibrio cholerae serogroups residing within the same biofilm on a chitin surface in nature whereby this ecological niche and horizontal gene transfer mechanism may provide for the serogroup switching described in this species. Such natural transformants possess the genetic background of VC O1 and a lipopolysaccharide and capsule characteristic of the novel the VC O139 strain. Vibrio cholerae O139-antigen gene cluster acquisition by the ancestral VC O1 El Tor strain is believed to have favored the recipient's growth and amplification in human beings already immune to the VC O1 serogroup to which its emergence as a principal cause of cholera may be attributable. Its continued persistence in environmental reservoirs may be due to a fitness advantage possessed by the VC O139 gene cassette in some environmental niches. One advantage may be its capacity to evade predation by bacteriophages. The prerequisites for

Competence For Natural Transformation-dependent

serogroup conversion consist of a recipient Vibrio cholerae capable of induction of competenc by chitin, an inducing chitin polysaccharide, a donor DNA source from a different

Vibrio cholerae serogroup existing in association with the recipient on the same chitinous surface, as well as the clonal expansion of the serogroup-converted transformant resulting from this process. Two natural elements consisting of chitin induction and bacteriophages function in Vibrio cholerae transformation. Chitin supports Competence For Natural Transformation and vibriophages act on susceptible bacteria providing a DNA source liberated from the bacteria.

The availability of chitin as an inducing substrate is believed to vary seasonally existing most abundantly during copepod blooms. Toxigenic Vibrio cholerae is believed to exist in a yet to be fully understood association with chitinaceous copepods during interepidemic periods, and these copepod blooms have the potential to result in cholera outbreaks. The clonal expansion of O139-serogroup convertants is believed to be attributable to the O139 side chain and capsule conferral of a selective advantage in environmental niche conditions conducive to VC growth and proliferation as an infectious agent to human beings as well as being a bacterium indigenous to aquatic habitats.

Chapter three: environmental factors influencing vibrio cholerae colonization, biofilm formation, virulence, and integration into the human host

3.1 Biofilm structural development and detachment in the aquatic environment

Vibrio cholerae bacterium also exists on abiotic surfaces. Biofilms are surface-attached microbial communities consisting of a thin layer of organic or inorganic microorganisms as well as their secreted polymers that adhere to surface structures. Transport and attachment of bacteria to a surface allows for the initiation of biofilm formation. Subsequent surface colonization is mediated by movement and growth of the attached bacteria as well as microcolony formation frequently surrounded by extrapolymeric substances. Additional bacterial growth as well as exopolysaccharide (EPS) production participate in the development of mature biofilm whose characteristic architecture consists of a pillar or mushroom-like cell structure separated by fluid-filled channels allowing nutrients to enter and wastes to diffuse out of the entire biofilm.

Bacteria swim toward the colonization surface utilizing their polar flagella establishing cell-to-surface interactions. Mature biofilm cells are capable of detaching and returning to the planktonic stage in a complete cycle of biofilm development. Detachment of mature biofilms depends on the degree of nutrient and oxygen availability as well as other stresses present in the environment. Extracellular or periplasmic polysaccharide lyases participate in biofilm detachment as well as cell dispersal in some biofilm-forming microorganisms by means of molecular mechanisms yet to be elucidated.

The presence of the Mannose Sensitive Hemagglutinin as well as the MSHA Type IV pilus and flagellum protruding from the surface of the Vibrio cholerae bacterium extending beyond its cell capsule are essential to its adherence to plankton and biofilm formation.

Transport and attachment of a bacterium to a surface initiates formation of biofilm. Flagella and pili are a requirement for the initial attachment of Vibrio cholerae cells to the surface. Bacteria swim towards the cell surface by means of their polar flagella forming cell-to-surface interactions, aided by the presence of the Mannose Sensitive Hemagglutinin type IV pilus. Upon cell surface contact, bacteria transit along the surface propelled by their flagella. They recruit more planktonic cells and divide forming microcolonies. Exopolysaccharide (EPS) production is necessary during subsequent stages of biofilm formation. Exopolysaccharide produced by the Vibrio polysaccharide (VPS) genes synthesized by attached cells is an essential component of the extracellular matrix stabilizing the biofilm and is essential for development of mature biofilm. Formation of mature biofilm consists of macrocolonies separated by water channels.

Surface colonization leads to microcolony formation usually surrounded by associated extrapolymeric substances. Additional bacterial growth and exopolysaccharide production result in the development of mature biofilm structures possessing pillars and channels.

Biofilm structural development is influenced by biomass growth rate, twitching motility, switching molecules and production of exopolysaccharides.

Vibrio cholerae attachment to planktonic detritus is believed to be its mechanism for escaping low nutrient conditions and increasing production of the VC bacterium. Vibrio cholerae is the cause of infectious outbreaks that are periodic and seasonal where it has established itself in the aquatic ecosystem. The characteristic surface of plankton has a mucilaginous polysaccharide that may also contribute to Vibrio cholerae adherence due to its nutritional and protective properties. The planktonic microenvironment provides a source of nutrition as well as protection from detrimental low or high salinity enhancing the ability of Vibrio cholerae to survive and grow in environmental waters. The Mannose Sensitive Hemagglutinin is believed to be ubiquitously expressed in the natural aquatic habitat of Vibrio cholerae.

Its only known role is that of facilitating VC persistence in the aquatic environment. This presence of MSHA as well as the presence of diverse filamentous phages using this receptor in the environment suggest that it has also contributed to the evolution of Vibrio cholerae. Contaminated shellfish from this aquatic habitat are harvested and enter the food chain causing Vibrio cholerae infection and illness in human beings.

VGJΦ is capable of transferring CTXΦ to Vibrio cholerae cells by a TCP-independent mechanism since its host receptor is the Type IV MSHA pilus bypassing the requirement for the toxin co-regulated pilus. The toxin coregulated pilus is a Type IV Vibrio cholerae pilus essential to intestinal colonization utilized by CTXΦ for infecting recipient cells. A bacterial flagellum is a long, movable, thin, whip-like projection functioning as a locomotive organelle consisting of proteinaceous flagellin strands woven into a helical filament attached to a cell wall basal body projecting from the free surface of a cell. The MSHA type IV pilus and flagellum aid Vibrio cholerae biofilm attachment to abiotic surfaces. Flagellation comprises flagellar movement required for surface bacterial spread. MSHA is also necessary for the colonization of biotic surfaces as is the case with the chitinous exoskeleton of zooplankton.

The co-regulated pilus as well as the TCP facilitate attachment to chitinous surfaces as well. The Maintenance of Biofilm Architecture A (MbaA) gene from the family of GGDEF- and EAL-domain regulatory proteins modulating second messenger c-di-GMP levels in cells are essential to the formation, maturation, and maintenance of the highly organized three-dimensional structure of Vibrio cholerae biofilms. The Vibrio cholerae gene MbaA codes for the inner membrane protein MbaA that down-regulates biofilm formation reducing the transcription of *vps* exopolysaccharide synthesis by a mechanism not fully understood. EPS production is necessary for biofilm development in the absence of MbaA.

The Vibrio cholerae gene *nspS* codes for the putative NspS binding protein believed to be a periplasmic sensor activated by the binding of the polyamine norspermidine. NspS which is also involved in the biofilm formation of Vibrio cholerae is thought to bind MbaA.

The *mbaA* gene is essential to biofilm maturation during which its highly organized three-dimensional architecture is formed and maintained. Biofilm architecture consists of a pillar- or mushroom-like structure separated by fluid-filled channels allowing nutrients to access every level of the biofilm as well as provide for the diffusion of toxic waste products out of the mature biofilm. The *mbaA* gene is believed to control the quantity of extracellular matrix within the biofilm and promote a greater amount of surface biomass during its later

developmental stages. The EAL domain or Domain of Unknown Function 2 (DUF2) is a signal transduction protein in bacteria that is ubiquitous.

It functions in the hydrolysis of the second messenger cyclic dimeric GMP (c-di-GMP). GGDEF proteins synthesize cyclic di-GMP (x-di-GMP) which is a protein activity regulator. The GGDEF domains in proteins possess the c-di-GMP synthase diguanylate cyclase (DGC) enzyme for c-di-GMP synthesis and the phosphodiesterase (PDE) enzyme for hydrolysis. c-di-GMP is believed to be the only substrate supporting this activity important in signal transduction and the second messenger process. c-di-GMP is a widely distributed bacterial signaling molecule controlling processes of bacterial surface life involving multicellular behaviors such as biofilm formation, surface motility, development, and regulation of gene expression. One DGC catalyzes c-di-GMP from two GTP molecules. The GGDEF provides for DGC activity when it exists as a dimer in a specific conformation which is dependent on the conformation of the sensory domains. TCP expression in the aquatic environment is presumed to be low since its only known essential role is in the intraintestinal environment. No apparent role for MSHA as an intestinal colonization factor has been found.

3.2 Vibrio cholerae biofilm matrix rugosity and structural genes

The response of Vibrio cholerae to environmental stresses is to undergo phase variation which results in smooth and rugose (wrinkled) colonial variants of distinct morphology.

The Vibrio cholerae rugose variant forms corrugated colonies and well-developed biofilms as well as having a higher level of resistance to osmotic and oxidative stresses than the smooth variant. Increased VPS production in the rugose variant results in these phenotypes which are mediated by proteins encoded by the *vps* genes. The *vpsR* gene encodes a necessary response regulator for *vps* gene cluster expression. VPS production in Vibrio cholerae is mediated by proteins encoded by genes in the *vpsI* and *vpsII* regions on the large chromosome separated by an intergenic region. The *vpsI* and *vpsII* refer to *vpsA-K* and *vpsL-Q* genes respectively.

VpsR and VpsT are positive regulators of *vps* gene expression in a manner homologous to the two-component superfamily system of regulatory proteins.

RbmB-F refers to Rugosity and Biofilm Structure Modulators consisting of five genes of the *vps* intergenic region. *RbmA* is the first gene in this region which is necessary for the fulldevelopment of corrugate rugose colony morphology as well as pillar and mushroom-like biofilm structures. The downstream *rbmB, rbmC, rbmD, rbmE,* and *rbmF* encode proteinsmodulating Vibrio cholerae rugose colony development and biofilm formation.Since *rbmA* expression is positively regulated by VpsR, it is believed to be coordinately expressed with the *vps* genes. Disruption of one or another of these genes in the rugose genetic variant prevents *vpsA* and *vpsL* expression, blocks VPS production, results in a smooth colonial morphology, and results in the loss of the characteristic three-dimensional biofilm structure formation.

The Biofilm Associated Protein *bapI* (or *vps*-coregulated protein) encodes a protein that also participates in Vibrio cholerae rugose colony and biofilm formation; it has a peptide

sequence similar to *rbmC*. *RbmB* influences rugose colony morphology, biofilm structure formation, as well as VPS accumulation. *RbmB* exists within the two Vibrio cholerae *vps* gene clusters subject to VpsR positive transcriptional regulation. It may act as a polysaccharide lyase by degrading the VPS composed of galactose, glucose, mannose, and *N*-acetylglucosamine necessary for colony corrugation development, biofilm formation, and Vibrio cholerae dissemination by means of yet to be fully identified mechanisms. A pellicle consists of a thin skin or film such as that found on a liquid surface. The pellicle consists of the biofilm form at the environmental air-liquid interface. The RbmC and BapI are secreted proteins essential for pellicle and biofilm formation whose transcription is positively regulated by VpsR. Hemolysis is the disruption of the red blood cell membrane. A hemolysin is a substance causing hemolysis. RbmC and its BapI homolog share peptide similarity with the Vibrio cholerae HlyA hemolysin *rbmC* and *bap1* transcription both being co-regulated in unison with the *vps* gene clusters, these genes putatively participate in colony morphology and biofilm formation, not as hemolysin proteins. RbmC and BapI may function as lectins by means of binding to extracellular matrix carbohydrates given their maintenance of rugose colony morphology as well as the stabilization of the pellicle and biofilm structures in Vibrio cholerae. RbmD, RbmE, and RbmF participate in cell aggregation as well as biofilm formation by means yet to be fully identified**.** Bile is a liver secretion that emulsifies lipids prior to their digestion in the intestine, also known to inhibit virulence genes in Vibrio cholerae. Bile is stored in the gallbladder. The term cholera is derived from the Greek word for bile “chole”. RbmABC as well as *bap1* levels are up-regulated in the presence of bile. These Vibrio cholerae extracellular matrix proteins stabilize the biofilm matrix.

3.3 Vibrio polysaccharide-independent biofilm formation and Dissolution in the aquatic environment

Vibrio cholerae are also capable of forming VPS-independent biofilms involving intercellular interactions between Ca^{2+} as well as the O-chain of the outer membrane lipopolysaccharide.

Specific control of biofilm formation and dissolution confers a selective advantage to Vibrio cholerae whereby certain environmental signals trigger biofilm formation and others are inhibitors that initiate its dissolution. The physicochemical conditions vary in natural aquatic ecosystems inhabited by Vibrio cholerae in the areas such as nutrient availability, salinity, temperature and Ca^{2+} levels. Ca^{2+} concentration varies from micromolar to millimolar levels in freshwater and marine aquatic environments respectively. Ca^{2+} is a negative regulator of gene transcription of VPS-dependent biofilm formation. The Calcium-Regulated Sensor (CarS) and Calcium Regulated Response Regulator (CarR) are constituents of this two-component system whose transcription decreases as a response to the increase in external Ca^{2+}. This CarRS two-component regulatory system is a negative regulator of *vps* gene expression and biofilm formation in Vibrio cholerae. CarR and HapR function in parallel pathways negatively regulating Vibrio cholerae biofilm formation. HapR does not regulate *vps* expression depending on Ca^{2+}. *Vps* gene transcription is positively regulated by the VpsT and VpsR transcriptional regulator. Ca^{2+} addition is believed to change either cell

to cell or cell to surface interaction creating more cluster yet less well attached biofilms.

Factors other than VPS may be involved in biofilm structural changes mediated by fluctuations in calcium levels. This is consistent with the ability of Vibrio cholerae to form VPS-independent biofilms requiring interactions between Ca^{2+} and the lipopolysaccharide outer membrane O-chain. Bacterial intracellular Ca^{2+} levels are governed by influx mechanisms involving ion channels, primary as well as secondary transporters, Ca^{2+} export systems, and Ca^{2+}-binding proteins (caBP). Increased Ca^{2+} is involved in decreased *vps* gene expression and VPS-dependent biofilm formation. Increased Ca^{2+} results in a decrease in Vibrio cholerae VPS-dependent extracellular matrix production. The effect of Ca^{2+} may be attributable to its function in extracellular polymeric matrix bridging, interactions with extracellular or cell surface Ca^{2+}-binding proteins, or its regulatory role on biofilm formation and surface attachment gene expression. Various rugose-associated phenotypes such as increased biofilm formation capability and **i**ncreased biocide resistance are partially mediated by *vps* gene functions. Vibrio cholerae rugose phenotypes involve production of an exopolysaccharide in which numerous bacteria may aggregate. Vibrio polysaccharide is the principal component of the Vibrio cholerae biofilm matrix essential to three dimensional biofilm structural development. Vibrio cholerae rugose forms are apparently resistant to chlorine conferring upon them the capacity to survive despite the stress placed upon them by chlorination treatment of the water in which they reside.

3.4 Environmental stresses influencing vibrio cholera Survival and biofilm formation

Survival and biofilm formation of Vibrio cholerae in an aquatic environment adapt to fluctuations in salinity and osmolarity through the production and transport of highly soluble small organic compatible solute molecules that counteract osmotic pressure. This transcriptional regulator or compatible solute regulator is referred to as CosR. Its expression is regulated by ionic strength rather than osmolarity. CosR represses genes functioning in ectoine biosynthesis and compatible solute transport in a salinity dependent manner. The term ectoine refers to a compatible solute found in microorganisms that are halophilic (pertaining to or having an affinity for salt or salty environments). CosR is an activator of biofilm formation and represses motility independent of its ectoine regulator function. This compatible solute regulator in Vibrio cholerae occurs in a manner in which the regulation of osmotic tolerance is related to biofilm formation and motility in the salt water environments in which they reside.

Oxidative stress refers to various pathological changes in living organisms in response to excessive cytotoxic oxidant and free radical concentrations in the environment. It results from production of such reactive oxygen species during the aerobic metabolism of bacteria or extracellular environmental conditions imposed on bacterial cells. AphA and AphB function in oxygen responsiveness. Vibrio cholerae represssion of virulence that is not needed anymore involves the oxidative modification of AphB. The adaptation of Vibrio cholerae to differing environments involves the reversible modification of AphB in the context of oxygen-rich aquatic environments and oxygen-limited human hosts. AphA and AphB will be discussed further in a subsequent section of this text.

3.5 Quorum sensing, hemagglutinin protease regulation, vibrio Polysaccharide, and biofilm formation regulatory genes

Quorum sensing (Autoinduction) is a process functioning in cell-to-cell communication used by bacteria to collectively regulate the expression of genes and coordinate group behavioral functions. This process allows for a synchronous alteration of Vibrio cholera activity in response to population density and species composition changes in the immediate bacterial environment. It involves an all-or-nothing switch from low to high or high to low cell density. The expression of genes for virulence and biofilm formation under conditions of low cell density promotes infection; the repression of these genes by autoinducers promotes bacterial dissemination under high cell density conditions in Vibrio cholerae.

Quorum sensing activating molecules are capable of repressing Vibrio cholerae virulence. Quorum sensing monitors cell-population density which is an important factor regulating the progression through the infectious cycle of Vibrio cholerae. Bacteria possess mechanisms that enable them to sense changes in the number of their given population and selectively change gene expression as a response to such cell density fluctuations. These mechanisms also confer upon them the capability of responding to changing environmental conditions such as those involving acidity, nutrient levels, and osmolarity. These multichannel signal-transduction circuits in Vibrio species regulate the expression of quorum sensing controlled genes.Quorum sensing functions in virulence factor production as well as biofilm formation.

Vibrio cholerae produces the Zinc (Zn)-metalloprotease Hemagglutinin Protease (HA) whichis an extracellular enzyme encoded by the hemagglutin protease regulator *hapA*. Hemagglutinin protease expression is regulated by quorum sensing. The hemagglutinin protease regulator (HapR) is a negative DNA-binding regulator repressing *vps* gene expression at high cell density and negatively regulates *rbmA* transcription. Thus, genes encoding the required proteins for VPS production and biofilm structure including *rbmA* are under coordinate regulation by VpsR and HapR. The function of HapR will be discussed further in a subsequent section of this text. The RbmA protein is produced in a higher amount in the rugose colony since its transcription is also higher in this variant. RbmA is necessary for the complete development of corrugated rugose colonial morphology and of the characteristic pillar structures of biofilms.

RbmA is a requirement for rugose colony formation in Vibrio cholerae as well as for structural integrity and fitness of the biofilm. VpsR and VpsT are transcriptional response regulators governing *vps* gene expression as well as the formation of corrugated bacterial colonies. They are positive regulators of VPS production as well as biofilm formation.
VpsR is the *vps* gene transcriptional regulator of VpsR, VpsT, and HapR located the furthest downstream. VpsT positively regulates *vpsR*.

It is believed that CarR is a negative regulator of *vpsT* and *vpsR* expression. *VpsT* expression is negatively regulated by the CarRS two-component system in Vibrio cholerae. Ca^{2+} functions as a signal from the environment having a negative effect on VPS-dependent biofilm formation. VpsR positively regulates RbmA production. *RbmA* exists in the *vps* region between the *vpsI* and *vpsII* clusters containing the required genes for VPS production.

Phase variation and the expression of associated properties among smooth and rugose variants are thought to coincide with Vibrio cholerae survival in aquatic environments through positive and negative regulation of multiple proteins. RbmA is necessary for the maintenance of rugose colony morphology and the development of wild-type biofilm structure; a requirement of which is VPS production. VPS consists of almost equal quantities of glucose, galactose, as well as lesser amounts of *N*-acetylglucosamine and mannose; suggesting that RbmA may be a sugar-binding protein whose mechanism of function has not yet been fully determined. Bacterial attachment to surfaces and the biofilm formation that follows constitutes a survival mechanism for bacteria, since surfaces absorb and concentrate nutrients from the surrounding water and protect against grazing predators as well as toxic compounds. The RbmA necessary for rugose colony formation as well as biofilm fitness enables cells to establish structured and detergent-resistant biofilms.

3.6 Quorum sensing/autoinduction genes and mechanisms, conditionally viable environmental cells, virulence factor expression, and the vibrio cholerae environment-human host-environment lifecycle

Quorum sensing is an adaptive response mechanism by which some bacterial species produce, secrete, and detect extracellular signaling autoinducer molecules to communicate with each other to control gene expression in response to cell density. Bacteria exist in two behavioral modes under the influence of quorum sensing. Under conditions of low cell density, bacteria function in a distinctly individual cell mode. Bacteria function in a more social mode under conditions of high cell density. Autoinducers are secreted under conditions of low cell density but do not accumulate to detectable levels. Increases in cell population density result in increased extracellular autoinducer concentration. Once autoinducers reach a sufficiently high level of concentration, they are recognized by and bind to cognate membrane sensors once this threshold is reached and are capable of transducing the signal to transcriptional regulators located downstream or functioning as transcriptional regulators mediating variations in gene expression. Binding facilitates signaling by means of a downstream phosphorelay pathway altering HapR transcriptional regulator expression. Quorum sensing autoinducers are typically Acylhomoserine Lactones (HSLs, AHL, or AHSLs) in gram-negative bacteria that are detected by either cytoplasmic LuxR or membrane bound LuxN receptors. The Luciferase (Lux) operon will be discussed further in subsequent sections of this text. Acylhomoserine lactones are diffusable signaling molecules whose production and reception occur during quorum sensing in gram-negative bacteria. It is a process of cell to cell communication amongst gram-negative bacteria relying on the detection and production of autoinducers functioning as extracellular signaling molecules. The LuxI-like autoinducer synthase gene catalyzes the formation and synthesis of the specific acylated HSL signal molecule. The AHSL synthase encoded by homologs of the AHSL synthase gene *luxI* produce the signal molecules. *LuxI* is expressed at a low basal level under conditions of low population density and the AHSLs accumulate. The respective signal generator, signal molecule, and signal receptor of quorum sensing systems consist of the LuxI homologue, AHSL, and the LuxR homologue. Quorum sensing target gene transcription is activated at a threshold population density proportional to the AHSL

concentration referred to as the quorum size which is the amount of bacteria required for quorum sensing system activation. AHSLs are perceived by the LuxR family of receptor/response regulator proteins.

The LuxR-like autoinducer diffuses through the cell membrane functioning to bind the AHSL autoinducer activating luciferase structural operon transcription under high cell density conditions. High autoinducer levels cause the LuxR-like protein to bind its cognate AHL autoinducer complex binding at target gene promoters functioning in AHSL autoinducer recognition and the transcriptional activation of the downstream target DNA genes.

The threshold concentration of AHSL results in complex formation between the signal molecule and the receptor protein and complex activation occurs. This activated complex forms dimmers or multimers with other LuxR-AHSL activated complexes functioning as transcriptional regulators of quorum sensing regulated target gene expression. The LuxR protein amino- terminal regions function in AHSL autoinducer binding; its C-terminal domains function in oligomerization as well as promoter DNA binding. Specific signal production results from an exact interaction of the LuxI-type with a certain acyl-acyl carrier protein (acyl-ACP). *Acf* is the gene code for the acyl carrier protein. LuxI-like enzymes couple the acyl-side chain of the acyl-ACP from fatty acid synthesis to the homocysteine moiety of 5-adenosylmethionine (SAM) for the production of a specific acyl-HSL. The biosynthesis of a particular acyl-HSL and oligopeptide autoinducer results in the production of a species-specific signaling molecule. TheLuxR/AHSL transcriptional activator signaling circuit is the mode of cellular communication between gram-negative bacteria whereby the acyl-HSL autoinducer diffusion through the bacterial membrane results in their increased environmental concentration concomitant with growth of the bacterial cell population. It is not a gradient based mechanism. Quorum sensing functions as an all-or-nothing switch from low to high or high to low cell density. Gene activation or repression result when the involved diffusible autoinducers reach a critical concentration as cell density increases at the particular location. Such cell-to-cell communication to coordinate gene expression takes place within and between various species and is believed to facilitate successful colonization and infection.

Pathogenic bacteria become virulent upon reaching a particular local concentration within the host.

The production of numerous extracellular virulence factors is regulated in a growth-dependent manner due to quorum sensing. Communication between quorum-sensing bacteria occurs by means of autoinducers functioning as extracellular signaling molecules facilitating community-wide gene expression synchronization. Quorum sensing is a means by which bacteria perform collective functions. Such quorum sensing pathways play a role in the regulation of cellular adaptation in response to changing environmental conditions including various forms of stress as well as starvation.

This type of bacterial cell-to-cell communication provides for population density-based regulation of gene transcription by the production, release, and sensing of signaling compounds.

A particular autoinducer concentration triggers a signal transduction cascade resulting in the expression of one or more target genes. Vibrio cholerae is a pathogenic bacterium causing acute disease that utilizes quorum sensing to repress the production of virulence

factor and formation of biofilm. Molecules activating quorum sensing in Vibrio cholerae are capable of controlling its pathogenicity.

Such quorum sensing systems serve to improve microbial access to nutrients and environmental niches, as well as enhance microbial defensive capabilities against other microorganisms or eukaryotic host defense mechanisms. Quorum sensing confers upon Vibrio cholerae the capability to essentially function in unison as would multicellular organisms to regulate their genetic competence, biofilm formation and virulence enhancing their ability to prevail.

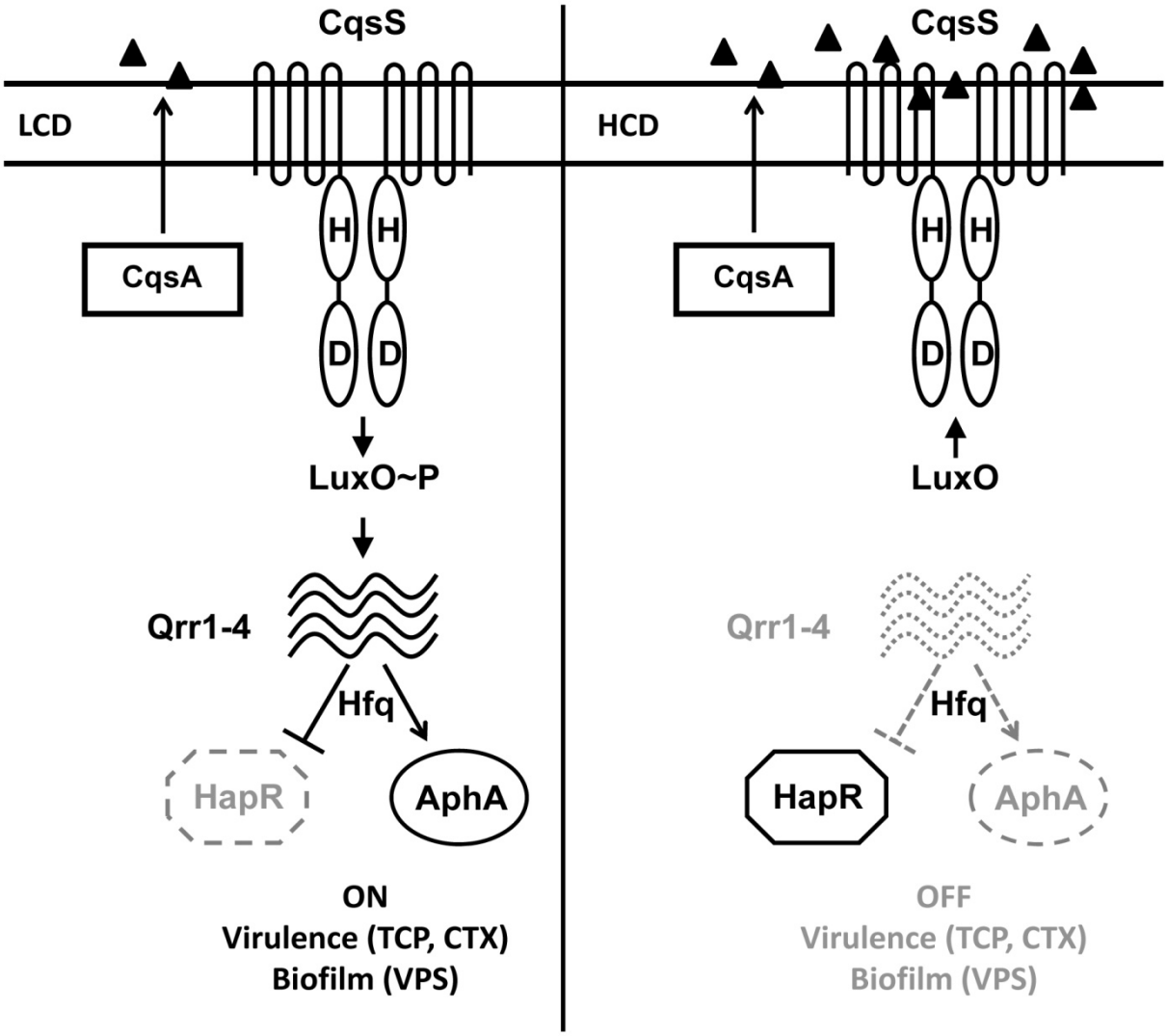


Figure 1. The Quorum-Sensing Circuit in Vibrio cholerae.

The CqsA/CqsS signal transduction system is shown as the example for the V. cholerae QS circuit.

(Left) At low cell density (LCD), the CAI-1 autoinducer concentration is below the detection threshold, and the membrane bound CqsA receptor functions as a kinase. The LuxO response regulator is phosphorylated and it activates the transcription of genes encoding the four Qrr sRNA genes. Aided by the RNA chaperone Hfq, the Qrr sRNAs activate and repress translation of the AphA and HapR proteins, respectively. (Right) At high

cell density (HCD), binding of the CAI-1 to CqsS inhibits its kinase activity. LuxO is not phosphorylated and transcription of the *qrr* genes is terminated.
Translation of AphA is inhibited and HapR is derepressed. Hundreds of genes are controlled by AphA and HapR, including genes required for biofilm formation and virulence. HapR also functions as a transcriptional activator of the heterologous *V. harveyi lux* operon [22], [24], [26]-[30]. Dotted lines denote components that are not expressed while solid lines represent those that are produced. (Ng, et al. 2012, 10.)

The Vibrio cholerae life cycle in the host involves a phase whereby the bacteria colonize the intestinal epithelium forming a thick cellular mat that resembles a biofilm. These clumps of Vibrio cholerae derived from quorum regulated biofilm can survive and exist in environmental waters on a long term basis as naturally occurring Conditionally Viable Environmental Cells (CVEC) that can assume a quiescent physiological state comprising a genetic clone closely related to local Vibrio cholerae isolates of the most recent epidemic. The human host can produce a hyper-infectious form of pathogenic Vibrio cholerae and excrete this Vibrio laden biofilm into environmental waters where they survive and proliferate. Under suitable conditions they no longer remain quiescent and infect subsequent human hosts who ingest water or food that came into contact with the contaminated environmental water contributing to the endemic or epidemic transmission of the pathogen. Environmental abiotic and biotic factors as well as its seasonality, as observed in the Indian subcontinent, are believed to contribute to the endemicity of cholera in a given region. The genetic regulators that provide for quorum sensing and biofilm formation provide for the development of CVEC as well.

The culmination of virulence factor expression in Vibrio cholerae contributes to the ability of this bacteria to escape from the host and re-establish itself in the environment. The extracellular enzyme hemagglutinin protease is secreted by way of the Eps pathway, as is cholera toxin.

MSHA needs another set of genes for its assembly and outer membrane translocation. CTX, HAP, and MSHA secretion requires the function of VcpD to process the MSHA subunit and the pilin-like proteins EpsG, -H, -I, -J, and –K required for the secretion of Ctx and HAP. Quorum sensing represses the expression of the Vibrio cholerae virulence factor Ctx exerting an antimicrobial effect in the following environmental example. The chironomids (Diptera; Chironomidae) are insects that are abundant in bodies of fresh water serving as natural reservoirs of Vibrio cholerae. Vibrio cholerae quorum sensing signals up-regulate hemagglutinin protease production which degrades chironomid egg masses preventing them from hatching,
serving as a virulence factor against chironomids as a source of pathogenic Vibrio cholerae toxin in the environment. This phenomenon is in contrast to the role of quorum sensing observed in other bacteria.

Free extracellular DNA abounds in aquatic environments. Although some of this DNA is degraded by nucleases secreted by the surrounding microbial community, some remain available to play a role in natural transformation of Vibrio cholerae, which possesses the extracellular nuclease-encoding genes Dns and Xds that are capable of degrading free DNA whereby it becomes unsuitable for transformation. Dns exists as a periplasmic enzyme catalyzing DNA hydrolysis (DNase) encoded by the *dns* structural gene that inhibits natural

transformation by hydrolysis of exogenous DNA in a cell-density dependent manner. A DNase is any nuclease that specifically catalyzes the hydrolytic cleavage of phosphate ester linkages in deoxyribonucleic acids. Dnase will be discussed further in a subsequent section of this text. Dns and xds gene products are responsible for the activity of extracellular DNase. Extracellular DNA is degraded when cell density is low. Vibrio cholerae growth on a chitinous surface involves an increase in transformation efficiency corresponding in time with increased cell density and the repression of *dns* expression by the HapR quorum sensing regulator, which is the principal regulator of the VC quorum sensing system. Dns degrades free DNA under low cell density conditions.

The absence of HapR at low cell density relieves *dns* repression and the corresponding degradation of free DNA occurs leading HapR to repress genes encoding virulence and biofilm formation as cell density increases, as well as inducing required genes for natural competence.

HapR negatively regulates *dns* expression at the transcriptional level. A regulatory cascade processes quorum-sensing signals. Present in Vibrio cholerae quorum-sensing circuits is the Hfq RNA chaperone protein which mediates interactions between small regulatory RNAs (sRNAs) and specific target messenger RNAs (mRNAs), altering the stability of target transcripts. sRNAs are post transcriptional regulators of genes involved in responses to adverse conditions such as cell surface and oxidative stress. Many sRNAs require Hfq for post-transcriptional regulation. Hfq binding to certain sRNAs as well as their mRNA targets promotes base-pairing and sometimes facilitates sRNA-mRNA complex degradation. The presence of Hfq is a requirement for virulence-factor expression. Hfq mediates the destabilization of the mRNA encoding the Vibrio choleae quorum-sensing master regulator HapR. HapR is a requirement for the expression of quorum sensing controlled genes. By implication, there is an sRNA in the circuit. Four sRNAs are present in Vibrio cholerae. The chaperone Hfq protein in concert with the four sRNAs creates a very sensitive regulatory switch controlling the transition into the high cell density quorum-sensing mode. Molecular chaperones comprise a diverse group of proteins overseeing the proper intracellular folding and assembly of polypeptides not comprising components of the final protein structure. The accumulation of autoinducers at high cell density results in the repression, not activation, of biofilm formation and virulence factor production
which are required for Vibrio cholerae to cause human infection. The administration of applicable autoinducers or other agonists of quorum sensing capable of repressing biofilm formation as well as virulence factor expression are in the process of being identified as possible therapeutic approaches for the treatment of cholera. Since *dns* repression occurs upon increased cell density via quorum-sensing regulator HapR dependent conditions, DNA substrate availability at the commencement of the competence process allows for *dns* expression and DNA degradation to take place at low cell densities before induction of the competence phenotype. Two-component quorum-sensing (autoinducer/sensor) pathways function in parallel regulating gene expression in Vibrio cholerae. In one pathway, Cholera autoinducer 1 (CAI-1, AI-1) is synthesized by the activity of cholera quorum sensing autoinducer (Cqs)A synthase and its cognate two-component Cholera quorum sensing sensor (Cqs)S. The Cholera Quorum-Sensing Autoinducer-A Enzyme Substrates (Cqs-A) forms the on-pathway intermediate for the production of the cholera quorum-sensing signaling

molecule Cholera Autoinducer-1 (amino-CAI-1). CAI-1 and amino-CAI-1 are natural ligands that vary in their respective hydroxyl versus amino moiety at the Carbon-3 (C3) position involved in signal recognition in the Vibrio cholerae membrane-bound CqsS quorum sensing receptor involved in CAI-1 detection produced by the CqsA aminotransferase enzyme. CqsS detects amino-CAI-1 as well.

Amino-CAI-1 subsequently converts to CAI-1 in a step involving a CqsA-independent mechanism yet to be fully identified. The enzyme CqsA is required for the biosynthesis of AI-1.

The second system consists of a Cholera Autoinducer-2 (AI-2, CAI-2) synthesized by the LuxS synthase and its LuxPQ cognate receptor. LuxP exists as a periplasmic protein binding AI-2 regulating the action of the two-component LuxQ protein. Thus, the two quorum sensing autoinducer molecules AI-1 and AI-2 are produced and detected by Vibrio cholerae. AI-1 and AI-2 detection takes place via their respective CqsS and LuxPQ transmembrane receptors. The two-component CqsS and LuxPQ proteins function as both kinase and phosphatase. Two-component histidine sensor kinases exhibit both histidine and phosphatase activities. Histidine is an essential amino acid whose decarboxylation yields histamine. Histamine is present in all body tissues influencing the cardiovascular system, nervous system, gastric secretion, muscle contraction, and allergic reactions.

Phosphorous is an essential part of the diet involved in most metabolic processes abundant in tissues and bone. A phosphataseis a hydrolase class enzyme catalyzing inorganic phosphate release from phosphoric esters.

The phospholipase enzyme catalyzes the hydrolysis of a specific ester bond in phospholipids. A phospholipid consists of a lipid containing phosphorous constituting the major lipid form in all cell membranes.

3.7 Qrr small quorum regulatory rnas in vibrio cholerae expression

Transduction of the information coded in the genes of AI-1 and AI-2 autoinducers in both systems enters the bacterial cell through a shared phosphorylation-dephosphorylation cascade affecting the expression of more than seventy target genes. AI-1 and AI-2 synthesis is catalyzed by the CqsA and LuxS enzymes respectively. The Cqs and LuxPQ autokinase domains become phosphorylated transferring phosphate through the LuxU phosphotransfer protein to the LuxO response regulator under conditions of low cell density. When the CqsS and LuxPQ receptors
are devoid of their ligands at low cell density, the prevailing kinase activity results in LuxO response regulator phosphorylation. LuxO-P is the phosphorylated form of LuxO acting as a transcriptional activator in concert with the alternative sigma factor σ^{54} subunit of RNA polymerase for the expression of the Qrr-1-4 small regulatory RNAs that in concert with the Hfq RNA-binding chaperone protein destabilize *hapR* mRNA. Repression of HapR synthesis results in Qrr-1-4 expression. The Qrr sRNAs are functionally redundant; any individual Qrr is sufficient to control *hapR* expression in Vibrio cholerae. HapR is the transcriptional regulator of quorum sensing. The Qrr sRNAs target mRNAs encoding the AphA and HapR quorum sensing master transcriptional regulators. Qrr-1-4 respectively

stabilize and destabilize the *aphA* and *hapR* mRNA transcripts facilitated by the Hfq RNA chaperone protein at low cell density.

This results in the production of AphA protein; no HapR protein is produced. Expression of quorum-sensing-repressed genes occurs and expression of quorum-sensing-activated genes does not occur in the absence of HapR. Under low cell density conditions when the concentration of AI-1 is below the threshold for detection, its CqsS functions as a kinase. CqsS directs phosphate to the LuxO response regulator. This phospho-LuxO promotes transcription of the genes encoding Qrr1-4 which inhibits the HapR master quorum-sensing regulator that is not produced under low cell density conditions. Under high cell density conditions in the presence of sufficiently accumulated AI-1, CqsS autokinase activity is inhibited reversing the flow of phosphate in the system resulting in dephosphorylation and LuxO deactivation. $Qrr_{1\text{-}4}$ transcription ceases resulting in HapR production and initiation of quorum sensing gene expression under conditions of high cell density. Upon the increase of AI-1 and AI-2 concentration above the threshold required for detection under conditions of high cell density, the binding of these autoinducers to their respective CqsS and LuxPQ cognate receptors results in their switch from kinases to phosphatases. The reversal of phosphate flow through the signal transduction pathway results in LuxO dephosphorylation and inactivation.

$Qrr_{1\text{-}4}$ is not transcribed under conditions of high cell density causing a cessation of *aphA* translation and derepression of *hapR* translation. The quorum sensing system ensures maximal AphA production under low cell density conditions, as well as maximal HapR production under high cell density conditions. AphH and HapR each control the transcription of numerous downstream target genes. Reciprocal AphA and HapR levels determine the nature of quorum sensing, high cell density, and low cell density gene expression. HapR and HA/protease are expressed in the high cell density state. HA/protease is encoded by *hapA*. HapR is a positive regulator of protease production. HapR production under high cell density conditions represses genes essential to biofilm formation and virulence factor production. The HapR regulator is essential to the regulation of virulence gene expression and biofilm development.

3.8 Quorum sensing, alternative sigma factors and the two Component system in vibrio cholerae gene expression, Virulence, and the bacterial life cycle

The AphA transcriptional regulator activates TcpP/H expression at low cell density and in tandem with the transmembrane regulators ToxR/S they activate the soluble Tox T regulator.

ToxT acts upon the *ctxA* and *tcpA* promoters activating cholera toxin production as well as the toxin co-regulated pilus. HapR represses *aphA* transcription diminishing cholera toxin and toxin co-regulated pilus production as well as repressing the exopolysaccharide (EPS) genes essential to biofilm formation and activating *hapA* under high cell density conditions. Thus, AphA functions in the ToxR virulence cascade activating *tcpP* and *tcpH* transcription that in concert with *toxR* and *toxS* activate *toxT* expression for the production of virulence factors such as cholera toxin and the toxin co-regulated pilus. The cAMP receptor protein (CRP) that mediates catabolite repression and Alternative Sigma Factor σ^S

(RpoS) for RNA polymerase are required for *hapA* transcription. Biosynthesis of CAI-1 requires the presence of this cAMP
receptor protein. The ability of pathogenic bacteria to survive and proliferate within living host cells depends upon their capability to monitor variations in their extracellular environment and respond through the expression of certain genes. Alternative Sigma Factors (σ,ς) provide for the regulation of gene expression as a response to extracellular changes. They can pay a role in bacterial virulence. Sigma factor genes are RNA polymerase subunit (*rpo*) designated in gram-
negative bacteria. The Alternative Sigma Factor σ^S participates in the general environmental stress response necessary for bacterial survival upon exposure to conditions such as low pH, starvation, and oxidative stress. RpoS is a protein family that functions in regulating the metabolism of nitrogen.

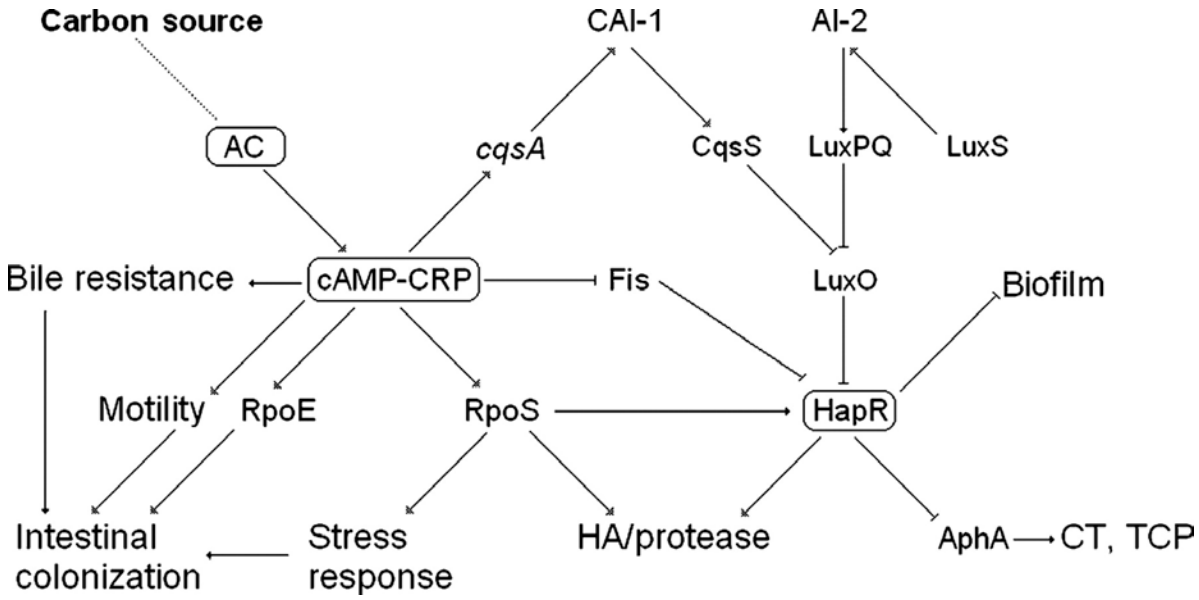
Catabolism is a metabolic process of destruction by which organisms convert substances excreting the resulting compound. The product of catabolism is a catabolite. The determinant of carbon catabolite repression is the CRP global regulator. Carbon catabolite repression is characterized by the inhibition of gene expression and/or protein activity in the presence of a quickly metabolizable source of carbon by means of adenylate cyclase activation via the phosphoenolpyruvate-dependent phosphotransferase system. This activation results in high intracellular cAMP levels as well as cAMP-CRP complex formation which binds to responding promoters activating or repressing transcription. This cAMP-CRP complex binds in the form of a dimer to the consensus sequence within, next to, or upstream of the responding promoters.

CRP controls CAI-1 expression to regulate HA/protease production. CRP modulates HapR expression by mechanisms including CAI-1, the nucleoid protein Fis, and RpoS regulation.

Fis is a DNA-bending protein that binds *qrr* promoters enhancing their transcription.

Fis expression is higher under low cell density conditions. Fis, as well as LuxO-P and σ^{54} are known *qrr*-promoter binding proteins. Such accessory factors enhancing σ^{54}-promoter activation function in the interaction of the LuxO-P response regulator with σ^{54} by means of DNA bending between their binding sites for DNA loop formation. CRP is believed to be essential to shifting Vibrio cholerae metabolism from a virulence mode characterized by the production of cholera toxin to an exit mode characterized by HA/protease enzymatic detachment activity. HapR induction by CRP negatively affects cholera toxin, toxin co-regulated pilus, and biofilm formation. CRP positively influences RpoS expression contributing to environmental stress resistance. CRP enhances motility, RpoE, and RpoS, and resistance to bile facilitating intestinal colonization. The Alternative Sigma Factor σ^E (RpoE) participates in the response to extracytoplasmic stress as well as contributes to the pathogenesis and virulence of Vibrio cholerae. RpoE gene expression in Vibrio cholerae depends upon promoters P_1 and P_2 that are upstream and downstream Promoters 1 and 2 (P_1 and P_2) respectively. P_1 is believed

to be σ^{70} dependent. σ^{70} is a bacterial enhancer-dependent transcription factor that is the



main σ factor in the expression of almost all genes during exponential growth. P_2 is σ^E dependent in a regulatory loop involving positive σ^E autoregulatory control of its own expression. σ^E also plays a role in the virulence of Vibrio cholerae and the response to environmental stress. σ^E is believed to control an extracytoplasmic response signaled by an accumulation of immature outer membrane protein precursors in the periplasm, believed to occur by means of a post-transcriptional mechanism in which anti-sigma factors sequester the Alternative Sigma Factor in a nonfunctional mode absent of the appropriate stimulus. Proper stimulus induction results in an increase of sigma factor activity by inactivation or removal of its cognate anti-sigma factor. Essential to the Vibrio cholerae lifecycle is the effect of CRP on signal transduction pathways functioning in response to changes in the environment. CRP affects quorum sensing as well as the various genes necessary for survival in the human host and the environment during the life cycle of Vibrio cholerae.

Figure 2. Model integrating the multiple regulatory interactions by which cAMP-CRP affectsthe *V. cholerae* life cycle. AC, adenylate cyclase; ↓, positive effect; ⊥, inhibitory effect.In this model, cAMP, by binding to CRP and activating *cqsA* expression, is proposed to link carbon catabolite repression and quorum sensing. (Liang, et. al. 2007, 2973)

The presence of HapR is a requirement for the expression of quorum sensing controlled genes. Another quorum-sensing system regulating virulence in Vibrio cholerae is the two-component VarS/VarA signaling system. This system acts on the phosphorelay pathway upstream of HapR regulating its level of expression by participating in the quorum sensing system at LuxO.

The VarS/VarA two component system indirectly modulates HapR expression by means of CsrB mediation in a manner independent of the quorum sensing regulation that associates the autoinducers to HapR as well. Carbon Storage Regulator (Csr) ABCD are small RNA-binding proteins involved in the translation of target genes and/or target mRNA transcript stability. CsrA is a global regulator of carbon metabolism that activates glycolytic

gene expression and represses genes essential for gluconeogenesis. Thus, CsrA is a post-transcriptional regulator capable of acting as an activator and a repressor.

All of the environmental signals sensed by this two-component sensor regulator system are yet to be fully identified. CsrA plays a role in several regulatory functions such as carbohydrate metabolism and motility. Motility is essential to the initial survival and virulence of pathogenic bacteria supporting their environmental persistence, enabling their adhesion to host cells, as well as supporting initial biofilm development. Putative Vibrio cholerae adhesion factors include VC O1 lipopolysaccharide, the GlcNAc-binding protein (GbpA), TcpF protein secreted by the toxin coregulated pilus biogenesis apparatus, OmpU, and cholera toxin. Entry of pathogenic bacteria into the host environment subjects them to cellular stresses such as increased temperature, increased gastric pH, osmolarity changes in the intestine, exposure to toxic nitrogen intermediates and reactive oxygen species, as well as nutrient starvation. CsrA post transcriptional regulatory systems control the activation of the infection related physiological response traits of pathogenic Vibrio cholerae to such varied stresses as well as changes in target gene expression. These traits respond to mechanisms of infection relating to colonization, pathogenicity, and persistence including antibiotic resistance, biofilm formation, motility, the transition from acute to chronic infection, as well as virulence gene expression. Csr-like post-transcriptional regulatory control functions to regulate virulence gene expression by changing the phenotype of pathogenic bacterial cells in response to prevailing environmental conditions so that they may adapt, persist and proliferate as infectious entities in the host whose progeny will subsequently be discharged back into the environment further perpetuating the cycle of infection in the environment and other hosts.

CsrA acts through LuxO regulating HapR expression levels. Lux refers to the Luciferase Operon genes such as *luxCDABE* involved in bioluminescence in gram-positive bacteria. However, gram-negative Vibrio cholerae does not possess functional luciferase operon genes capable of expressing the degree of visible light production characteristic of bioluminescent gram-positive bacteria . *LuxCDABE* expression is activated by HapR in Vibrio cholerae. Vibrio cholerae possesses the necessary *luxS*, *PQ* ,and *OU* genes for the production and response to the quorum sensing signal AI-2. Along with the Qrr1-4 small quorum regulatory RNAs regulating virulence gene expression, the CsrB, CsrC, and CsrD non-coding RNAs (ncRNAs) have been identified in Vibrio cholerae.

CsrBCD are regulated by the VarS/VarA two component sensor/regulator system and act via control of CsrA activity also influencing Qrr RNA expression. The Two Component System (TCS) ubiquitously senses and responds to environmental stimuli in bacteria. TCSs are a means by which bacteria sense signals from the environment and engage in intracellular transmission of signals so that this input can be processed resulting in the necessary change in gene expression affecting virulence.

The TCS possesses a sensor kinase in the cell membrane as well as a response regulator whereby signals from the environment are monitored by the sensor kinase that detects environmental stimuli phosphorylating the response regulator modulating gene expression through this phospho-relay system.

The phosphorylated response activator adjusts its activity in support of various functions including the binding of DNA and control of transcription. Two component

systems serve as a mediator between signals in the bacterial environment and such elaboration of bacterial function. The Vibrio cholerae VarS/VarA TCS as well as the CsrA system control expression of regulatory RNAs controlling the entire quorum sensing regulon. VarS and VarA activate CsrB, CsrC,and CsrD that encode small RNAs capable of binding to and inhibiting CsrA activity. The signals activating VarS and VarA systems are yet to be clearly established. Pathogenic Vibrio cholerae commonly resides in estuarine environments. The quorum sensing mechanism of Vibrio cholerae translates cell density changes in its environment into changes in gene expression. Vibrio cholerae sense cell density by means of the production and detection of small molecular autoinducers whose accumulation occur during conditions of increasing cell density. An attribute of this regulatory pathway is that HapR is solely expressed at sufficiently high levels to mediate its downstream effects at high cell density. HapR exists as a Tet-R family DNA-binding transcriptional regulator of a number of phenotypes essential to virulence and environmental survival. Such proteins generally possess a repressor function. HapR is capable of repressing the production of virulence factor as well as the formation of biofilm. Quorum-sensing-controlled gene expression occurs in the absence of HapR. Under high cell density conditions, dephosphorylation inactivates LuxO ending sRNA production which relieves *hapR* repression enabling downstream quorum-sensing-controlled target gene regulation. VarS and VarA regulate HapR expression by entering the quorum sensing system at LuxO. VarA and VarS also influence HapR activity independent of LuxO. VarS and VarA regulate the HapR regulon by alteration of HapR activity as well.

VarA modulates HapR regulation of its target genes independent of its role in the post-transcripional regulation of HapR levels by means of the quorum sensing system. VarA activates CsrB transcription to regulate the activity of the transcriptional activator HapR in a downstream pathway other than that involving the quorum sensing system. Thus, CsrB expression is capable of restoring the activity of HapR when VarA is not present in the system. VarA is located downstream of VarS. VarA regulation of HapR levels also occurs by means of the quorum sensing system. CsrB expression results in the restoration of HapR activity in the absence of VarA. The global regulator CsrA is capable of being titrated away from its target mRNA transcripts by CsrB. CsrA acts through LuxO regulating levels of HapR expression. HapR regulation by CsrB is not dependent upon the Vibrio cholerae quorum sensing system. CsrB represses CsrA which represses PckA. *PckA* encodes the phosphoenolpyruvate carboxykinase essential to carbon metabolism and the gluconeogenesis process. PckA is highly expressed during CsrB expression. It is possible that PckA influences HapR activity through the regulation of carbon-metabolism intermediate levels that binds to and induces HapR conformational change. Alternatively, PckA may affect the expression of a second transcriptional regulator necessary for HapR activity at its target promoters.

Upon Vibrio cholerae penetration of the intestinal mucosa during colonization, its flagella detach and a corresponding increase of FliA occurs. FliA is an activator gene of flagellar synthesis whose genetic transcription is encoded by sigma factors σ^{28} and σ^{54} that is essential to the motility and virulence of Vibrio cholerae. FliA is a repressor of HapR expression. This motility pathway functions in concert with the quorum sensing pathway maximally repressing HapR expression at the beginning of intestinal colonization under

conditions of low cell density allowing for maximum virulence gene expression at the initiation of the cycle of infection in the host. The LuxR family protein VqmA is also a *hapR* transcriptional activator gene that functions under low cell density conditions and is auto-regulated stimulating quorum sensing activity in a manner independent of LuxO regulation.

It is believed to respond to a yet to be identified environmental signal.Vibrio cholerae alternates between a planktonic and biofilm community existence in order to cycle between aquatic and human host ecosystems. Vibrio cholerae senses its population density by producing autoinducer signaling molecules and gauging nutrient availability in order to adapt to changes in the environment. Population density as well as nutrient availability constitute interdependent environmental signals. The existence and integration of various signal transduction pathways and global regulatory networks enable responses to these stimuli.

Mediation of LuxO-P repression is indirect. LuxO functions as either a transcriptional activatoror repressor. LuxO-P is a *qrr*-promoter binding protein. This activator type response regulator requires the alternative sigma factor σ^{54} for the activator response to occur. σ (ς^{N}) bacterial enhancer dependent transcription factors are proteins that provide for the formation of various holoenzymes capable of distinguishing groups of promoters necessary for a variety of cellular functions. RNA polymerase containing σ^{54} requires the presence of an activator protein for the initiation of transcription.

Vibrio cholerae virulence gene expression and biofilm formation take place under conditions of low cell density absent of autoinducers; they are repressed under high cell density conditions upon the accumulation of sufficient quorum system signaling molecules. It is believed that the quorum sensing system functions in the detachment of planktonic cells once they have transited through the acidic environment of the host stomach. In the intestinal setting, the planktonic growth process is believed to be essential to colonization of the intestinal epithelium of the human host by Vibrio cholerae. Biofilms are more resistant to acid than planktonic cells.

Quorum sensing signal deficient biofilms are less capable of colonization. Biofilms possess increased resistance to host defenses, exist as a reservoir of infectious bacteria, and are essential to intracellular replication. Bacterial growth is very efficient in the sessile matrix-enclosed communities of biofilms protecting bacteria from host immune system defenses contributing
to antibiotic resistance and the persistence of pathogenic bacteria.

As previously stated, a bacterial flagellum is a long, movable, thin, whip-like projection functioning as a locomotive organelle consisting of proteinaceous flagellin strands woven into a helical filament attached to a cell wall basal body projecting from the free surface of a cell.

Flagellar Regulatory Protein ABC (*flrABC*) genes must be present for flagellar synthesis to occur at this flagellar regulatory locus. *FlrA* and *flrC* gene products encode σ^{54}-activators forming a flagellar transcription cascade. Genes for flagellar synthesis and probably cell division during the free-swimming phase of the life cycle of Vibrio cholerae are transcribed by the σ^{54} holoenzymes, *flrA*, and *flrC*. *FlrA* and σ^{54}-holoenzyme transcribe *flrB* and *flrC*. *FlrB* and *flrC* possess amino- and carboxy-terminal domains existing as members of the two-component bacterial signal transducer family; whereby bacterial signal transduction takes place involving phosphotransfer between a sensor protein that detects an

environmental signal transmitting this information by means of phosphorylation to a response regulator. *FlrB* promoter transcription
depends on functional *flrA* and *rpoN* genes in Vibrio cholerae. The five flagellin genes of Vibrio cholerae are *flaA, flaB, flaC, flaD,* and *flaE*. *flaA* is the essential core flagellin gene for motility and the sole flagellin gene transcribed by the σ^{54}-holoenzyme. *FlaA* promoter transcription depends on functional *flrA, flrB, flrC,* and *rpoN* genes. *FlaB, flaD,* and *flaE* are transcribed by RNA polymerase possessing the flagellar sigma factor σ^{28}. *FlaC* possesses a σ^{54} and a σ^{28}-independent promoter.

Small RNAs modulate the response of bacteria to stress by regulating the expression of the alternative sigma factor σ^{S}(RpoS). σ proteins are associated with the regulation of nitrogen metabolism. The binding of σ factors is reversible. σ protein factors are essential to double- stranded DNA promoter recognition and binding, promoter melting, the inhibition of nonspecific initiation, targeting for activator function, as well as the control of premature transcription via promoter clearance and release from RNA polymerase. σ^{54}-RNA polymerase is targeted by signal transduction pathways in which activation occurs by means of remote enhancers. The activation of σ^{54}-RNA polymerase involves specific bacterial enhancer-binding proteins activated by nucleotide hydrolysis. Rates of initiation are controlled by regulation of the DNA melting step necessary for open promoter complex formation. σ^{54}-RNA polymerase is subject to independent regulation by a variety of genes by a specific family of sequence-dependent enhancer proteins having binding sites that are promoter-specific.

Individual protein control occurs by means of its own signal transduction pathway enabling a single type of σ^{54} polypeptide to mediate transcriptional responses to various physiological needs.

The products of σ^{54}-dependent transcription enable such functions as RNA modification, utilization of nitrogen and carbon sources, energy metabolism, chemotaxis, flagellation, development, response to heat and phage shock, electron transport as well as the expression of alternative σ factors in proteo- bacteria. Transcription initiation at a σ^{54}-dependent promoter requires a functional cognate
activator protein. Transcriptional efficiency at a particular promoter may vary over a wide range without the use of a separate repressor due to the σ^{54} protein function. Gene transcription by the σ^{54} polymerase may be silent or expressed upon activation as influenced by prevailing environmental or physiological conditions. σ^{54}-dependent activator binding to DNA sites at long distances from the initiation of transcription involve the looping method of activation.

A closed complex is formed by the σ^{54}-holoenzyme occupying the promoter prior to activation. The stable closed complex is an easy target for the looping of activators that are bound to remote sites. Looping-out of intervening DNA enabled by the bending protein integration host factor (IHF), may stimulate σ^{54}-polymerase recruitment at least at one promoter. The result of looping is an activator-dependent isomerization of the closed complex into an open complex leading to initiation of transcription. σ^{54} binding to RNA polymerase results in a blocked initiation pathway in which open-complex formation referred to as DNA melting may be independently controlled from closed-complex formation referred

to as DNA binding. σ^{54} essentially converts the RNA polymerase to an enhancer-requiring enzyme. σ^{54}-RNA polymerase activation occurs by diverse signal transduction pathways whose common terminal mechanisms consist of an output involving triggering or hidden ATPase action within an enhancer-binding activator protein, which overcomes the block to DNA melting (open-complex formation) within the closed transcription complex allowing initiation of transcription. There is a dependance on enhancers and ATP hydrolysis in the σ^{54} transcription mechanism.

Upon removal of the physiological stimulus, activators no longer undergo a triggering of their ATPase action and their cognate promoters revert to an inactive closed complex (DNA binding) state.

Various activators experience a triggering of their ATPase action by a phosphorylation cascade as is the case when the nitrogen regulator NtrC is present. Nitrogen Regulator Protein C (NtrC), Nitrogen Regulator I, (Nr_1, NRI or NTRC) response regulators act as either transcriptional activators or repressors for the Ntr regulon. NtrC functions as a prokaryotic enhancer-binding protein activating transcription by means of the σ^{54} holoenzyme form of RNA polymerase. The function of NtrC will be further elucidated in a subsequent section of this text. Phosphorylation of the N terminus of the protein occurs during changes in physiological conditions. Changes in conformation may influence changes in activator affinity for enhancer DNA sites, DNA multimerization, and assembly of a DNA-bound complex in the presence of ATPase activity. There is an interaction between σ^{54}-RNA polymerase and activation of NtrC in this enhancer-dependent transcription process. The three regions of σ^{54} are where the primary DNA-binding functions exist in region III with a cross-linking region with associated motifs, a helix-turn-helix motif, and an RpoN box in close proximity to the C terminus. Adjacent sequences have a modulating effect. Other sequences comprise the minimal core-binding domain. Region II varies and may participate in DNA melting during transition from the closed to open complex and in helping σ^{54} binding to homoduplex and heteroduplex DNA. In the region I domain, inhibition of polymerase isomerization and initiation in the absence of activation and stimulation of initiation in response to activation occur. Complete σ^{54} function occurs due to genetic cross-talk among domains. Primary DNA-binding activity enabling recognition of double-stranded promoter DNA exists in the C-terminal domain. σ^{54} is capable of binding to particular promoters when the core polymerase is absent. Holoenzyme provides for tighter binding than σ^{54} alone. The N terminal region plays multiple roles in binding to duplex and melted DNA.

The segment between the C terminal region and the major core binding determinant exerts an influence upon binding affinity. There is not necessarily direct DNA contact in these regions. Variation exists in the DNA sequences of σ^{54}-dependent promoters since virtually every sequence deviates from the consensus sequence. The interface between σ^{54} and the core RNA polymerase is believed to be substantial. Core polymerase binding is also affected by the C-terminal DNA binding domain as well as N-terminal region I. σ^{54} possesses determinants of interaction with the core and DNA when they are in close proximity indicative of a coordination of function between domains during transcription initiation. There is a gradual conformational change in the holoenzyme indicative of a stabilization of σ^{54}-core interactions after initial binding. For initiated complexes, N-terminal region I conformation is seemingly changed regarding closed complexes. The region is

important in the control of DNA melting. Interaction of region I with the core is believed to contribute some specialized properties of the holoenzyme since it can be controlled at the DNA-melting step. Opening of DNA in the closed complex of σ^{54} holoenzyme at the promoter occurs during activation by means of ATPase activity of the activator that loops from the enhancer site. The role of σ^{54} in activation and DNA melting by holoenzyme indicates it may be the main target of the activator. σ^{54} specifically recognizes DNA at the upstream fork junction where local DNA opening occurs. σ^{54} bound to heteroduplex DNA in the presence of the junctions changes conformation independent of core polymerase in a reaction in the presence of activator and nucleotide hydrolysis. In an isomerized complex, σ^{54} interactions result in an extended Dnase I footprint approaching the start site. DNA melting occurs in the region.

Thus, σ^{54} conformational changes are believed to be events triggered by activators associated with holoenzyme conformational changes. The σ^{54} DNA cross-linking pattern in holoenzyme changes upon activation with changes occurring specifically at the upstream junction fork. The resulting cross-link requires activator and ATP establishing interactions with the melted nontemplate strand next to the upstream junction fork. The activator utilzes ATP to effectuate conformational changes.

The holoenzyme is organized by σ^{54} in order for its conformational changes to be prevented absent of activator. The σ^{54} N-terminal region is essential to this process. Bacterial promoter melting is dependent upon engagement of single-stranded DNA within the open complex. Region I controls melting and related activities and are believed to originate in σ^{54} and the core. σ^{54} holoenzymes act to bind upstream fork junctions and bind the single-stranded DNA. The LuxO-P-σ^{54} complex activates sRNA transcription at low cell density. Repressors have no such requirement. LuxO-P activates repressor expression controlling downstream target genes. The repressor is comprised of redundant small regulatory RNAs (sRNAs) as well as the Hfq sRNA binding protein. The Hfq (HF-I) molecule is an RNA chaperone that exerts control upon cellular behavior and physiological traits in bacteria.

Hfq controls sRNA stability, mRNAs, and positively or negatively regulates target mRNA translation through small ncRNAs in a target dependent manner functioning in the control of bacterial virulence. This Hfq-sRNA repressor complex destabilizes LuxR and HapR mRNAs. Hfq action occurs downstream of LuxO and upstream of HapR. Hfq binds various sRNAs promoting their interactivity with their target mRNAs in many bacteria such as Vibrio cholerae. Quorum-sensing is an all or nothing switch from high to low cell density conditions or low to high cell density states. It is not a gradient. Quorum-sensing repression is a post-transcriptional occurrence.

Multiple sRNAs provide for very fine tuned transitions between high and low or low and high cell density states; acting downstream of LuxO-P destabilizing *luxR/hapR* mRNA and regulating quorum-sensing dependent gene expression in Vibrio cholerae. LuxO-P and σ^{54} do not control *hfq* transcription. σ^{54} promoters initiate transcription in the presence of an activator protein as well as the σ factor. A stable closed complex with DNA promoters is formed by σ^{54} and RNA polymerase.

An activator possessing latent ATPase activity activated by inducer binding or phosphorylation activate transcription by means of the resulting conformational change in σ^{54} thereby allowing open complex formation by the σ^{54}-RNA polymerase complex.

LuxO is a homolog of the activator class of Nitrogen Regulator C response regulators. LuxO functioning as a NtrC-type response regulator binds to σ^{54}-dependent promoters activating transcription. The LuxO NtrC family two-component response regulator consists of an N-terminal regulatory receiver domain, a central ATPase domain, and a C-terminal DNA-binding domain. The NtrC protein family of σ^{54}-binding transcriptional activators rely on ATP hydrolysis to promote open complex formation, have a conserved basic structure, often bind far upstream from σ^{54}, and activate from a distance. NtrC response regulators involve an N-terminal receiver domain and cross-regulation. The transcription factor NtrC is a transcriptional activator for nitrogen-regulated promoters in the presence of the σ^{54} factor existing as the promoter that responds to the nitrogen status in a bacterial cell.
This response regulator, in concert with the histidine kinases, are members of the protein family of two-component systems in prokaryotes and eukaryotes. NtrB is an autokinase sensor protein. In the presence of NtrB there is a sensor and a transmitter C-terminus domain and auto- phosphorylation of histidine residues occurs. In the NtrB/NtrC two-component sensor-response regulator system functioning in the control of gene expression, the glnA gene is part of an operon including the NtrB and NtrC genes that regulate the operon. Glutamine synthase is encoded by the *glnA* gene. Activation of NtrC transcription from the *glnA* promoter involves its binding to an enhancer composed of two binding sites three turns of the DNA helix apart which functions downstream or upstream of the promoter. This enhancer bound NtrC directly interacts with σ^{54} holoenzyme activating transcription catalyzing closed complex isomerization between the polymerase and the promoter opening complexes in a process requiring ATP hydrolysis and NtrC dephosphorylation in its amino-terminal regulatory domain. Phosphorylated NtrC (NtrC-P) hydrolyzes a nucleotide for closed-complex isomerization between σ^{54} holoenzymes as well as a promoter to open complexes. NtrC dimer phosphorylation is necessary for it to possess functional ATPase for transcriptional activation. NtrC dimers in an oligomer require functional ATPase for the activation. The dephosphorylation which occurs by means of the NtrB auto-kinase increases when combined nitrogen limits conditions whereby there is high *glnA* transcription.

NtrC phosphorylation is not necessary for *glnA* binding to DNA in the helix in its carboxy-terminal region. *GlnA* enhancer maintains high NtrC concentrations in close proximity to the promoter increasing its potential contact with σ^{54} holoenzyme. The *glnA* enhancer also enables complex formation containing a minimum of two NtrC protein-protein dimer interactions increased by NtrC phosphorylation (NtrC-P) and oligomerization mediated outside of its carboxy-terminal DNA binding region for activation of transcription to occur. Phosphorylation activates NtrC under nitrogen-limiting conditions altering the protein for oligomerization to occur. Oligomerization stimulates ATP hydrolysis. The DNA ligand functions as an enhancer mediating NtrC-P and NtrC dimer interactions enabling activation at the correct nitrogen-regulated promoter sites on the chromosome. Ntr sensitive operons can have direct and indirect effects. These σ factors involve distinct consensus DNA-binding sequences. Promoters are DNA segments that are generally upstream from a gene coding region that control expression of the gene functioning as a recognition signal for RNA polymerase where initiation of transcription occurs. A promoter may also be a substance in a catalyst increasing the rate of catalytic activity. Promoter subscripts are of successively increasing strength P_1 being of weaker strength than P_2 which is stronger and so on. Nitrogen

sufficiency exists when the level of glutamine synthesis is low. Expression of P_1 is at a base level and there is termination at the rho terminator.

P_2 expression requires NtrC-P for binding to the enhancer in the presence of σ^{54}. Ammonia assimilation is a factor influencing high expression of nitrogen. P_2 expression requires NtrC-P binding of enhancer and NtrB and NtrC upregulation with rho termination in the presence of σ^{54} RNA polymerase. Reading occurs through the terminator resulting in upregulation of NtrB and NtrC. NtrB and NtrC are elements in a signal transduction pathway in which the P_2 promoter and GlnD are also present. Cellular glutamine (Gln) levels may be limited or abundant.

Microbial nitrogen is derived from glutamine containing sources such as pyrimidines, purines, amino acids, amino sugars, NAD^+, and para-aminobenzoic acid (PABA,PAB) which is a B vitamin complex compound required for folic acid synthesis. The products of glutamine are ATP, cytidine triphosphate (CTP), carbamyl phosphate, glucosamine, histidine, and tryptophan. Glutamine is a nitrogen donor.

The P_1 promoter provides for glnA expression for glutamine synthesis even under low nitrogen conditions. P_1 functions in the presence of σ^{70} . σ^{70} (ς^{70}) is the second of the two classes of the bacterial enhancer-dependent transcription factors being the main σ factor in the expression of almost all genes during exponential growth. Bacteria utilize the major housekeeping factor σ^{70} as well as other alternative sigma factors to confer various promoter specificities upon the core RNA polymerase. Alternative sigma factors coordinately regulate expression of gene sets that contribute to a common physiological response. The alternative sigma factor σ^{54} is different from other alternative sigma factors because it transcribes genes possessing a diversity of physiological roles in different bacteria.

An accessory-activating protein required by the σ^{54}- holoenzyme enables this RNA polymerase to transcribe genes as a response to differing input from the environment. An individual σ^{54} activator is capable of directing transcription of an appropriate gene set for an individual physiological response.

Multiple responses by σ^{54} holoenzyme can occur in the presence of a multitude of activators responding to various cues from the environment. In Vibrio cholerae, the presence of multiple σ^{54}-activators provides for σ^{54}-holoenzyme expression of different gene sets as a response to changes in environmental conditions throughout its pathogenic cycle during its free-swimming and colonization phases. Although $\sigma^{54}$and σ^{70} differ in amino acid sequence and transcription mechanism, they both bind the same core RNA polymerase producing holoenzymes possessing different properties. Activators bind next to the polymerase site touching the enzyme without looping in σ^{70}-holoenzyme regulation. Highly stable closed complexes and looping occur during activation in σ^{70}-holoenzyme regulation. σ^{70}-RNA polymerase activation also involves signal transduction pathways involving many diverse activators. Transcriptional activation by σ^{54} involves core RNA polymerase complexed with σ^{54} holoenzyme as well as an accessory activator protein to initiate transcription. σ^{54}-activators bind to an enhancer in the promoter region activating transcription via contact with σ^{54}-holoenzyme bound at the promoter. σ^{54}-activator nucleotide hydrolysis is coupled to σ^{54} -holoenzyme-promoter closed complex isomerization to a transcription-competent open complex. Transcription of the homologous σ^{54}-activator transcription domain is modulated in a response to environmental signals, frequently via an amino terminal regulatory domain,

ensuring proper temporal control of initiation of σ^{54} dependent transcription. P_2 functions in the presence of σ^{54}. P_3 provides for NtrB and NtrC expression making the cell capable of sensing and responding to prevailing nitrogen levels. P_3 functions in the presence of σ^{70}. Under low glutamine conditions, P_2 retains uridine monophosphate (UMP) and cannot bind to NtrB which phosphorylates itself transferring the phosphate to the transcriptional activator NtrC.

Under high glutamine conditions, GlnD removes UMP from P_2 which binds to NtrB preventing NtrC activation. The degree of enzyme activity is influenced by the presence of ammonia (NH_3).

The degree of ammonia assimilation influences the degree of nitrogen expression. Under low ammonia conditions, additional glutamine synthetase encoded by the (*glnA*) gene is required. GlnA expression in Vibrio cholerae involves σ^{54} in enteric bacteria. σ^{54} functions in polar flagellar synthesis, glutamine synthetase expression, and colonization in Vibrio cholerae. The complex regulation of glutamine synthetase plays a key role in nitrogen metabolism. NtrC-P are capable of activating transcription of the GlnA-NtrB-NtrC operon.

These promoters of this operon activate transcription at three distinct start sites. Two-component systems function in signal transduction activities during chemotaxis, sporulation, virulence, as well as in adaptive responses to changes in osmolarity and phosphate or nitrogen supply.

Response regulators are the final target of the signal transduction machinery, transmitting a signal from the environment through a phosphorylation cascade by means of a sensor histidine kinase to the N-terminal receiver domain of the response regulator protein. In response to an environmental signal, the sensor histidine kinase phosphorylates itself at a histidine. This phosphorylation cascade initiated by auto-phosphorylation of the conserved transmitter domain of the histidine kinase is the first part of two component sensory systems which respond to environmental changes such as osmolarity and pH. Two-component sensors such as CqsS possess membrane-spanning domains enabling them to function as membrane-bound receptors.

The autoinducer AI-1 is an example of a ligand defined for histidine kinases. The response regulator subsequently transfers the phosphate from the sensor kinase to itself at an aspartate.

The phosphate group transferred from the histidine kinase to the conserved aspartate residue in the response regulator's N-terminal receiver domain induces activation of the response regulator.

Phosphorylation of a specific amino acid is capable of activating a response regulator.

The response regulator is usually an activator or repressor of transcription whose action depends on its phosphorylation state. The activated response regulator proteins are mostly transcriptional activators. Other proteins participate in the transfer of phosphate between the sensor kinase and the response regulator providing for additional regulation points in this phosphorelay system.

Such intermediates can affect the possibility of a response. Two-component systems employ phosphorylation to regulate the activity of the response regulator protein turning genes on and off in response to environmental changes. NtrC is phosphorylated and

activated under conditions of nitrogen starvation by the histidine kinase NtrB (also referred to as the nitrogen regulator II (NR_{II}). Phosphorylated NtrC (NtrC-P) is a transcription activator at promoters dependent on σ^{54} (also referred to as RpoN). NtrC-P binds to its specific DNA binding sites upstream of the protein consensus sequence, contacts sigma factor σ^{54} RNA polymerase, stimulating the isomerization of the closed RNA polymerase complex to the open complex. Cooperative NtrC-P binding to a sufficient amount of crosslinked DNA occurs during induction of ATPase activity at low protein concentrations. NtrC-P possesses ATPase activity essential to transcription activation. The NtrC-type response regulator central ATPase module is essential since ATPases function in protein unfolding and degradation, organelle function and maintenance, replication and recombination, as well as intracellular transport. NtrC-P can also aggregate under high protein concentrations in the absence of DNA in the presence of strong protein-protein interactions. The first step in the activation of NtrC is its phosphorylation to NtrC-P. The second step consists of the interaction of two NtrC-P dimers that induce the conformational change in NtrC-P to the active conformation if NtrC cooperatively binds to DNA. The two-component system functions influence the ability of Vibrio cholerae to adapt and survive in the environment as well as its potential virulence as a pathogen. Various NtrC proteins homologous to LuxO function in two-component signaling systems influencing processes such as nitrogen metabolism, motility, and virulence. NtrC-type response regulators such as LuxO engage in three distinct biochemical activities consisting of phosphoryl-group accepting, DNA-binding, and ATP hydrolysis. It is believed that LuxO-P activates the expression of a repressor controlling downstream target genes at low cell density.

Multiple redundant small regulatory RNAs (sRNAs) in concert with the sRNA binding Hfq protein function as the repressor. Vibrio cholerae *hapR* mRNAs and LuxR are destabilized by the Hfq sRNA repressor complexes at low cell density. Thus, Hfq is a requirement for quorum sensing repression. Quorum sensing supports virulence gene expression at low cell density in Vibrio cholerae. TcpA is the major subunit in the VC toxin-coregulated pilus whose presence when produced at low cell density results in the quorum sensing initiated cascade leading to the TcpA production noted at high cell density. Virulence factor expression requires the presence of the Hfq protein cascade. The quorum sensing signal transduction circuit of Vibrio cholerae involves the LuxO-σ^{54}complex regulated sRNA. There must be a σ binding site upstream of the locus. The LuxO-P-σ^{54} complex activates sRNA transcription. Most σ^{54} binding sites occur in intergenic regions and possess a terminator being independently transcribed loci referred to as a Quorum Regulatory RNA *(qrr)* genes *qrr1, qrr2, qrr3,* and *qrr4* in Vibrio cholerae regulated by and being epistatic to LuxO-P regulation of TcpA. Epistasis consists of the interaction of genes at different loci whereby one gene masks the expression of the other gene. The opposite reactions of HapR and TcpA are reciprocally controlled by quorum sensing. The non-coding *qrr* genes function in quorum sensing regulation in Vibrio species. These quorum regulatory RNAs under Hfq direction function in the destabilization of quorum sensing master regulator LuxR and HapR mRNAs in Vibrio cholerae. The quorum regulatory RNA genes are elements of a negative feedback loop regulating cell state transition from low density to high density populations. Negative Feedback (Negative Regulation) refers to the maintenance of

constant system output by exertion of an inhibitory control on an essential step by a product of the system.

HapR is capable of activating *qrr* gene transcription initiating such a negative feedback loop in the quorum sensing circuit. HapR can directly and indirectly repress its own production.

Direct *hapR* promoter repression by HapR occurs under high cell density conditions preventing HapR overproduction in the social mode. HapR is a virulence factor repressor under high cell density conditions. The genes encoding the Vibrio cholerae virulence factors CTX toxin and the toxin co-regulated pilus are HapR repression targets under high cell density conditions. Post-transcriptional *hapR* repression by means of the Qrr sRNA feedback loop occurs in the presence of LuxO-P and HapR upon the transition from high to low cell density conditions in a negative feedback loop accelerating the transition of Vibrio cholerae cells from the social mode into the individual cell mode. The removal of the autoinducers results in a HapR induced increase of the individual cell mode promoting Qrr1-4 sRNAs leading to the rapid degradation of *hapR* mRNA. Functional HapR protein is necessary in this feedback loop; base-pairing between *hapR* mRNA and Qrr1-4 is not required. This feedback is believed to be indirect at the *qrr* transcription level, specific to the qrr promoters instead of being attributable to a general effect exerted on Lux O-P or σ^{54}-regulated promoters. The sRNA-mediated feedback on functional HapR occurs independent of direct transcriptional HapR autorepression. This sRNA-mediated feedback loop occurs at the transition from high to low cell density. Autorepression influences the transition from low to high cell density mode. This loop controls the *hapR* steady-state level under high cell population density conditions. The transition from low to high active regulator concentrations is accelerated by direct autorepression by transcriptional regulators since this autorepression provides for the combination of a strong promoter with controlled steady-state gene product levels. *HapR* autorepression controls the transition into high cell density mode.

This transition is not attributable to *HapR* feedback on the *qrr* genes. *HapR* autorepression also provides a definitive response to autoinducers avoiding steady state *hapR* overproduction. Transcriptional autorepression influences the increase, usually not the termination, of the activity of a regulator since autorepression has no effect upon the degradation rate of the particular encoded gene product. The sRNA loci are believed to be regulated by LuxO-P with σ^{54} and essential for LuxO-P binding. Several sRNAs act downstream of LuxO-P destabilizing *hapR* mRNA regulating quorum-sensing gene expression in Vibrio cholerae. The sRNA chaperone Hfq is the repressor and the *qrr1-4* sRNAs along with LuxO-P and σ^{54} activate loci expression encoding the sRNAs with repression occurring from Hfq-sRNA-mediated destabilization of the *hapR* mRNA transcript. Any one of the four sRNAs is capable of completing quorum sensing repression. One sRNA activated by LuxO-P can effectuate the transition between low and high states of cell density. Hfq action occurs downstream of LuxO and upstream of HapR. Simultaneous inactivation of the four sRNAs is required to eliminate Hfq-mediated quorum-sensing repression. Hfq binds sRNAs promoting interactivity between them and their target mRNAs. LuxO-P as well as σ^{54} do not control *hfq* transcription. HapR is the master quorum sensing regulator in Vibrio cholerae that is autoregulated by two negative feedback loops acting on the same gene product. These feedback loops provide for the responsiveness to

extracellular autoinducer concentration changes and set the concentration thresholds at which behaviors regulated by quorum sensing are initiated or terminated.

The direct transcriptional feedback loop supports a strong response to autoinducers transitioning into the social mode and the prevention of runaway *hapR* expression under high cell density conditions. The HapR auto-repression loop involves HapR protein binding to a site immediately downstream from the transcriptional start site of *hapR* repressing its own transcription.

HapR accumulates to a sufficient level regulating its target genes at high cell density. Since HapR also binds to its promoter, it prevents further *hapR* transcription preventing over-accumulation of HapR. The HapR auto-repression loop provides for the correct timing of the quorum sensing response since it is necessary for the HapR pool to remain at a sufficiently low level to allow HapR to be sufficiently eliminated during the transition of Vibrio cholerae from a high to low cell density pattern of gene expression. Conversely, the sRNA-mediated feedback loop accelerates exit form the social mode. The HapR-Qrr Feedback Loop provides for HapR enhanced transcription of the Vibrio choleae *qrr1-4* genes. Due to the absolute requirement for LuxO-P initiation of *qrr* transcription, this feedback loop functions solely when Vibrio cholerae cells shift from high to low cell density conditions. HapR-Qrr feedback results in a *qrr* transcription surge when this transition takes place accelerating the required alterations in gene expression required under conditions of Vibrio cholerae low cell density. Redundant Qrr sRNAs compensate for each other. The absence of any individual Qrr causes up-regulation of the others. *LuxOU* mRNA is another Qrr sRNA target that along with *hapR* mRNA becomes destabilized upon pairing with Qrr sRNAs repressing LuxO and HapR production. LuxO and HapR are also *qrr* transcription activators. This regulatory capability generates the LuxO-Qrr Feedback Loop and the aforementioned HapR-Qrr Feedback Loop providing for the fine-tuning of Qrr levels essential to the process of Qrr Gene Dosage Compensation. This involvement of multiple redundant genes in a negative feedback loop is characteristic of Gene Dosage Compensation. Negative feedback regulation of genes serves to reduce gene product fluctuations and participates in the maintenance of homeostasis. HapR is produced under high cell density influencing *qrr* transcription in its transition to low cell density conditions. Thus, the HapR-Qrr feedback loop is not believed to participate in Qrr Gene Dosage Compensation under conditions of low Vibrio cholerae cell density. LuxO-P occurs under conditions of low Vibrio cholerae cell density and is capable of mediating Qrr Cell Dosage Compensation. The LuxO-Qrr and HapR-Qrr feedback loops in concert provide for a mechanism of *qrr* transcription adjustment based on the total activity of present Qrr sRNAs at a given time based on prevailing conditions of high or low cell density. This gene dosage compensation mechanism allows for responses to physiologically relevant changes in functional Qrr sRNA levels, as well as the calibration of these levels for the timely activation and termination of quorum sensing controlled behaviors. Quorum sensing is a post-transcriptional event involving one or more sRNAs. Targets of quorum sensing include *ctxA*, *tcpP*, the required *vps* operon for biofilm formation, the *hapA* protease necessary for individual cell detachment from high cell density biofilms, and the required virulence factors for host intestinal cell lining colonization that induces the severe watery diarrhea characteristic of infection by Vibrio cholerae. The Vibrio cholerae infectious cycle involves transitions between the individual mode and the social mode. Vibrio cholerae cells

in a high cell density biofilm structure have a greater chance of surviving the transit through the acidic gastric environment of a new host than individual VC cells. After arrival in the intestine, the quorum sensing activated biofilm dispersal is necessary for individual cells to colonize the intestinal epithelium and initiate the production of virulence factor. Vibrio cholerae exists as individual cells in the water column or as biofilm associated cells attached to surfaces. The intestinal cell population density increases and quorum sensing signals result in the dispersal of individual cells obviating the repetition of this
pattern later in the infection process. These cells are in a quorum sensing individual cell mode to reinitiate further Vibrio cholerae colonization of the intestine.

3.9 Genetics and chitin for natural competence for genetic transformation in vibrio cholerae

Exposure to chitin induces a state of competence whereby a switch from nuclease-mediated degradation of extracellular DNA and uptake of DNA by the VC bacteria occurs.

Competence entails the transfer of free extracellular DNA occurring in the environment into the cell. The production of the extracellular nucleases Dns and Xds secreted by Vibrio cholerae must be terminated during the competence process to allow uptake of intact DNA. It is believed that low-density Vibrio cholerae populations produce the nucleases participating in rapid growth by providing nucleotides that replete the available supply of nucleotides. In the presence of static high-density populations, nuclease production ends allowing for the uptake of intact DNA coinciding with a potential phase of genome diversification. Chitin is a nutrient polymer promoting horizontal gene transfer between Vibrio cholerae genetic variants through the induction of natural competence for genetic transformation consisting of the uptake of environmental free DNA as well as its internalization and recombination into the bacterial chromosome for natural transformation to occur. This is a form of horizontal gene transfer. Planktonic exoskeletons are comprised of Chitin which induces natural competence for genetic transformation. Exposure to chitinous surfaces induces natural competence in Vibrio cholerae enabling the internalization of environmental free DNA. The natural transformation of DNA functions in genome maintenance for repair of damaged genes as well as the acquisition of new genes and alleles essential to genetic diversity and evolution. Chitin-induced natural transformation is believed to mediate the acquisition of multigene DNA clusters coding for functions such as the induction of the structure and antigenic characteristics of Vibrio cholera O serogroup determinants or the utilization of specific carbohydrates as a means of nutrition.

Such acquisition involves conserved gene flanking whereby incoming DNA incorporates into the recipient chromosome via homologous recombination. This induced horizontal gene transfer is believed to be functionally restricted to aquatic niches as well as seasons characterized by environmental abundance as would occur during copepod blooms. This manner of gene acquisition is likely the result of natural selection by factors in the habitat whereby Vibrio cholerae variants arise. Natural selection attributable to phage predation is believed to influence the acquisition of novel types of O antigens. The Vibrio cholerae O139 serogroup variant is virtually undetectable in endemic regions. The emergence of the Vibrio cholerae O139 strain occurred via horizontal gene transfer.

Researchers are concerned that the occurrence of Vibrio cholerae O139 may be the onset of an eighth cholera pandemic. New Vibrio cholerae strains emerge by means of horizontal gene transfer. The presence of chitin or other nutrient sources, high DNA concentrations in the environment, as well as periods of fast growth during nuclease secretion facilitate rapid vibrio growth at times when conditions are favorable in the aquatic environment. Novel catabolic functions arising in the natural environment may promote occupation of new niches in a location of abundance of an essential nutrient. As previously indicated, *Dns* is downregulated before transformation occurs. *Dns* downregulation before the onset of transformation suggests that nuclease production as well as natural competence may occur in the same bacterial strain when they are expressed at different times. This capability is made possible in Vibrio cholerae by the same regulatory quorum sensing circuit. The evolutionary processes discussed led to the creation of this system. *Xds* is also a structural gene for DNase having relatively no effect as a modulator of natural transformation in Vibrio **c**holerae. The *dns* and *xds* genes encode extracellular nucleases playing a role in the fitness of Vibrio cholerae in aquatic habitats. The presence of at least three interconnected extracellular and intracellular signaling molecule pathways are required for the initiation of natural compe- tence and transformation in Vibrio cholerae. Vibrio cholerae attach to chitinaceous copepods and metabolize chitin. The first pathway involves the dependency on a chitin surface or chitin oligomers such as $GlcNAc_{2-6}$ for competence induction since these compounds lead to potential competence gene upregulation. Amongst them are the *pil* competence genes encoding a type IV pilus believed to be involved in the process of DNA uptake. The presence of chitin also leads to induction of the gene encoding the TfoX gene which is the main regulator of natural transform-ation. The nucleoside scavenging cytidine repressor (cytR) is a Vibrio cholerae biofilm repressor that is positively regulated to TfoX not known to be involved in transformation. Under conditions of high Vibrio cholerae conditions on chitin, CytR-cAMP receptor protein interactions may render multiple genes inactive negatively controlling DNA uptake. This is a CytR-dependent anti-
activation mechanism in which natural competence in Vibrio cholerae is controlled by a nucleoside scavenging response in which nutrient stress and cell-to-cell signaling are involved in natural transformation. The chitin oligomers lead to increased *tfoX* transcription and translation.

A chitin-induced sRNA TfoR activates TfoX mRNA translation contributing to natural competence induction. Chitin is sensed by the ChiS chitin sensor due to the presence of the chitinase-released GlcNAc di-/oligomers resulting in signal transfer via TfoR for the production of TfoX comprising the flow of genetic information in this chitin sensing regulatory circuit. The second CCR pathway involves the intracellular accumulation of the secondary messenger cAMP that in concert with the cAMP receptor protein CRP participates in colonization of the chitinous surface, chitin degradation, and natural competence. TfoX-induced *pilA* and *comEA* expression requires cAMP.

The third pathway in the natural competence induction process is quorum sensing. HapR is a negative regulator for *dns* transcription and its repression of the nuclease is involved in the influence of quorum sensing on natural transformation. The *comEA* bitopic membrane protein is a DNA receptor in bacteria whose regulation is dependent upon quorum

sensing. C*omE* is an operon comprising the open reading frames *comEA, comEB,* and *comEC,* as well as a *comER* overlapping open reading frame.

The *comE* locus proteins *comEA* and *comES* are necessary for genetic transformation to occur. C*omEC* is believed to be a polytopic membrane protein necessary for DNA transport. *ComEA* participates in DNA binding to the competent cell surface and transport.

The C-terminus of ComEA possesses a domain essential for DNA binding located outside of the membrane. *PilA* is not believed to be regulated by quorum sensing in this process. cAMP is required for TfoX-induced expression of *pilA and comEA.* There is a link between HapR concentrations, nuclease repression, and *comEA* induction. ComEA is required for DNA uptake when high cell density occurs on the chitin surface. Transformation requires ComEA expression. Cytidine is a nucleoside component of ribonucleic acid and its nucleotides essential in the synthesis of various lipid derivatives. Cytidine triphosphate is a nucleotide precursor in ribonucleic acid and cytidine monophosphate (CMP) and cytidine diphosphate synthesis which function in glycoprotein and and phospholipid synthesis respectively. *ComEA* regulation is dependent on quorum sensing. *PilA* is not is not regulated by quorum sensing.Competence is a physiological state at the end of the exponential growth phase driven by the expression of a set of competence genes solely transcribed in the presence of the ComK competence transcription factor. The proteins encoded in these late competence genes mediate the binding and uptake of DNA and include *comCFG* products. *ComG* proteins are membrane associated and function in providing exogenous DNA access to the ComEA binding domain as well as in the presentation of ComEA at the cell surface for accessibility to DNA. ComEA is a DNA binding protein having an affinity for double-stranded DNA existing as a late competence protein functioning as the DNA receptor during transformation. The ComG operon, and ComC are also necessary for DNA binding to competent cells. ComGA and ComGB are believed to be nucleotide-binding and integral membrane proteins respectively. ComGC, ComGD, ComGE, and ComGG are proteins having hydrophobic N-termini with cleavage site for processing in the manner of type IV prepilins. They associate with the cytoplasmic membrane before processing.

ComC gene products are involved in the processing of proteases and in the pilin proteins that are pili structural components in gram-negative bacteria. ComGC, ComGD, and ComGG are believed to be translocated to the outer surface of the membrane after their cleavage by the ComC protease. ComGF is an integral membrane protein.

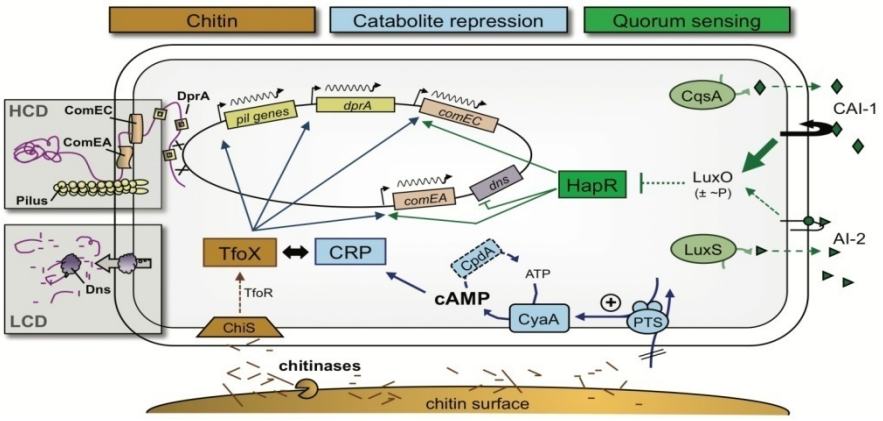


Figure 3. Model of the regulatory network of natural competence and transformation of V. cholerae.

At least three extracellular and intracellular signaling molecules must be present to allow natural transformation to occur in *V. cholerae*. 1) Chitin degradation products such as chitin oligomers,which lead to the induction of the sRNA TfoR and the main regulator of transformation TfoX(chitin pathway shown in brown). 2) The secondary messenger cAMP, which has to accumulate within cells (CCR pathway shown in blue). 3) Extracellular autoinducers, with an emphasis on the stronger autoinducer CAI-1, which feed into the quorum sensing circuit (shown in green). Whereas chitin- and TfoX-dependent induction and the requirement for cAMP and CRP are universal for all, so far investigated, competence genes, the QS-dependent circuit regulates only a subset of those, such as *comEA* and *comEC*.

Therefore, QS acts as a switch in gene expression and is responsible for the final fate of the surrounding DNA (boxed areas). At a low cell density (LCD), the DNA (shown in purple) is degraded by the nuclease Dns. As a consequence, the cells are non-transformable. At a high cell density (HCD) and, therefore, high abundance of the autoinducer CAI-1, the nuclease gene *dns* is transcriptionally repressed, whereas *comEA* and *comEC* are activated. ComEA as well as ComEC then contribute to the DNA uptake process, probably due to their ability to shuffle the DNA through the periplasmic space and the inner membrane, respectively. (Liang, et. al. 2007, 2973)

3.10 Biofilm transition state regulatory genes

Microorganisms are capable of transition from a planktonic free-swimming state to a sessile colonial biofilm state that enhances resistance to environmental stress. This transition involves an increase in the level of the cyclic di-guanosine monophosphate prokaryotic second messenger in a manner yet to be fully understood. The VpsT transcriptional regulator senses c-di-GMP inversely controlling extracellular matrix production and motility. VpsT is a master biofilm formation regulator. VpsT is a FixJ/LuxR/CsgD family prokaryotic transcriptional response regulator acting as an effector in two signal transduction using phosphoryl transfer from upstream kinases modulating the protein activity of the response regulators.

The phosphorylation site may be conserved within the VpsT receiver domain; other phosphotransfer-dependent signaling residues or cognate kinases have yet to be discovered. VpsT and VpsR have been associated with signal transduction using the c-di-GMP bacterial second messenger in a process involving yet to be fully identified nucleotide direct targets. VpsT is comprised of an N-terminal receiver (REC) as well as a C-terminal helix-turn-helix (HTH) domain which mediates DNA binding. Dimerization of c-di-GMP is mediated by extension of the VpsT $(\alpha/\beta)_5$-fold canonical (classical) receiver domain structural motif defining an extensive class of response regulators including CsgD and other LuxR family proteins. CsgD is necessary for the production of the extracellular matrix components cellulose as well as curli fimbriae essential to the development of corrugated rugose colonies and biofilm formation. A change in oligomerization upon c-di- GMP binding is essential for the function of VpsT; rather than regulation by means of phosphorylation. Regulation of gene expression is believed to be independent of VpsT phosphorylation. Interaction between the two VpsT monomers dependent on c-di-GMP is required for DNA recognition and transcriptional regulation. Corrugated rugose morphology involves increased

exopolysaccharide levels induced by VpsT. The rugose variant of Vibrio cholerae requires c-di-GMP for increased *vps* and VpsT gene expression suggesting the involvement of VpsT in a positive feedback loop integrating c-di-GMP for the production of a sufficient transcriptional response. Extracellular matrix and biofilm formation occur by means of the mutual dependence of VpsT and VpsR involving VpsT introduction of c-di-GMP- ensitivity to the regulatory circuit. VpsT is a transcriptional regulator inversely regulating biofilm formation and motility by means of direct integration of c-di-GMP signaling.

Vibrio cholerae VpsT extracellular matrix production and motility is regulated by its direct sensing of cyclic-di-GMP.

3.11 Vibrio cholerae biosignaling, g proteins, and intracellular signaling

In the fundamental life process of biosignaling, cells receive and act on signals from beyond the plasma membrane. Amino acids (alpha-amino-substituted carboxylic acids) are the building blocks of protein. A protein is a macromolecule composed of long sequences of amino acid chains linked by peptide (a compound of two or more amino acids) bonds comprising three-fourths the dry weight of most cells as a component of structures, enzymes, hormones, muscle contractions, immunological responses, as well as other essential life functions. Bacterial cells receive input from membrane proteins acting as information receptors that sample the surrounding medium for pH, osmotic strength, nutrition, oxygen, light, as well as the presence of harmful chemicals, predators, or competitors for available nutrition. Such input signals elicit appropriate responses from the bacterium. Animal cells exchange information such as ion and glucose concentrations in extracellular fluids and the interdependent metabolic activities occurring in different tissues. The signals contain information detected by different receptors which are converted to a cellular response involving a chemical process during signal transduction. The cell is often required to sense environmental change and respond accordingly employing various sensors causing a response to a signal in signal transduction systems. G proteins (Gs, guanosine nucleotide-binding proteins, GTP) are a family of binding proteins acting in intracellular signaling pathways that activate an effector enzyme; also referred to as small G proteins or small GTPases . A ligand is a small molecule that specifically binds to the specific protein receptor of a larger molecule. Ligand binding to a G-protein coupled receptor induces the exchange of GTP (Guanosine 5’ triphospate) for bound GDP (Guanosine 5’ diphosphate). The G proteins cycle between an active GTP-bound and an inactive GDP-bound form. In the GTP bound configuration, the G protein exposes previously buried Switch I and Switch II regions that interact with downstream proteins in the signaling pathway until G protein in activates itself by hydrolyzing its bound GTP to GDP. This enables the G protein toactivate a downstream enzyme in a signaling pathway. There is intrinsic GTPase activity in G proteins enabling them to self-inactivate. Since all G proteins have the same core structure and use the same mechanism, the active conformation occurs when GTP is bound and the inactive conformation results when GDP is bound. G protein-coupled receptors (GPCRs) are a familyof plasma membrane receptor proteins that transduce an extracellular signal into a change in cellular metabolism (also referred to as serpentine or heptahelical receptors). Since GPCRs are implicated in many human diseases, they are targeted by some drugs. Signal

transduction through GPCRs involves the plasma membrane receptor, an effector enzyme within the membrane generating an intracellular second messenger, and a G protein activating the effector enzyme. Stimulated by the activated receptor, the G protein exchanges bound GDP for GTP which dissociates from the occupied receptor binding to a nearby enzyme altering its activity. G proteins essentially serve as molecular binary switches possessing a timing capability affecting human wellness and disease. Pathogenic bacteria causing cholera produce a toxin that targets G proteins which interfere with normal signaling in host cells.

Chapter four: Mechanisms of vibrio cholerae pathogenesis, intestinal colonization, virulence, proliferation and disease in the human host

4.1 Vibrio cholerae bacteremia and ctx ab bioenergetics

Bacteremia has been observed in Vibrio cholerae O139 and O141, and rarely for VC O1. This bloodstream infection may represent retention of the invasive capability of the non-O1 serogroups of Vibrio cholerae even after acquisition of genes coding for Ctx. The A subunit which is responsible for direct toxic activity comprises parts of A1 and A2. The physiologically inactive B subunit responsible for cell binding consists of five binding B peptides.

Cholera toxin is a protein secreted by Vibrio cholerae in the intestine of an infected person. Subunit B recognizes and binds to specific gangliosides (sphingolipids) containing complex oligosaccharides as head groups on the intestinal epithelial cell surface providing an entry route to these cells by subunit A. Subunit A is broken into the A1 and A2 fragments after entry.

The transfer and expenditure of energy play a vital role in the elaboration of cholera toxin as is the case in other biological processes. Adenosine Triphosphate (ATP) transfers the energy liberated in energy releasing reactions that break down complex organic compounds into simple substances. ATP consists of the purine base adenosine as well as the five-carbon sugar ribose to which three phosphate (PO_4^{3-}) groups are arranged in a linear fashion. This energy powers cellular activities including transport of substances across cell membranes. The enzyme ATPase catalyzes the hydrolysis of ATP. The addition of a water molecule to ATP results in the removal of the third phosphate group resulting in an energy liberating reaction. ATP and the addition of H_2O in the presence of ATPase yields ADP, the third PO_4^{3-} group, as well as energy.

The energy supplied by the break down (catabolism) of ATP into ADP is in constant use by cells. Given the limited supply of ATP at any given time, ATP synthase catalyzes the addition of a phosphate group to ADP. ADP and the addition of the third PO_4^{3-} group and energy in the presence of ATP synthase yields ATP and H_2O. The necessary energy for the attachment of the phosphate group to ADP for the production of ATP is primarily supplied by the catabolism of glucose in the two phase process of cellular respiration. The anaerobic phase does not require oxygen for the partial catabolism of glucose into pyruvic acid which yields two ATP molecules. The aerobic phase requires oxygen for the complete catabolism of glucose into carbon dioxide (CO_2) and H_2O generating heat and thirty-six to thirty-eight ATP molecules. Translation is another term for protein synthesis. ADP-ribosylation is a post-translational protein modification involving the addition of one or more ADP-ribose moieties in the process of cell signaling. A moiety consists of one of two or more parts into which an entity can be divided. ADP-ribosylation is responsible for the action of some bacterial toxins. *CtxAB* exotoxins ADP-ribosylate proteins within intoxicated intestinal epithelial cells. The cholera toxin proteins are ADP-ribosyltransferases modifying target proteins in human cells. Cholera toxin ADP-ribosylates G proteins causing the life

threatening physiological derangement manifested as massive fluid secretion from the lining of the small intestine, massive diarrhea, loss of electrolytes, as well as severe prolonged shock observed in all but the mildest cases of the disease. A1 associates with the ADP-ribosylation factor ARF6 which is a G protein in host cells. This occurs through residues in its Switch I and II regions being accessible solely when ARF6 is in its active GTP-bound form. This ARF6 association activates A1 catalyzing the transfer of ADP-Ribose (the pentose in ribonucleic acid) from Nicotinamide Adenine Dinucleotide (NAD^+ oxidized form) to the Arginine (Arg) residue in the P loop of the alpha subunit of G proteins. NAD^+ is a co-enzyme functioning as a carrier of hydrogen atoms and electrons in certain oxidation-reduction reactions. Arginine is an amino acid that occurs among the hydrolysis products of protein. Hydrolysis occurs when a compound is cleaved into two or more simpler compounds involving the uptake of the H and OH (hydroxyl atom group or radical) portions of a molecule on either side of the cleaved chemical bond by the action of acids, alkalies or enzymes. P loop refers to a folded region in the protein structure of the Ras signal transduction protein which is the prototype for all G proteins.

The movement of GDP and GTP occurs at the nucleotide-binding pocket of the Ras protein. ADP-ribosylation blocks GTPase activity of Gs rendering it permanently active.

The result is continuous adenylate cyclase activation of intestinal epithelial cells, chronically high cAMP and chronically active cAMP-dependent protein kinase A (PKA) which phosphorylates the CFTR Cl-channel and a Na^+ H^+ exchanger in the intestinal epithelial cells.

Adenylate cyclase, the cyclic AMP synthesase enzyme acting on ATP to form cyclic AMP, is a protein found in the plasma membrane whose active site is on the cytoplasmic face. It is a second messenger molecule in eukaryotic signal transduction being activated or inactivated by G proteins that interacts with and regulates other proteins such as the cAMP-dependent protein kinase A and cyclic nucleotide-gated ion channels. Protein kinases specifically regulate enzymes by phosphorylation that catalyze important reactions such as amino acid transformation, glycogen turnover, and cholesterol biosynthesis.

4.2 The intestinal secretory response to vibrio cholerae infection

Vibrio cholerae is a heat-labile (capable of alteration or destruction by heat) enterotoxin that causes an aberrant intestinal secretory response acting through the stimulation of adenylate cyclase. The result is NaCl efflux triggering massive loss of water through the intestine as cells respond to the osmotic imbalance that ensues. Active transport involves the passage of ions or molecules across a cell membrane against an electrochemical gradient. Electrolyte compounds separate into ions in water and conduct electricity. Negatively charged bicarbonate and chloride ions can be actively transported or follow Na^+ via passive transport which does not require the expenditure of metabolic energy. The sodium pump is a mechanism using metabolic energy from ATP to achieve active transport of sodium across a membrane in biological systems.

Basolateral sodium-potassium pumps involving Na^+/K^+ ATPase actively transport sodium ions out of absorptive cells after they have moved into absorptive cells by means of diffusion and secondary active transport. Most of the Na^+ in gastrointestinal secretions are

reabsorbed and are not lost in the feces. The Na^+ pumping NADH-ubiquinone oxidoreductase (NQR) enzyme creates a sodium motive force across the bacterial membrane. Sodium pumps expel sodium, sometimes coupled with other substances, from most cells of the body. The sodium pump can also move sodium across multicellular membranes such as the intestinal mucosa or the walls of renal tubules. The enormous stool volume of cholera diarrhea results from sodium pump inhibition of the intestinal mucosal cells by metabolic products of Vibrio cholerae. A sodium pump inhibitor has been found in the stool water of cholera patients. This inhibitor is absent in the stool water of healthy subjects. A sodium pump inhibitor was found in the plasma of cholera patients. There are individuals classified as carriers who have Vibrio cholerae in their gastrointestinal tracts but do not get cholera diarrhea. Either the carrier does not provide the Vibrio cholerae with the metabolic ingredients necessary to produce the sodium pump inhibitor; or an X factor exists in the individual acquiring cholera which allows the sodium pump inhibitor to penetrate the cells of the intestinal mucosa. Cholera toxin is a heat-labile endotoxin.

Since the sodium pump inhibitor is also heat-labile, it is unlikely to be an endotoxin because Ctx inhibits the sodium pump. Ling believed that the term sodium pump just implies that there exists a cellular barrier to massive fluid and electrolyte movement by normal mucosa.

The barrier appears to be disrupted in cholera so that the cell cannot accumulate sodium against a concentration gradient (Love, 1969, 66). Ussing and Windhager stated that such leakiness of the intestinal mucosal cells may involve changes in the cellular shunt path and not be transcellular (Idid.). Epithelia possess an intrinsic permeation barrier capability being selective for the absorption of positively charged solutes controlled mainly by the tight junctions of cells.

The permselectivity of the barrier refers to the ability of the epithelia to discriminate or preferentially transport molecules of different charges. Epithelia have certain charge characteristics involving an electro-chemical potential which the solutes experience as they pass across cell membranes. Barrier permselectivity combines passive contributions from electrostatic shunt path activity due to membrane-fixed charges as well as the contribution from cell membrane activity; which is a reflection of intrinsic cell membrane carriers and pumps such as the active sodium pump. A characteristic of the permselectivity barrier is that epithelia are selective to positively charged solutes as compared to their negatively charged counterparts under physiological conditions. Transepithelial transport of charged solutes is greatly influenced by epithelial charge selectivity as well as membrane shunt path permeability. The intestinal epithelium has a large absorptive area. Intestinal shunt path permeability is high. The protein cholera toxin is composed of five CT-B subunits that bind cholera toxin to the host GM_1 gangliosides of the Ctx receptors on the mucosal epithelial cell membranes, as well a single catalytic CT-A subunit that enters the epithelial cells of the upper small intestine of the host activating adenylate cyclase and triggering Ctx holotoxin uptake into these cells by endocytosis conferring cholera toxin activity. GM_1 is an oligosaccharide, membrane phospholipid and a ligand. Reduction of the proteolytically nicked cholera toxin A (CTA) subunit leads to endoplasmic reticulum release of the CTA_1 active toxin component. This fragment is translocated to the cytoplasm catalyzing the NAD-dependent ADP-ribosylation of the $G_{5\alpha}$ protein for the

constitutive activation of adenylate cyclase. The increase in cyclic AMP that follows induces secretion of fluids and electrolytes into the small intestinal lumen producing the voluminous rice water stool characteristic of cholera. The A_2 peptides of the Vibrio cholerae (and Escherichia coli) family of AB_5 heat-labile enterotoxins of which Ctx is part of, penetrate the central pore of corresponding B pentamers. The A_2 domain B pentamer interactions that occur within the upper pentamer pore are hydrophobic. The interactions occurring in the lower pentamer pore are mostly hydrophyllic. This hydrophobic surface in the B pentamer pore is mostly buried in the holotoxin structure due to hydrophobic interactions between subunits A and B composed of CtxB subunit residues that interact with $CtxA_2$ domain residues in cholera toxin. Although CtxAB interact at the AB_5 holotoxin interface, the B pentamer is highly stable in its own right and able to bind GM_1 with or without the presence of CtxA. CtxA solely associates with CtxB, while the five CtxB subunits are forming a pentamer. The conserved hydrophobic ring in the upper pore of the AB_5 toxins may be important in promoting CtxA-CtxB interactions as well as the increase of the rate of cholera holotoxin assembly beyond that of the B pentamer. The concentration of A and B proteins in the periplasm of Vibrio cholerae determines the rate of cholera holotoxin assembly. The A subunit enzyme mediates the transformation of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The increase of adenylate cyclase with the corresponding increase in cAMP affects membrane permeability by disrupting the normal ionic flow of the small intestines resulting in decreased absorption of sodium chloride ions, active chloride and bicarbonate secretion, and the massive loss of fluid and electrolytes.

Covalent modification of a $G_{\alpha s}$ protein is catalyzed by the A subunit resulting in α subunit modification by attachment of an ADP-ribose to an arginine receptor; stabilizing the GTP-bound form of $G_{\alpha s}$ trapping it in the active conformation. This G protein activates protein kinase A (PKA) continuously opening a Cl^- channel inhibiting sodium absorption by the Na^+-H^+ exchanger by channel and exchanger phosphorylation, resulting in excessive NaCl loss as well as loss of a large quantity of water into the intestine. Thus, cholera results from the trapping of a G protein in the active conformation causing perpetual stimulation of the signal transduction pathway resulting in the voluminous secretion of electrolytes and fluids from the intestines.

The volume of the secretions exceed the normal absorptive capacity of the bowel.

The secretions originate mostly from the small intestine; although cholera toxin also inhibits colonic water absorption. The diarrhea contains large quantities of sodium, chloride, bicarbonate and potassium. Little protein or blood cells are present. Symptomatic cholera patients may eliminate as much as twice their body weight in fluid should they survive four to six days in the absence of proper therapeutic glucose-electrolyte solution rehydration therapy. Glucose-mediated sodium chloride and water absorption can be reestablished in actively purging cholera patients receiving such therapy.

4.3 The physiological response of the small intestine to vibrio cholerae infection

In addition to the role of the epithelial cells, the intestinal wall has a muscular layer, and an enteric nervous system which can function independently of the central nervous system. The intestine contains various cells that can produce hormones and neuropeptides

with effects on intestinal secretion such as the enterochromaffin cells producing serotonin. This vasoconstrictor inhibits gastric secretion. There are immunologically active cells, such as mast cells, located among the polar epithelial cells and underneath the epithelium of the lamina propria. These cells conceivably respond with cAMP formation when exposed to Ctx which could indirectly stimulate secretion by acting on a receptor cell and from factors released from the primary cells affecting the fluid transport processes of the intestinal epithelial cells. Another pathophysiological role for the enteric nervous system in enterotoxin-induced secretory states may be that Ctx first adheres to a receptor cell and triggers the release of amines and/or peptides stimulating intramural nervous reflexes by activation of dendrites adjacent to receptor cells. The stimulated reflex(es) contain(s) at least one cholinergic synapse that by definition employs acetylcholine as the neurotransmitter. Vasoactive Intestinal Polypeptide (VIP), an intestinal secretion inducing neurotransmitter, may be the final mediator on the polar epithelial cells to start the electrolyte and fluid secretion in response to Ctx and other enterotoxins. Thus, the effects of Ctx may indeed be mediated through intracellular messengers other than cAMP including prostaglandins that stimulate intestinal smooth muscle, the vasoconstrictor serotonin and neurotransmitters such as acetylcholine. Prostaglandins are strong mediators of various physiological processes that will be discussed further in subsequent sections of this text.

4.4 Virulence factors, genes, proteins, toxins, the type i secretion system, and mechanisms of vibrio cholerae pathogenesis and colonization of the human intestinal epithelium

Additional toxins and other virulence factors are involved in cholera pathogenesis. The RXT toxin family consists of exotoxins produced by pathogenic gram-negative bacteria such as Vibrio cholerae secreting the toxins into the extracellular space via the type I secretion system. Three proteins residing in the inner and outer membrane of Gram-negative bacteria function in a one step process not involving a periplasmic intermediate in the type I secretion system pathway.

RTX refers to “Repeats In Toxin” given its sequence of repeated peptides. Repeat toxin is virulence associated encoding a protein having cytotoxic activity. RTX toxins are hemolysins and leukotoxins. A leukotoxin is a substance causing degeneration and necrosis of leukocytes (white immunocompetent blood cells). Any substance from a living agent causing the lysis of a red blood cell and the liberation of its hemoglobin is a hemolysin. The cytotoxic activity of the RTX toxin gene cluster possess hemolytic, leukotoxic, and leukocyte-stimulating functions. *RtxA* is the presumptive cytotoxin. RTX activity is dependent on the acetyltransferase *RtxC* which encodes the activator protein as well as *RtxB* and *RtxD* proteins functioning in toxin transport from an associated ATP-binding cassette transmembrane protein ABC transporter system.

This ATP-binding cassette (ABC) transport system involves a high affinity binding protein found in the periplasm between the cytoplasmic membrane and the cell wall that carries the substance to be transported to the membrane spanning transporter. This active transport across the membrane is fueled by the energy resulting from the breakdown of ATP by an ATP hydrolyzing protein. The type I secretion system is dependent upon an ABC

transporter and is involved in the secretion of various toxins or hydrolytic enzymes from a variety of mammalian pathogens.

This system can secrete specific proteins when it carries the specific type I secretion signal. It is independent from the Sec protein secretion system functioning in protein escort across the cytoplasmic membrane or its integration into the membrane. Sec substrates possess specific topographic signals targeted to an embedded SecYEG translocon which is a polypeptide-conducting channel functioning co-translationally by a Signal Recognition Particle (SRP) in lipid bi-layer membrane integration or in a post-translational manner by SecB for complete translocation.

A translocon is a transmembrane protein complex of the endoplasmic reticulum (ER) whereby nascent polypeptides move into the ER lumen when protein synthesis occurs which regulate interactions between ribosomes and the ER. The bacterial SRP receptor functions in membrane binding. The SecY protein plug helix that clogs the unused channel as well as the center constriction sealing around the translocation chain provide for translocational function compatible with the membrane permeability barrier. The translocon possesses a lateral gate accepting newly synthesized protein permitting its hydrophobic segment to enter the lipid phase. Bacterial system post-translational translocation is empowered by SecA ATPase interacting with SecY and a preprotein for conformational transitions connected with the ATPase cycles. The type I secretion system translocator consists of the two inner membrane ABC transporter and Membrane Fusion Proteins (MFP) encoded by genes that are co-transcribed and closely linked to the gene for this transport substrate also referred to as the Allocrite, which is an unprocessed substrate for the ABC transporter. Comparatively, the ATP is a processed substrate which is hydrolyzed. A third Outer Membrane Protein (OMP) completes the translocator providing the conduit for Ctx secretion into the extracellular space**.** *TolC* is an example of a multifunctional outer membrane protein exhibiting an extended periplasmic domain.

Bacterial secreted proteins are important in bacterial pathogenesis. In the context of genomic sequencing and organization, Open Reading Frames characteristically encode functional viral genes. ORFs possess a modular genomic organization in which genes having similar functions are grouped. The RTX gene cluster comprises open reading frames for RTX toxins being cytotoxin *RtxA*, as well as *RtxC* and *RtxB*/*RtxD* of the associated ABC transporter system. The *RtxC* gene occurs solely in the El Tor biotype. The RTX gene cluster is linked to the CTX element of the Vibrio cholerae genome. Its activity is independent of the CTX element. Genes signaling for toxin synthesis and secretion are in one operon plus one additional unlinked gene in most RTX toxins. The order of the genes in the operon is CABD. Gene A encodes the toxin. Two secretion proteins are encoded in genes B and D. The toxin activator is encoded in gene C. *TolC* may act as an open reading frame in theVibrio cholerae genome. The CTX element is the neighboring gene cluster of RTX. The Vibrio cholerae RTX toxin is encoded within a gene cluster linked to the VC prophage. RTX toxins may induce production of proinflammatory substances such as Interleukin-1 (IL-1), tumor necrosis factor (TNF), Nitric Oxide, and Eicosanoids that would play a role in the manifestation of the inflammatory diarrhea characteristic of cholera. Eicosanoids are derived from arachidonic acid which include leukotrienes and prostaglandins. Leukotrienes are regulators of allergic and inflammatory reactions. Nitric Oxide (NO, Endothelium-derived

relaxing factor) is a short-lived dilator released from vascular endothelial cells in response to vasodilator binding to endothelial cell receptors causing guanylate cyclase activation in vascularsmooth muscle resulting in cyclic GMP increase inhibiting muscular contraction producing relaxation.

Since genes of common pathologic origin may be genetically linked, the Vibrio cholera e RTX toxin may be a gastrointestinal virulence factor as is the case with cholera toxin.Cholera pathogenesis is a multifactorial process involving genes encoding virulence factorsaiding the colonization, coordinated expression of virulence factors, and toxigenic actionat the molecular level. The sequencing of the entire genome of Vibrio cholerae has resulted in the discovery of twenty-four isolates. The CTX genetic element corresponds to the genome of CTXΦ, a lysogenic filamentous bacteriophage carrying structural genes encoding cholera toxin, as well as the Vibrio Pathogenicity Island (VPI) carrying genes for the pilus colonization factor toxin coregulated pilus. The Vibrio Pathogenicity Island I and the CTX prophage that encodes cholera toxin are two large DNA regions containing the genes and/or gene clusters encoding the major virulence factors in epidemic Vibrio cholerae O1 and O139.The receptor for the cholera toxin encoding filamentous phage CTXΦ is the toxin-coregulated pilus which is part of the VPI in toxigenic Vibrio cholerae isolates. The epidemic Vibrio cholerae pathogenicity island possesses a phage-like integrase (*int*) from the family of site-specific recombinases at its termini, a *vpiT (*formerly named *orf1*) transposase-like gene, and *att* phage- like attachment sites. It is inserted at the *ssrA* tRNA-like locus that flanks the right junction of the VPI. The VPI chromosomal integration site is located at the 3' end of the *ssrA* gene encoding a small RNA having the tRNA-like tertiary structure as well as an acceptor stem having a terminal Cytosine-Cytosine-Adenine (CCA) end for 3' end aninoacyl-tRNA synthetase catalyzed covalent bonding for aminoacylation with the amino acid alanine. It is also referred to as a tmRNA due to its function as an alanine-specific tRNA and possesses a short reading frame coding for ten amino acids. The resulting eleven amino acid tag (ten encoded amino acids + alanine) added to polypeptides translated from mRNAs devoid of a termination codon function to mark a protein target for specific proteolysis. Insertion of pathogenicity islands into tRNA or tRNA-like genes occurs in numerous pathogenic bacteria and this mechanism is also sharedamongst lysogenic and temperate phages. The G-U base pair is conserved in cytoplasmic alanine-specific tRNAs as well as *ssrA* RNA. Vibrio pathogenicity island excision resulting in the deletion of U is capable of changing acceptor stem structure inactivating acceptor activity preventing aminoacylation. This deleterious effect on the cellular function of *ssrA* RNA can interfere with the homeostasis of host cells to target protein degradation in a manner not yet fully understood. An intact VPI is believed to preserve *ssrA* RNA integrity providing a selective advantage for pathogenicity. A vibrio pathogenicity island-encoded virulence factor Mop gene (formerly referred to as Orf4) has been identified as a putative protease modulating the virulence of Vibrio cholerae. Mop is a putative Zinc-metalloprotease since it contains such a motif. Zinc-metalloproteases are frequently involved in the virulence of pathogenic bacteria. The Mop protein is associated with biofilm formation, cholera toxin, intestinal colonization and motility. A pleiotropic function for the Mop gene in the pathogenesis and persistence of Vibrio cholera has yet to be fully identified. The Vibrio Pathogenicity Island of epidemic Vibrio cholerae classical and El Tor biotype strains is capable of excision from the

chromosome at the terminal *attL* and *attR* sites to form an extrachromosomal circular excision product (pVPI) that is joined at a particular *attP* site. The extrachromosomal circular excision product forms upon the joining of its left and right ends. The *int-att-vptT* junction as well as the core *attP* of pVPI is the same as the chromosomal VPI *attR* site. The mosaic genomic structure of the Vibrio Pathogenicity Island contains conserved and divergent regions *vpiT* and *hutA* where gene translocation is believed to occur. Vibrio cholerae contains two circular chromosomes. There is an association between regions of the Vibrio Pathogenicity Island on chromosome I and *hutA* on some strains; the particulars of which are yet to be fully elucidated. The Vibrio Pathogenicity Island encodes the TCP receptor for CTXΦ and is essential for cholera toxin gene acquisition.

The toxin co-regulated pilus, the receptor for CTXΦ entry into the bacterium functioning in the colonization of the human intestinal epithelium, and the Accessory Colonization Factor (ACF) have been acquired by the Vibrio cholerae genome by horizontal gene transfer from other bacterial donors. The TCP and accessory colonization factor gene clusters borne on the vibrio pathogenicity island DNA region are solely present on toxigenic strains observed during epidemic and pandemic Vibrio cholerae outbreaks. Integrease and transposase genes as well as *att* flanking sites present on the Vibrio Pathogenicity Island may be of phage origin. The evolution of toxigenic Vibrio cholerae strains from non-toxigenic strains requires the acquisition of the VPI as the initiator of this multi-step process; leading to TCP expression facilitating CTXΦ acquisition providing the genes for Ctx. The Vibrio cholerae Vibrio Pathogenicity Island-encoded Toxin Coregulated Pilus gene cluster involves genes encoding type-IV pilus and intestinal colonization factor formation. The acquisition of virulence genes and pathogenicity islands are factors in the improved evolutionary fitness of Vibrio cholerae as a pathogen as well as a more efficient seeding of the environment and enhanced endemicity. TCP and Ctx genes may be found in environmental strains containing neither the O1 nor O139 Vibrio cholerae antigen. Thus, virulence genes are not exclusive to clinical strains; they are also found on environmental strains that can be reservoirs for Vibrio cholerae virulence genes. An operon is a sequence of DNA bases containing one or more structural genes with the operator controlling their expression; the operator is the DNA segment to which the repressor protein binds controlling the expression of adjacent genes. The toxin-coregulated pilus operon consists of a cluster of fifteen genes. CTXΦ uses TCP as its receptor in this process. Vibrio cholerae requires the coordinately regulated virulence factors Ctx and TCP for colonization of the intestinal mucosa and infection to occur. In Vibrio cholerae, the CTXΦ genome can exist as a replicating plasmid (a double-stranded DNA molecule integrated with or existing independently of the chromosome being stably inherited but not required for host cell growth and reproduction) or a prophage (a latent form of a bacteriophage remaining in the lysogen usually integrated into the host chromosome). As a filamentous phage, CTXΦ has the unusual capacity to integrate into and reside in the host chromosome as a prophage. CTXΦ integration into the Vibrio cholerae genome is irreversible. The CTXΦ acquired in the process of integration is vertically transmittedalong with the rest of the genome. The major virulence genes of Vibrio cholerae required for pathogenesis exist in clusters capable of lateral propagation and disperse among different strains. The core genes of the CTX genome were previously believed to primarily encode the *ctxA* and *ctxB* operon encoding toxin. The work of Waldor and Mekalanos revealed the

role of the core genes of the CTX genome is that of encoding the morphogenesis and structural proteins of the phage rather than toxin production. Notwithstanding this discovery, the CTX nomenclature remains in use constituting a misnomer unless the morphogenesis and structural proteins are found to serve a dual function in this context. The CTXΦ core is generally flanked by the Repeat Sequence 2 (RS2) region containing three open reading frames encoding required genes *RstA* for phage DNA replication, *RstB* for site-specific CTX phage integration into the host genome, and *RstR* for repression of *RstA* expression being transcribed in a direction opposite to that of the other phage genes. The *RstR* gene encodes a repressor regulating transcription of the *RstA* initiator replication protein and seemingly regulates the expression of all phage genes.

The *RstR* gene is flanked by the intergenic *ig1* and *ig2* regions of RS2. No definitive role has yet to be established for the *ig1* intergenic region. The *ig2* intergenic region carries the *RstA* promoter and the *RstR* operator. The *RstA*, *RstB*, and *RstR* genes are open reading frames as are the *ig1* and *ig2* intergenic regions for regulation of CTXΦ. These three genes as well as both intergenic regions are components of the related aforementioned RS1 genetic element found adjacent to CTX prophages in numerous Vibrio cholerae strains. The *RstR* gene encodes a repressor protein regulating the lysogeny of CTXΦ in the RS2 region. *RstR* is the main repressor of CTX genome replication being produced in the phage lysogenic state at high levels repressing virion production. RstR binds to the *RstA* promoter (*PrstA*) repressing gene expression in the RS2 and core regions. The *PrstA* required for CTXΦ virion synthesis is located upstream of *ctxA* in the gene sequence and can extend into *ctxAB*. (*PctxAB* is a promoter located upstream of *ctxAB*.) There is an *OPrstA* (operator/promoter region) and *PrstR* overlap indicative of *RstR* autorepression. The CTX prophage may hypothetically produce virions on occasion. *RstR* proteins may be biotype specific. An example would be classical biotype *RstA* being repressed by classical *RstR* but not by El Tor biotype *RstR*. CTXΦ classification is based upon *RstR* gene sequence variation. CTXΦ from classical Vibrio cholerae isolates carry $RstR^{class}$ genes and are designated as $CTX^{class}\Phi$. El Tor isolates carrying $RstR^{ET}$ are designated CTX El Tor Phi ($CTX^{ET}\Phi$). Vibrio cholerae O139 isolates carry either $CTX^{ET}\Phi$ or CTX Calcutta Phi ($CTX^{calc}\Phi$) whose *RstR* is designated as $RstR^{calc}$. O139 Calcutta Vibrio cholerae strains refer to those linked to VC outbreaks that occurred in that area of India during 1996. Vibrio cholerae O139 strains carry both $CTX^{ET}\Phi$ and $CTX^{calc}\Phi$ prophages. The VC Calcutta strains arose due to infection of a Vibrio cholerae O139 CTX^{ET} lysogen by the previously unknown $CTX^{calc}\Phi$. A prophage encoding $rstR^{calc}$ presumed to be integrated $CTX^{calc}\Phi$ in a non-VC O1, non-VC O139 strain was detected in Vibrio cholerae Calcutta environmental strains, indicating that CTX^{calc} may be within the estuarine environment. Since the CTX^{ET} and CTX^{calc} prophages give rise to infectious virions, they are both vectors for *ctxAB* transmission. Nonpathogenic Vibrio cholerae strains lack *ctxAB*. $RstR^{calc}$ is an allele-specific phage repressor regulating replication of $CTX^{calc}\Phi$ by inhibiting the activity of the $RstA^{calc}$ promoter. $RstR^{calc}$ exerts no inhibitory effect upon the classical and El Tor Vibrio cholerae *RstA* promoters that are regulated by their own cognate *RstR*s. The transcriptional repressor activity of $RstR^{calc}$, $RstR^{ET}$, and $RstR^{class}$ is sequence specific. $RstR^{calc}$ does not repress $RstA^{class}$ or $RstA^{ET}$ promoter activity. $RstR^{class}$ and $RstR^{ET}$ in turn do not repress $RstA^{calc}$ promoter activity.

The production of *RstR* allows the CTX^{calc} prophage to control its replication as well as inhibit *RstA*-mediated replication of newly introduced DNA relying upon the $RstA^{calc}$ promoter. $RstR^{calc}$ production confers immunity to the CTX^{calc} lysogen from superinfection by identical CTX^{calc} phages but confers heteroimmunity (or susceptibility) to infection by $CTX^{ET}\Phi$. Each *RstR* functions as a biotype-specific repressor of its cognate *RstA* given the variations in the sequences and binding sites of the repressor proteins. *LacZ* is a *lac* operon functioning in the transport and metabolism of lactose in some enteric bacteria. Expression of the $RstA^{class}$ reporter construct $RstA^{class}$-*lacZ* is repressed by classical, not El Tor *RstR*. Expression of the $rstA^{ET}$ reporter construct $RstA^{ET}$-*lacZ* is repressed by El Tor, not classical *RstR*. Such repression enables integrated phages to inhibit replication of novel infecting phages of the same biotype conferring immunity to secondary infection. This diversity of the CTX phage repressor *RstR* constitutes heteroimmunity amongst the various CTX phages.
Tcp is the gene code for the cholera toxin protein. Vibrio cholerae O141 strains also carry the CTX^{class} prophage as well as the classical allele of the *tcpA* gene indicative of genetic exchanges between VC O141 and VC O1 El Tor classical strains. Vibrio cholerae O141 strains possibly acquired CTX in a TCP-dependent manner from infection with a CTX^{class} bacteriophage despite having been believed to now be defective and unable to produce infectious particles given the possible extinction of classical Vibrio cholerae O1 strains. The genes encoding *ctxAB* are located in the CTX prophage in toxigenic Vibrio cholerae. The *rstR* repressor genes of CTX phages are different in CTX^{ET} and CTX^{class} although most other elements of the CTX phage genomes are similar in the classical as well as El Tor Vibrio cholerae O1 biotypes. *RstR* genetic diversity is the molecular basis for heteroimmunity amongst the various CTX phages. Novel variants of the Vibrio cholerae O1 El Tor strain have emerged carrying the classical CTX^{class} type prophage. Such strains referred to as hybrid variants display most VC O1El Tor biotype traits, but possess the CTX^{class} prophage and have been isolated from distant environmental locations. These novel hybrid variants are believed to have originated by means of horizontal (also referred to as lateral) gene transfer and recombination occurances. Acquisition of the CTX^{class} prophage from Vibrio cholerae O141 by VC O1 El Tor strains are attributable to lytic phages as well as chitin-induced competence.

4.5 Vibrio cholerae virulence genes, toxins, mechanisms of bacteriophage proliferation, and the infection of the mucosa of the human small intestine

The lack of homoimmunity for phages such as KSF-1Φ may allow superinfection of the same host by various KSF-1-derived phage particles carrying different elements of DNA capable of accumulating and resulting in further evolution of Vibrio cholerae. An example is El Tor Vibrio cholerae that would resist superinfection with another El Tor derived CTXΦ. The CT-converting phage CTXΦ carries the *RstR* phage repressor gene that encodes the *RstR* protein providing immunity to superinfection by the same phage. Any genetic difference between the various CTX phage types is the difference in the *RstR* gene. A site-specific recombination system allows lysogenic phage to integrate at a specific site on the host chromosome in the RS2 region. CTXΦ integrates into the Vibrio cholerae genome forming stable lysogens similar to lysogenic

phages. The core region of CTXΦ consists of an RS2 DNA replication module including the genetic retinue *zot*, *ace*, *psh*, *cep*, $pIII^{CTX}$ (formerly named orfU), and $pIII^{CTX}$ phage coat proteins (whose functions will be further elucidated shortly in the context of the RS1 filamentous self-transducing phage that generally flanks CTXΦ) as well as *ctxA* and *ctxB*. The $gIII^{CTX}$ gene encodes the minor capsid protein *pIII* that recognizes and interacts with the receptors and coreceptors of the CTXΦ phage. *CtxA* and *ctxB* genes encode cholera toxin which is the key virulence factor of Vibrio cholerae. The RS2 module of the phage is immediately preceded by the similar RS1 element. Contemporary toxigenic Vibrio cholerae contains the CTXΦ related RS1 filamentous phage inserted adjacent to their CTX prophages. The four encoded proteins of the RS1 region are associated with the CTX prophage in Vibrio cholerae O1 El Tor biotype and O139 isolates. RS1 is a satellite filamentous phage of CTXΦ encoding a site specific recombin- ation in Vibrio cholerae capable of horizontal propagation exploiting CTXΦ morphogenesis genes to exist as a phage itself. RS1 is dependent on the CTXΦ for capsid proteins, packaging, as well as transmission. *RstC* is an antirepressor in the phage replication process under the control of *PrstA* that can bind directly to *RstR* preventing it from binding to *PrstA* counteracting the activity of the *RstR* gene so that CTXΦ gene expression may take place. The *RstC* gene presumably encodes an acylation enzyme activating cholera toxin. *RstC* contributes to virulence in Vibrio cholerae by inducing expression of *ctxA* and *ctxB*. The *RstC* antirepressor carried by the RS1 satellite phage is often present adjacent to the CTXΦ prophage in toxigenic Vibrio cholerae O1 El Tor and O139 strains. Horizontal transfer of cholera toxin and CTXΦ encoding genes as well as the origination of novel toxigenic Vibrio cholerae strains from non-toxigenic progenitors have been established. The vibrio pathogenicity island is capable of excising from the cell to form circular intermediates (CIs) as a first step in its horizontal transfer from cell to cell. Other Vibrio cholerae encoded pathogenicity islands are also capable of genetic excision and formation of circular intermediates for this purpose. RS1 relies on CTXΦ coat and excretion proteins for the packaging of its genome. RS1 propagates as a filamentous phage aided by the core genes of CTXΦ. The RS1 module of the phage comprises three genes whose sequence is identical to the corresponding genes in the RS2 phage module. Besides carrying these respective *RstA1*, *RstB1*, and *RstR1* genes for replication, integration, and repression common to the RS2 region, the RS1 genome encodes the antirepressor *rstC* gene possessing one additional open reading frame having no counterpart in the RS2 phage module. The *RstR* gene of RS1 provides immunity to infection by CTXΦ carrying the same phage repression gene. The site specific integration system integrates into the Vibrio cholerae chromosome at the *attRS* attachment site forming stable lysogens. Filamentous phages are capable of lysogenizing their host. In toxigenic Vibrio cholerae strains particularly El Tor and O139, the CTXΦ genome integrates into the chromosome flanked by the RS1 repetitive sequence closely related to its RS2 region.CTXΦ and RS1 are phages having identical packaging, secretion, and infection processes.CTX is integrated on both chromosomes never containing an RS1 element in Vibrio cholerae classical biotype isolates.

The remaining CTXΦ genome encodes *psh*, *cep*, $pIII^{CTX}$ (formerly named *orfU*), *ace*, and*zot* phage coat proteins essential for assembly and secretion of new CTXΦ phage virions; aswell as cholera toxin that does not contribute to virion formation. The RS1 genome contains genes encoding the necessary proteins for *RstA* replication, *RstB* integration, as well

as *RstR* and *RstC* regulation of gene expression; but lacks the genes encoding the proteins *psh, cep, pIII*^{CTX}*, ace,* and *zot,* necessary for the assembly and secretion of viral particles as well as Ctx. The RS1 satellite phage is capable of autonomous replication depending on its CTXΦ helper phage for assembly as well as secretion of RS1 viral particles.

On the other hand, RS1 encodes the *RstC* antirepressor protein not present in the CTX prophage which promotes the transcription of the CTXΦ genome and RS1 genes by counteracting *rstR* phage repressor activity.RS1Φ particle production is capable of occurring independently of CTXΦ using functions encoded by the aforementioned KSF-1Φ filamentous phage. The RS1Φ produced in this mannercan infect recipient VC strains by a mechanism independent of the CTXΦ receptor toxin-coregulated pilus. *Cep* and *zot* are major coat proteins. *Psh*, *ace*, and *pIII*^{CTX} minor coat proteins are based on the relatively few copies present per virion, their sizes, positions within the phage genome, and occasionally their homology. *Cep*, *zot*, *psh*, *ace*, *gIII*^{CTX}structural proteins and *pIII*^{CTX} are core region proteins; the *ctxAB* toxin genes which do not contribute to phage production are also present in this region. The zonula occludens toxin (*zot*) increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junctions (zonula occludens). *Zot* is a CTXΦ-encoded assembly protein that mediates phage secretion playing a role in phage assembly not being part of the virion in and of itself. The pilin-like factor Core-Encoded Pillin (*cep*) is CTXΦ's major coat protein enhancing colonization. Accessory Cholera Toxin (*ace*) is an enterotoxin related to increased transmembrane ion transport believed to play a role in phage assembly. The enterotoxic effect of *ace* and *zot* found by Fasano et al, as well as Trucksis et al, may be attributable to their expression during bacterial invasion of the human host (as cited in Boyd, 2008, p. 50). The *gIII*^{CTX}gene functions in phage infection and replication processes. A particular phage initiates the infection process by the binding of one of its ends to the pilus of the the bacterium carrying its particular episome. An episome is an accessory extrachromosomal replicative genetic element existing autonomously or integrated with the bacterial chromosome functioning as a plasmid. The pilus is recognized by the minor capsid protein *pIII* located at one end of the bacteriophage particle. The phage is believed to then be brought to the bacterial surface by retraction of the pilus. The subsequent translocation of phage DNA into the cytoplasm requires the presence of *tolQRA* operon complex genes. *pIII*^{CTX} is believed to be a minor coat protein that mediates CTXΦ infection and replication being required for the assembly and/or release of these virions by Vibrio cholerae. *TcpA* interacts directly with *pIII*^{CTX}.

The life cycle of CTXΦ consists of its attachment and entry into Vibrio cholerae, CTXΦ integration into the VC genome, CTXΦ DNA replication, and CTXΦ secretion from Vibrio cholerae, as well as the regulation of these life cycle stages. The CTXΦ receptor on theVibrio cholerae cell surface is a motile contractile type IV pilus, the toxin co-regulated pilus, functioning in the presence of the periplasmic and inner cell membrane protein *TolQ, TolR, TolA* operon complex (*TolQRA*) of Vibrio cholerae. The translocation of the filamentous bacterio- phage DNA requires the products of the chromosomally encoded bacterial *tolQ*, *tolR*, and *tolA* cytoplasmic membrane proteins as well as its binding to the pilus for its movement into the cytoplasm of the Vibrio cholerae bacterium. *TolQ* is a membrane spanning protein. *TolR* is a membrane protein. *TolA* is a membrane anchored protein. Insertion of the major capsid protein from the infecting bacteriophage into the

cytoplasmic membrane requires the presence of *TolA*. The functions of the *TolQRA* proteins have not yet been clearly elucidated. Uptake of CTXΦ particles into the Vibrio cholerae bacterium does not always require their pilus receptors if they contact the *TolQRA* complex upon contact with the VC outer membrane.A virion is an elementary virus particle composed of a nucleoid (central core) containing DNA or RNA surrounded by a capsid. This nucleocapsid (nucleic acid-protein complex) may be a complete virus or be surrounded by an envelope. CTXΦ may possess a virion coat protein that interacts with the TCP and the *TolQRA* complex enabling it to infect Vibrio cholerae. $pIII^{ctx}$ protein residues may bind to Vibrio cholerae co-receptor *tolA* as a mediator of this infection process by CTXΦ in the presence of the toxin-coregulated pilus. *pIII* is a structural minor capsid protein encoded by gene *gIII* that recognizes and interacts with phage receptors and coreceptors. The $pIII^{ctx}$ protein of CTXΦ may bind to the major pilin protein TcpA of the toxin co-regulated pilus.

The proper sequence of CTXΦ $pIII^{ctx}$ alleles must interact with TcpA/ *TolA* proteins in order for co-receptor *tolA* to bind CTXΦ $pIII^{ctx}$ in the presence of TcpA. $pIII^{ctx}$ is required for CTXΦ assembly and release by Vibrio cholerae. Helpern and Waldor have found that the role of $pIII^{ctx}$ in CTXΦ infection mimics the action of the *pIII* mediator of Ff phage infection of E. coli enterotoxin. This finding that $pIII^{ctx}$ functions as a CTXΦ *pIII* analog has caused some researchers to rename the *OrfU* protein of CTXΦ as $pIII^{ctx}$ (Helpern & Waldor 2002). The function of the aforementioned Mannose Sensitive Hemagglutinin as a receptor requires a counterpart in the phage for direct interaction to occur. The absorption protein *pIII* is the MSHA's counterpart in filamentous phages.

4.6 Rolling circle rplication, type ii secretion, general secretion pathway, and dnase i/ii

The genes of a filamentous phage consist of functionally related groups organized in a modular structure. Rolling Circle Replication consists of the replication of circular DNA molecules in which linear daughter DNA molecules containing repeated DNA sequences (concatemers) are produced from the parent which are cleaved forming unit length genomes which circularize. These genes include those of the replication module that contains genes coding for the rolling circle replication protein and the single-stranded DNA-binding protein, the structural model containing major and minor coat protein-encoding genes, as well as the assembly and secretion module that contain the genes for the morphogenesis and extrusion of the virus particles. Rolling Circle Replication commences by means of an initiator protein encoded by bacteriophage DNA or a plasmid. This type of replication will be discussed further in a subsequent section of this text. The inovirus group consists of three functional modules. The replication module contains genes encoding the capsid structural proteins of the filamentous phages as well as those coding for rolling-circle replication and single-stranded DNA binding proteins. The major and minor coat protein-encoding genes are contained in the structural module. Genes for the morphogenesis and extrusion of the phage particles are contained in the assembly and secretion module. The VGJΦ phage possesses an an additional regulatory module in which transcriptional repressor genes are putatively grouped which may also be present in other lysogenic filamentous phages such as CTXΦ of Vibrio cholerae. CTXΦ encodes the transcriptional repressor *rstR* which regulates the expression of other phage genes. Eps refers to the extracellular host protein pathway

consisting of 13 gene products including the open reading frames *epsC* through *epsN* as well as the prepilin peptidase VcpD required for toxin secretion and outer membrane biogenesis in Vibrio cholerae. CTXΦ uses the *EpsD* secretin of the type II secretion system of Vibrio cholerae for phage particle extrusion. Secretin is a strong basic polypeptide hormone secreted by the duodenal and upper jejunal mucosa upon the entrance of acid chyme into the intestine; stimulating the release of pancreatic juice and bile by the pancreas and liver respectively containing bicarbonate changing the duodenal pH from acid to alkaline to facilitate the action of intestinal digestive enzymes. VGJΦ also uses the *EpsD* host protein for phage morphogenesis. In the type II secretion pathway, the individual Ctx subunits are initially produced as precursor proteins with characteristic N-terminal signal peptides. Subsequent to translocation through the cytoplasmic membrane by way of the sec pathway, removal of the signal peptides occurs and the mature subunits are released into the periplasm assembling noncovalently into an AB_5 holotoxin complex assisted by the disulfide isomerase DsbA (TcpG). The assembled toxin complex traverses the outer membrane in a second step requiring VcpD and the remaining twelve gene products of the Eps host protein.. The B subunit pentamer contains the genetic information for outer membrane translocation. The A subunit is secreted due to its association with the AB_5 holotoxin complex. The periplasmic toxin complex is similar to the secreted form of the toxin.

It is believed that proteins are secreted in their fully or nearly fully folded configurations by way of the type II secretion pathway; with folding being prerequisite for outer membrane transloc- ation. The General Secretion Pathway (GSP) consists of Eps host protein genes required for extracellular secretion of certain proteins including chitinase, cholera toxin, heat-labile enterotoxin, endoase (ChiA), and protease. The General Secretion Pathway of Vibrio cholerae provides for the extracellular secretion of various proteins including chitinase, cholera toxin, and protease. The GSP may be involved in hemolysin and polysaccharide secretion in Vibrio cholerae. The GSP is believed to be essential to the pathogenicity and survivability of Vibrio cholerae in the environment. Cholera toxin is also involved in the process of dissemination being the major causative component of clinical disease. Secreted chitinase and protease play a role in the detachment of Vibrio cholerae from waterborne chitinous surfaces as well as from the intestinal epithelium of the host respectively providing for its dissemination.

The Eps secretion apparatus is believed to be constitutive actively secreting cholera toxin in the human intestine as well as in the aquatic environment secreting chitinase and protease. The Vibrio cholerae colonization surface can be the intestinal epithelium where Ctx is secreted; or an environmental example is the exoskeleton of crustaceans where chitinase is released.Some species encoding the type II secretion apparatus may be isolated from various ecological niches. Vibrio cholerae can live in the aquatic environment as a free-swimming organism, associated with plankton, or as a constituent of biofilm attached to chitinous or abiotic surfaces.Vibrio cholerae undergoes a phenotypic transition from its growth on plankton to its existence on a formed biofilm. Polysaccharide biosynthesis is essential to biofilm formation which takes place in Vibrio cholerae, apparently not involving the Eps host protein pathway. The General Secretion Pathway EPS genes are believed to participate in soluble protein secretion through the outer membrane as well as in the assembly of the outer membrane. Cholera toxin secretion occurs by means of a two step pathway specifically

requiring a set of EPS genes for outer membrane translocation. Since several Eps open reading frames overlap each other possibly as part of a single operon, *eps*D, -H, -I, -J, -K, and –N are seemingly required for Ctx secretion. The *epsD* gene of the type II secretion apparatus is believed to form the outer membrane translocation pore. Secretion of the filamentous bacteriophage CTXΦ,which is actively secreted without lysing the host cell, requires *epsD. EpsD* participates in the pathogenesis of the Vibrio cholerae bacterium as well as the bacteriophage through active Ctx secretion and phage release allowing for horizontal gene transfer. The secretory pathway for Ctx and CTXΦ are located at the *epsD* pore site. GSP functions are also required for transport of OmpT and OmpU into the outer membrane or for transport of other required proteins for proper assembly and anchoring of affected GSP proteins in the outer membrane. OmpT and OmpU are believed to have major stabilizing effects on the outer membrane since they are major outer membrane proteins in Vibrio cholerae. Another pathway for extracellular Ctx secretion exists in Vibrio cholerae involving the extracellular protein deoxyribonuclease (DNase). A DNase is any nuclease that specifically catalyzes the hydrolytic cleavage of phosphate ester linkages in deoxyribonucleic acids. An endodeoxyribonuclease is a DNase that cleaves internal bonds at the terminus. DNase I is an endonuclease that catalyzes the endonucleolytic cleavage of single and double stranded DNA producing dinucleotides and oligonucleotides with 5'-phosphate groups yielding 5'-phosphodinucleotides and 5'-phosphooligonucleotides. It is secreted by exocrine glands such as the pancreas and parotid gland into the alimentary tract. DNase is also present in other tissues including the kidney, thymus, lymph node, small intestine, heart, liver, and epididymis where its participation in various cellular functions is being further investigated. The optimal pH for DNase I as an enzyme is neutral. Ca^{2+} and Mg^{2+} are necessary for its enzymatic activity. Paneth cells are epithelial cells in the fundus of the small (and large) intestinal glands (Crypts of Lieberkuhn) containing granules secreting antimicrobial substances such as defensins that are cationic peptides occurring in neutrophils and macrophages binding fungal and bacterial membranes increasing membrane permeability. Paneth cell secretory activity is directly affected by the bacterial environment of the intestinal lumen. These tubular glands of the small intestinal mucous membranes open between the bases of the villi. Paneth cells are believed to play a role in the regulation of intestinal flora due to their ability to phagocytose microorganisms as a participant in host defenses in the small intestine where Vibrio cholerae colonization and infection occur. DNase I is distributed primarily along the secretory pathway of Paneth cells; being synthesized in the rough endoplasmic reticulum (rER), modified in theGolgi apparatus, stored in secretory granules, and secreted into the intestinal lumen possibly along with other exocrine secretory materials. The presence of DNase I along the secretory pathway in Paneth cells of the human small intestine is indicative of its importance as a component of the host intestinal immune defense system. DNase also plays a role in transformation in gram-negative bacteria such as Vibrio cholerae in a multistep process. Dubnau found that DNA binds to the cell surface, DNA uptake into the periplasm occurs via an outer cell membrane pore composed of secretins, and transport of a DNA strand across the inner cell membrane occurs as the other strand is degraded (Jajosky, 2007, 18). Chen and Dubnau found that DNA uptake specifically consists of DNA transition from a DNase-sensitive to a DNase-protective state generally occurring in gram-negative bacteria by passage through the outer

cell membrane (Ibid.). DNase II is an endonuclease catalyzing the endonucleolytic cleavage of single and double stranded DNA yielding nucleoside 3'-phosphates and 3'-phosphooligonucleotide as dinucleotide and oligonucleotide end products. It occurs in tissues such as the liver, pancreas, thymus, and gastric mucosa whose activity is cation-independent and optimal pH is acidic around 5.0.

The DNase II enzyme is believed to participate in the engulfment-mediated degradation of exogenous DNA occurring during phagocytosis. It is also believed to play a role in DNA fragmentation anddegradation in the process of apoptosis (genetically programmed cell death). DNase I and II are capable of digesting single and double-stranded DNA. Vibrio cholerae is believed to secrete these two distinct DNases into the extracellular environment by a mechanism that has yet to be fully elucidated.

Extracellular secretion of proteins is an important virulence mechanism of bacterial infection.For proteins to reach the extracellular environment of gram-negative bacteria, they must cross two membranes of the bacterial cell envelope. Translocation across the cytoplasmic membrane via the periplasmic compartment and the peptidoglycan layer to the outer membrane occurs. There are six specialized pathways for extracellular secretion known to date. The type II secretion pathway is encoded by at least 12 genes specifically supporting the transport of agroup of seemingly unrelated proteins across the outer membrane that are essential to Vibrio cholerae secretion and pathogenesis. The species dependent genes essential to type II secretion are designated by the letters A through O as well as S; being relatively well conserved in a single operon with several genes overlapping with variations usually located at the 5' and 3' ends of the gene clusters. The O gene being the last gene of the type II secretion operon bears homology to the required prepilin peptidase gene for type IV prepilin subunit processing. In species in which the O gene is not linked to the remainder of type II secretion genes, it is associated with a required subset of genes for type IV pilus biogenesis. Their prepilin peptidase is of dual function being required for type IV prepilin subunit processing and methylation as well as that for the prepilin-like components encoded by the type II secretion genes G, H. I, J, and K. These genes that are components of the type II secretion apparatus may be capable of forming a pilus-like structure. It is yet to be confirmed if the pilus structure supports secretion per se. The cytoplasmic membrane-anchored type II secretion pilus may function through extention and retraction to push secreted proteins through the gated pore in a piston-like fashion. The VcpD (PilD) of Vibrio cholerae is a requirement for Ctx secretion via the Eps secretion apparatus and processes the precursor form of *epsI* and the prepilin forms of the type IV pilin subunits PilA and MSHA. It is postulated that the Vibrio cholerae VcpD gene has been acquired by horizontal transfer since Eps genes have existed for a relatively long time. The Eps cluster may have originally contained an *epsO* gene at the distal end having become redundant and then lost upon VcpD gene acquisition. Protein D of the secretin family possesses proteins required for type IV pilus biogenesis and filamentous phage extrusion that are present in the outer cell membrane where they are believed to form gated secretion pores. The secretins are of oligomeric structure exhibiting ion-conducting properties. The remainder of the type II secretion apparatus components also appear to be associated with the cytoplasmic membrane where some remain intact, although they are required for outer membrane translocation. The proton motive force of the cytoplasmic membrane is required for outer membrane

translocation to occur. The secretion apparatus comprise a multiprotein complex spanning the cytoplasmic and outer cell membranes. Protein entrance to the type II secretion pathway involves protein translocation acrossthe cytoplasmic membrane by way of the Sec system as well as subsequent folding into a translocation competent conformation in the periplasm. Cellulases, lipases, pectinases, phospholipases, proteases, and toxins are proteins associated with the destruction of various tissues that contribute to cell damage and disease secreted by the type II pathway. Secreted proteases, lipases, phospholipases, and toxins may participate in colonization or tissue destruction by defeating host defenses and/or serving as a nutrient source. Ctx and Tcp are the principal virulence factors in Vibrio cholerae required for infection of the intestinal mucosa.This pathway also supports the secretion of cholera toxin and the extracellular enzyme Hemagglutinin Protease (HAP) in Vibrio cholerae. The expression of the genes corresponding to these proteins and sometimes those functioning in secretion are either under quorum-sensing control or under environmental regulation at the site of colonization.Bacterial gene expression is regulated by a variety of intracellular and extracellular signals.

Certain phage-encoded and host-encoded factors are required for CTXΦ integration into the Vibrio cholerae host genome as outlined in the following table.

Table 1: CTXΦ and Vibrio cholerae-encoded proteins required for CTXΦ life cycle

Stage	CTXΦ	V.cholerae
Attachment	$pIII^{CTX}$	*TcpA, TolQRA*
Infection	$pIII^{CTX}$	*TolA*
Integration	*RstB*	*XerC, XerD*
Replication	*RstC, RstA*	*ToxT*
Secretion	*Zot*	*EspD*
Regulation		
Repression	*RstR*	*LexA*
Activation	*RstC*	*ToxT*

Source: (Boyd 2008, 50)

4.7 The genetics and mechanisms of ctxφ integration into the vibrio cholerae genome, and type ii ctxφ secretion in vibrio cholerae

Subsequent to CTXΦ DNA absorption to the cell wall of Vibrio Cholerae, viral single-stranded DNA (ssDNA) is injected into the cytoplasm of the cell. Tandem arrays of prophage DNA interspersed with the related RS1 genetic element are site-specifically integrated into the chromosome in toxigenic Vibrio cholerae isolates. Such arrays may yield hybrid virions composed of DNA from two adjacent prophages or a prophage and a downstream RS1 genetic element. The source of coding sequences are from the 5' prophage. Most of the intergenic region 1 sequence is derived from the 3' prophage. Since tandem elements are required for virion production, CTX virions rarely result from Vibrio cholerae strains possessing a lone prophage; the few resulting virions are from imprecise prophage DNA excision. Generation of pCTX, the replicative form (RF) of the CTXΦ phage genome, in a step preceding virion production, is not dependent on the reversal of site-specific CTXΦ DNA integration into the Vibrio cholerae chromosome. pCTX production is not dependent

on RecA-mediated homologous recombination amongst adjoining prophages. CTXΦ specific proteins required for pCTX replication are capable of functioning on a chromosomal substrate not requiring prophage excision from the chromosome, maximizing vertical prophage DNA transmission as well as enabling CTXΦ dissemination to new hosts. Linear ssDNA circularizes forming pCTX when it enters the cell cytoplasm remaining as an extrachromosomal element or more likely integrating into the Vibrio Cholerae genome at site-specific attachment sites. CTXΦ depends upon the host- encoded tyrosine recombinases XerC and XerD in the integration process. CTXΦ integration occurs at site *dif*1 on Vibrio cholerae chromosome I requiring XerC and XerD binding. There is an *attP* integration site on the CTXΦ genome in the Ig1 intergenic region. It is believed that XerC and XerD binding occurs at two different sites within *attP* at the core NtrC XerCD binding site spanning the recombination region as well as a site required for integration possibly serving as an architectural or stabilizing factor. An alternative CTXΦ integration model suggests that ssDNA CTXΦ genome is involved in integration into the genome of Vibrio cholerae. There are two pairs of binding sites for XerC and XerD in inverted orientation on the single stranded pCTX genome that fold into a hairpin structure that create a recombination targetfor XerCD. XerC is capable of catalyzing a single pair of DNA strand exchanges between this target and *dif*1 in the presence of XerD that results in CTXΦ integration not having functional Xer sites. Xer recombinases may promote direct insertion of ssDNA genome into the *dif*1 site of Vibrio cholerae. The Xer recombinase systems as well as the prevalence of *dif* sites among filamentous phages illustrate the process of phage integration into the bacterial genome that is irreversible. CTXΦ DNA integration depends on the XerCD host recombinases. This contrasts with the classical model of phage integration of ssDNA phage conversion to double stranded DNA (dsDNA) prior to integration.

The ssDNA forms a stem-loop structure producing a duplex DNA region upon which Xer recombinases act. Chromosomally integrated CTX prophage can function as a template for ssDNA phage production; requiring either a tandem array of CTX prophages or the aforementioned CTX-RS1 array. Rolling circle DNA replication occurs when one DNA strand is cleaved and the free 3' hydroxyl end extends aided by replication enzymes. The 3' end lengthens as the replication fork rolls around the circular DNA template producing a 5'end. The single stranded tail in the process of complementary DNA strand synthesis may be converted to a new double DNA strand. Rolling circle replication allows for rapid and continuous production of numerous genome copies from a single event initiating this process; being important in viral replication. The CTX plasmid form is produced by rolling circle replication of tandemly repeated CTX-CTX or CTX-RS1 arrays. Infectious CTX particle production requires either of two CTX prophages or a CTX-RS1 array and that the CTXΦ DNA be a hybrid sequence derived from the aforementioned elements. *RstA* is required for pCTX replication. *RstA* is believed to make a single-stranded nick in the CTX prophage in *Ig-1* resulting in a 3'-end serving a a template for new DNA synthesis by host encoded DNA polymerase. Strand displacement takes place and replication continues into downstream CTX prophages until *Ig-1* is reached during DNA replication. *RstA* nicks the displaced strand which yields single stranded CTX genome. This is why classical CTXΦ do not produce infectious phage particles because these isolates do not possess complete CTXΦ arrays or RS1 prophages being the substrate for replication of ssDNA. CTX is secreted out

of the CT cell in the absence of Vibrio cholerae cell lysis.CTXΦ uses the EspD secretin of the type II extracellular protein secretion system. The EspD outer membrane protein appears to be the sole component of the type II system required for CTXΦ secretion.

4.8 Acquisition of genes regulating vibrio cholerae pathogenization, colonization, and virulence

Colonization of the human intestine by Vibrio cholerae is a complex process involving proteolytic enzymes, hemagglutinins (antibody responsible for aglutination of red blood cells), colonization pili and the coordinated expression of chemotactic and motility functions. Although production of Ctx, encoded by the *ctxAB* genes, is directly responsible for the manifestation of diarrhea, cholera pathogenesis relies on this synergistic action of other genes including those for the colonization factors. In Vibrio cholerae, the major virulence genes appear to exist in clusters. In at least two regions of the VC chromosome, genes encoding virulence factors are clustered. These include the ctx element, the toxin-coregulated pilus and the accessory colonization factor gene cluster more commonly referred to as the TCP pathogenicity island. The accessory colonization factor of Vibrio cholerae regulated by *ToxR* is immediately present downstream of the TCP group of genes. The principal colonization factor of brush borders in the small intestine is the toxin-coregulated pilus necessary for the adherence of toxigenic Vibrio cholerae to the intestinal epithelium. This bundle-forming toxin- coregulated pilus is an intestinal surface organelle of Vibrio cholerae coordinately expressed with cholera toxin. The genes for TCP are part of the VPI segment usually absent from non- epidemic strains. TCP biogenesis requires the action of at least eleven accessory proteins mostly encoded by genes in the TCP operon. Structurally, the VPI includes groups of virulence genes as well as their regulator, a transposase gene and specific *att*-like attachment sites on each end of the pathogenicity island. The presence of an integrase on the VPI with homology to a phage integrase gene suggests that the island was also derived from a bacteriophage. The CTXΦ virion uses TCP as a receptor when infection takes place. Colonization is aprerequisite to the establishment of a productive infection. *TcpA*, the protein constituting the pilus structure, is homologous to the bacterial type-IV pilins of colonization.The TCP gene cluster consists of genes regulating the biogenesis, secretion, and export of *TcpA*. An allele is any one of two or more different genes occupying the same locus on a specific chromosome. The *TcpA* gene of the TCP cluster possesses specific alleles for Vibrio Cholerae O1 classical and El Tor biotypes. *TcpACDE* and *F* genes are believed to encode assembly and transport proteins required for pilus biogenesis. The *tcpA* gene of VPI-I encodes the major pilin protein of *TcpA* involved in Vibrio cholerae colonization in the host intestine; also acting as the receptor for CTXΦ to infect VC strains. *TcpG* is thought to be involved in disulphide bond formation within *TcpA*. *TcpH* may play a positive regulatory function. *TcpI* may be involved in the negative regulation of pilin production. *TcpJ* is believed to encode the secretory apparatus for Tcp export. The pilus (fimbria) is a protein filamentous surface organelle that mediates specific binding (adherence) and colonization of host tissue. Production of pili by Vibrio cholerae parallel the expression of cholera toxin. All strains of Vibrio cholerae known to date produce a colonization pilus of the same antigenic type possessing an adhesive component which

facilitates attachment to the intestinal mucosa. Vibrio cholerae O1, irrespective of the biotype or serotype, adheres to the human small intestinal mucosa more extensively than its adherence to the villous surface of the human small intestine (Yamamoto and Yokota 1988, 2753). A microarray consists of a hybridized arrangement of sample known or unknown DNA target spots to a large set of oligonucleotide probes on a substrate in an automated high-throughput matching process for the purposes of DNA sequence determination, detection of gene sequence variations or expression, or gene mapping. The Vibrio cholerae microarray or "cholera chip" as a tool by which different strains already sequenced can be genetically compared. The genetic sequence of classical, other El Tor, and non-toxigenic environmental isolates has been compared to Vibrio cholerae strain N16961 characteristic of the seventh VC pandemic. The Vibrio cholerae microarray based analysis revealed that certain genes or gene groups are not present in some of the El Tor strains compared with the seventh pandemic strain studied yielding the following four categories.

1. Genes absent in classical VC strains present in all El Tor strains.
2. Genes present exclusively in pandemic strains.
3. Genes present exclusively in the seventh pandemic El Tor strains.
4. Genes not present in individual strains.

Seven genes absent in the classical strains were present on the large chromosome in the region encoding the RTX toxin. The classical strains were believed to lack the *rtxC* gene that may encode an acylation enzyme activating the toxin. The microarray analysis indicated that the gene deletion extends into the *rtxA* and *rtxB* genes. The remaining five genes are located on the smaller chromosome, three of which being hypothetical and the other two being putative acetyl transferases residing on the integron island. The classical and El Tor strains have few genes uniquely distinguishing one from another. Although both strains may have evolved from separate lineages, the ancestral environmental strain from which they evolved seems to more greatly resemble the El Tor strain. Thus, foreign gene acquisition events have been instrumental in aiding the pathogenization process of Vibrio cholerae (Uma et al. 2003, 1543).

4.9 The genetics of vibrio cholerae pandemic strain emergence and pathogenicity

Some genes differentiate the classical and seventh pandemic strains from environmental and prepandemic El Tor strains. The classical biotype caused the fifth and sixth pandemics.The current seventh pandemic initiated in the Sulawesi Islands of Indonesia in1961 was caused by the El Tor biotype which is capable of generating genetic variants during a short time span. Early El Tor and prepandemic strains carry TCP island genes. A non-toxigenic Gulf Coast $TCP^{+}CTX\Phi^{-}$ isolate exists. Twenty-two genes absent in classical, prepandemic TCP^{+} El Tor isolates as well as a prepandemic environmental El Tor isolate exist in seventh pandemic strains. They occur as a block of eleven genes as clusters having a low GC content designated as Vibrio Seventh Pandemic Island I (VSP I). Vibrio Seventh Pandemic Island II (VSP II) is another chromosomal island also having a low GC content solely found in seventh pandemic isolates. VSP I and VSP II may have acquired the seventh pandemic strains by horizontal gene transfer. Other genes are being studied that may influence the pathogenicity of Vibrio cholerae.

HlyA encodes a Vibrio cholerae hemolysin protein cytotoxic for some mammalian and red blood cells. Toxin-linked cryptic (TLC) is a DNA fragment having a yet to be fully understood relationship to CTXΦ. Many integrating filamentous phages seemingly exploit the aforementioned XerCD recombination system to integrate into a site overlapping the *dif* site of the bacterial chromosome, which in Vibrio cholerae is an important spot for multiple-phage integration. Several integration events contributing to the evolutionary history of current circulating toxigenic Vibrio cholerae strains may have occurred. Complete analysis of a genomic sequence of a VC EL Tor strain revealed two tandem imperfect repeats of the toxin-linked cryptic element next to the CTXΦ prophage flanked by *att*-like sites. The TLC element is closely associated with the CTXΦ prophage in toxigenic Vibrio cholerae strains having filamentous phage-related DNA sequences capable of producing a cryptic plasmid. TLC elements may be vestiges of an ancestral TLCΦ phage that infected Vibrio cholerae, integrated at the *dif* site, and lost its morphogenesis and structural modules becoming defective. The result was the impairment of new transduction events of the TLC element by itself. It is alternatively speculated that the TLC element experienced deletion of its morphogenesis and structural modules in other hosts, from which a foreign capsid transferred it into Vibrio cholerae. Upon establishment of the TLC element, the bacterium became infected by CTXΦ which integrated into the *attR* site of the TLC element. A subsequent similar integration event with the RS1 satellite phage resulted in the current structure of the VC EL Tor strain studied (Campos 2003, 5693). *PilE* putatively encodes a fimbrial assembly protein. *Intl4* encodes an integrase recognizing a family of Vibrio Cholerae Repeated Sequences associated with gene-VCR organization.

VCR functions will be further explained in subsequent paragraphs. Sixth-pandemic, seventh-pandemic, and recent United States Gulf coast isolates are clones believed to have independently evolved from environmental, non-toxigenic, Vibrio cholerae non-O1 El Tor organisms. The seventh pandemic has accumulated variation possibly involving random genetic drift and recombination. Genetic drift involves random fluctuations in the frequency of appearance of a gene variant (allele) in a population. Recombination consists of the intermolecular exchange of chromosomes combining genetic information from two different genomes of a species. O139 isolates evolved from seventh-pandemic isolates. Horizontal transfer of O antigen genes plays a role in the process. Substantial genetic exchange and a relatively low level of clonal activity occurs in Vibrio cholerae. Since most environmental Vibrio cholerae isolates do not possess cholera toxin genes, a heat-labile enterotoxin, or a toxin coregulated pilus, the acquisition of virulence and toxigenicity causing disease in humans are attributable to the aforementioned clonal and genetic exchange phenomena. Cholera was an unknown disease to nomadic Paleolithic humans who were hunter-gatherers circa 2,000,000-10,000 B.C. Vibrio cholerae is commonly found in marine and estuarine environments associated with crustaceans and mollusks. Vibrios from the gut of such infected shellfish are excreted into the environmental waters. Human hosts who ingest shellfish such as prawns or oysters from environmental waters infected with pathogenic Vibrio cholerae will also excrete vibrios from the gut back into the environment where they reenter their normal life cycle. A single cholera patient is capable of excreting up to twenty liters of “rice water” stool containing 10^7 to 10^8 toxigenic Vibrio cholerae cells per milliliter each day into the environment. When non-itinerant societies became established remaining in

a geographic location, the local communal water supply would become infected by the excreted Vibrio cholerae that arose independently from environmental strains contaminating their water supply perpetuating the circle of infection, morbidity and mortality of cholera. Human-to-human infection occurs in the presence of environmental Vibrio cholerae contamination, poor hygiene, and crowded living conditions. Such infection has been made possible by sequential acquisition of the necessary Vibrio cholerae genes.

The virulence gene cassette and toxin coregulated pilus are both necessary for virulence, although separated on the Vibrio cholerae chromosome. Isolates containing cholera toxin contain the complete virulence gene cassette. Transposons are DNA segments carrying required genes for chromosomal DNA movement. Sixth-pandemic, seventh-pandemic, and Gulf Coast clonal isolates possess the same virulence genes (to be further elucidated in a subsequent section of this text) on an element functioning as a transposon with the tcp maping elsewhere in the gene sequence on the chromosome. These genes are usually not present in nonpathogenic Vibrio cholerae isolates. The *asd* (aspartate-semialdehyde dehydrogenase) gene functions in the common pathway for the biosynthesis of the essential branched-chain amino acids threonine, lysine, and methionine. The *asd* locus on the Vibrio cholerae chromosome is a housekeeping gene concerned with basic cell functions.

Genetic recombination events may occur attributable to the *asd* gene. Vibrio cholerae O139 and seventh-pandemic isolates possess identical *asd* gene sequences. The Vibrio cholerae O139 isolates evolved from an early seventh-pandemic isolate having acquired enough genetic information resulting in a novel O antigen. Vibrio cholerae isolates having virtually identical *asd* sequences had different O antigens; and isolates possessing the O1 antigen that did not form a gene cluster but were present in different lineages are seemingly attributable to the horizontal transfer of the O antigen.

Although the Vibrio cholerae O1 antigen is present in pandemic isolates, it is not necessary for pathogenesis because VC O139 isolates possess pandemic potential as well. The *asd* gene is one of the few Vibrio cholerae chromosomal housekeeping genes elucidated to date. The exchange of genetic material and the emergence of new strains are why Vibrio cholerae is described as an “emerging pathogen”.

Table 2: the chronology of cholera

At home	To 1817	Endemic on the Indian sub-continent	
Historic Pandemics	1817-1823	First Pandemic	John Snow and the Broad St. Pump,1854
	1829-1850	Second Pandemic	
	1852-1860	Third Pandemic	
	1863-1879	Fourth Pandemic	
Classical Pandemics	1881-1896	Fifth Pandemic	Robert Koch and the comma bacillus,1884
	1899-1923	[Sixth Pandemic]	
El Tor Pandemic	1961 to date	Seventh Pandemic	De and Chatterjee Cholera toxin,1953
O139 Bengal	1992 to date	Eighth Pandemic?	

(Drasar and Forest 1996, 9)

The table refers to the British physician and epidemiologist John Snow who by means of epidemiological surveillance discovered that the 1849 outbreak of cholera in the vicinity of

the Broad Street Pump in London was attributable to human consumption of water contaminated by cholera laden raw sewage from the well at that location. The well was rendered unusable as a water source. Subsequent to the removal of the pump handle, a marked decrease of cholera cases in that area was observed. During the cholera outbreak of 1854 in London, epidemiological surveillance by Dr. Snow revealed that two distinct companies supplied water to the affected area sourced at two distinct locations along the Thames River. His surveillance revealed that most of the affected cholera patients purchased their water from the Southwark and Vauxhall Company that sourced its product from a location along the river in London below which raw sewage was discharged. However, the Lambeth Company sourced its product from an upriver location outside of the city before it reached downriver raw sewage discharge points in London.

Households supplied by this company experienced an over eightfold lower death rate from cholera compared to those supplied by the Southwark and Vauxhall Company (Prescott 2002, 848).

4.10 The mechanisms and genetics of the toxin-coregulated pilus and cholera toxin virulence

Toxin-coregulated pilus (TCP) and cholera toxin (ctx) are the two virulence factors produced by Vibrio cholerae that cause disease as well as the colonization of the upper small intestine of the host. TCP and Ctx expression are controlled by a transcriptional cascade that culminates with the expression of the AraC/XylS-family transcriptional regulator ToxT; which also activates the transcription of five other Vibrio cholerae pathogenesis genes *acfA, acfD, tcpI, aldA,* and *tagA* located within the vibrio pathogenicity island whose functions have yet to be fully elucidated. The AraC/XylS family of regulators are capable of binding DNA and activating transcription. The ToxT regulon includes the genes encoding cholera toxin and the toxin co-regulated pilus. The Tox T protein is the direct transcriptional activator for most Vibrio cholerae virulence genes. Cholera toxin, *ctxAB*, and the toxin co-regulated pilus component *tcpA* and *tcpJ* encoding genes are contained in the operons downstream of the DNA binding sites for transcription of the ToxT transcriptional activator. The Vibrio cholerae chromosome I possesses some phage-like properties, the vibrio pathogenicity island, as well as the type IV toxin co-regulated pilus including the TCP biogenesis genes *tcpA* and *tcpJ*. The toxin co-regulated pilus is the CTXΦ receptor. ToxR and TcpP are membrane-localized transcriptional activators, functioning in concert with their respective ToxS and TcpH cofactors, are required for the activation of transcription of the *toxT* gene. Positive Feedback (Positive Regulation) refers to a continual increase of system output due to the exertion of a stimulatory effect on an essential step by a product of the system. Production of the Tox T protein enables the activation of its own expression in a positive-feedback loop since it is located within the *tcpA* operon.

The transcriptional activators AphA and AphB regulate the transcription of the *tcpPH.* AphB is a LysR-family transcriptional regulator. AphB is a required transcriptional activator for the expression of the virulence regulator TcpP essential to virulence factor production. Vibrio cholerae virulence gene expression is influenced by the ambient oxygen concentration.

Anaerobiosis is a host environmental factor modulating virulence factor production by enhancing the activity of AphB which functions in oxygen responsiveness. The oxygen concentration in the intestine of the host is presumed to be low. AphB proteins are more active under anaerobic rather than aerobic conditions. Viable Vibrio cholerae cells that have survived the acidic gastric environment enter the small intestine producing virulence factors such as cholera toxin and the toxin co-regulated pilus whose direct primary transcriptional activator or virulence genes is ToxT regulated by ToxRS as well as TcpPH. AphA and AphB encoded by unlinked genes are the additional activators regulating tcpPH transcription. AphB senses oxygen concentration modulating its own activity. TcpP expression is higher under anaerobic conditions in an AphB dependent manner. Oxygen-limiting conditions are required for virulence gene expression in the host intestine. Vibrio cholerae is believed to detect oxygen-limiting conditions in the small intestine inducing the expression of the required genes for virulence and colonization. Increased production of virulence factor under microaerophillic and anaerobic conditions is attributable to increased transcriptional activation of the tcpP promoter leading to an increase in TcpP and downstream virulence genes. AphB oxidative sensing is believed to be an essential factor in oxygen-related virulence induction.

Bacteria are capable of growth in a variety of environments and have developed regulatory systems enabling them to take advantage of elements in their habitats supportive of their growth, proliferation, and survival. ArcA/ArcB is a genetic regulatory system believed to function in anaerobic metabolism. The two component ArcA/ArcB system represses a variety of aerobic enzymes under anaerobic conditions. ArcA is required for the activation of virulence gene expression in Vibrio cholerae by means of a mechanism yet to be elucidated. ArcA/ArcB does not function in AphB regulated oxygen-dependent activity.

4.11 Toxt activation of tcp gene transcription for tcp pilus synthesis

ToxT is an independent activator for the transcription of the divergently transcribed and presumably co-regulated Accessory Colonization Factor encoded *acfA* and *acfD* genes that are necessary for intestinal colonization in a process yet to be full described. *AcfA* and *acfD* respectively encode a putative outer membrane protein and a putative lipoprotein.A methyl-accepting protein (MCP) may be encoded by *tcpI* which is divergently transcribed from the *tcpPH* operon. TcpP and TcpH are necessary for ToxT expression. Tox T activates *tcpI* expression. The role of TcpI in pathogenesis is unknown. The regulatory cascade provides for the synchronous function of the ctx module in response to environmental stimuli; controlling virulence in response to factors such as temperature, pH, and concentration. This regulation involves ctx gene expression, TCP genes, and metabolic genes such as *aldA* and *tagA*. Aldehydedehydrogenase is encoded by *aldA*. A putative lipoprotein is encoded by *tagA*.ToxT independently activates the divergent transcription of *tagA and aldA* genes.
The function of *aldA* and *tagA* in Vibrio cholerae pathogenesis is unknown. *ToxR, toxS*, and *toxT* genes are part of the regulatory cascade. The *ctx* and *tcp* operons are elements in the regulon whose expression is influenced by identical environmental signals. The ToxR, ToxS, and ToxT transmembrane proteins are involved in controlling this regulon. ToxR is a regulatory protein that induces positive feedback control. ToxR interacts with ToxS sensing

environmental change signaling the chromosome inducing genetic transcription for pilus formation and attachment as well as toxin production. The ToxS periplasmic protein is involved in the response to environ- mental signals, conformation changes, and the activation of ToxR transcription of the operon.

The ToxR and ToxS regulatory system involves ToxS as the sensor protein that phosphorylates converting ToxR to the active DNA-binding configuration. The cytoplasmic protein ToxT is a transcriptional activator of the tcp operon. ToxR activates ToxT expression. ToxT activates tcp gene transcription for tcp pili synthesis.

4.12 Toxr activation of vibrio cholerae virulence gene expression in response to environmental signals, heat shock proteins, and alternative sigma factors

Expression of CT and *tcpA*, as well as other proteins, are coregulated by the *toxR* regulatory system which includes the membrane spanning *toxR* protein. ToxR is an integral transmembrane deoxyribonucleic acid-binding regulatory protein with a cytoplasmic and periplasmic domain that activates the expression of a number of virulence genes in response to appropriate environmental signals. The Vibrio cholerae ToxR gene encodes a transmembrane DNA-binding protein exerting positive control of genetic transcription for cholera toxin, TCP pili, as well as various proteins essential to cholera pathogenesis. *ToxR* alkaline phosphatase fusion proteins are capable of regulating toxin expression in response to pH, temperature and amino acids.

The amount of *toxR* messenger ribonucleic acid (mRNA) is also regulated. The heat shock protein gene G (htpG) located upstream of the *toxR* promoter is divergently transcribed from *toxR* and is homologous to the Hsp90 family of heat shock proteins (HSPs). Neither *toxR* nor *htpG* expression is regulated by ToxR. HSP loci encoding the heat shock proteins are the sitesof gene transcription on the chromosome. HSPs are molecular chaperones of antigenic peptides serving as helper proteins that aid in the proper folding of proteins and protect against cell damage expressed in response to stressful conditions such as high heat. The peptide binding activity of certain heat shock proteins occurs in a peptide binding pocket on the HSP chromosome. Regulation of ToxR expression by temperature may be under the control of Alternative Sigma Factor -32 **(**σ^{32}) RNA polymerase subunit H (RpoH) genes. Expression of the ToxR transcriptional activator of virulence factors in Vibrio cholerae is modulated by the heat shock protein response.

4.13 The role of the major histocompatibility complex, antigen presenting cells, dendritic cells, sigma factors, and the tox regulon on vibrio cholerae pathogenicity and virulence

The HSP-antigenic peptide complex is immunogenic. Major Histocompatibility Complex (MHC) antigens also referred to as Human Leukocyte Antigens (HLAs) are surface proteins specific to and located on white blood cells as well as other nucleated cells unique to each person (except identical siblings) utilized to type tissues and in the prevention of transplanted tissue redemption.

The Major Histocompatibility Complex genes determine the major histocompatibility antigens consisting of a group of multiallelic closely linked genes located in a small region in one chromosome referred to as MHC antigens or HLAs. Macrophages are derived from a particular white blood cell known as a monocyte capable of phagocytosis; a process that ingests and destroys microbes, cellular debris, and foreign matter. Antigen Presenting Cells (APCs) process and present antigens to T-cells during an immune response. APCs include B-cells, macrophages and dendritic cells (long branch-like APCs) present in the skin, mucous membranes, and lymph nodes. Dendritic cells (DCs) are immunocompetent antigen presenting cells present in peripheral tissues normally and are derived from blood marrow precursors. DCs function in the initiation of the primary immune response.

Dendritic cells recognize pathogens and present pathogen-derived peptides to T-cells. DCs are found in the germinal centers of the lymph nodes that are capable of retaining antigen-antibody complexes for long periods of time. Chemokines are responsible for DC differentiation and migration. Dendritic cells consist of long tentacle-like structures that probe the cellular environment for infectious particles presenting them to T-cells inducing an antigen-specific immune response. They are distinct from monocytes, which are mononuclear phagocytic leukocytes (white blood cells) formed in the bone marrow that are transported to tissues where they develop into macrophages. Phagocytes are cells capable of ingesting microorganisms as well as other particulate antigens. Macrophages are mononuclear phagocytes residing in tissues.

Classical dendritic cells reside in the lymph nodes. Monocyte-derived dendritic cell (Mo-DC) antibodies attach to the Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin-1 (DC-SIGN) C-type lectin particular to the Mo-DC surface. It occurs on macrophages and dendritic cells. DC-SIGN regulates adhesion processes involving DC trafficking, T-cell synapse formation, and antigen capture. Some DC-SIGN C-type lectins can conversely bind a pathogen, protect it in endosomes transporting the infectious element via DCs to lymphoid tissues enhancing infection of T-cells as is the case in the Human Immunodeficiency Virus. DC-SIGN mediates dendritic cell migration from the blood into tissues and functions in phagocytosis. DC-SIGN is a DC specific antigen-recognition receptor expressed on the dendritic cell surface. DC-SIGN is believed to mediate the endocytosis of pathogens which are then degraded in lysosomal compartments. The receptor returns to the surface of the cell membrane.

The pathogen-derived antigens are presented to T-cells by means of MHC class II proteins initiating the adaptive immune response. When monocytes are confronted with a gram-negative bacterial infection such as Escherichia coli or their cell wall lipopolysaccharide component in the blood, they migrate to lymph nodes where they develop into monocyte-derived lymph node dendritic cells stimulating T-cells to fight the infection. Thus, monocyte-derived dendritic cells are recruited from blood monocytes into the lymph nodes by lipopolysaccharide or live or dead gram-negative bacteria. The blood monocyte reservoir becomes the principle APC in response to certain microbial pathogens that yield DC-SIGN cells having essential functions for dendritic cells. The extent to which Mo-DCs may develop and function in humans is yet to be fully elucidated. Both types of dendritic cells are capable of presenting antigenic proteins as well as live gram-negative bacteria to Major Histocompatibility Complex (MHC) classes I and II.

A regulatory cascade comprising σ^{32} and σ^{70}, ToxR, ToxS, ToxT, and other putative transcriptional factors function at different stages of the infection cycle of Vibrio cholerae enabling an effective response to the host environment. Lymph is fluid contained in lymphatic vessels that flows through the lymphatic system that is returned to the blood. The lymphocytes found therein are immunologically competent white blood cells. T-cells are immunocompetent lymphocytes (white blood cells) that differentiate into helper T-cells (T_H-cells) or cytotoxic T-cells (T_C-cells) functioning in cell-mediated immunity. Helper T-cells recognize exogenous antigen fragments associated with MHC-II molecules on the APC surface. The CD (Cluster Designation) system classifies lymphocyte expressed cell-surface markers observed by computer analysis of monoclonal antibodies against human leukocyte antigens. Antibodies possessing similar specificity characteristics are grouped and assigned a number such as CD1, etc. CD numbers are applied to specific antigens recognized by different monoclonal antibody groups as well. Aided by the CD4 protein, the T_H-cell and APC interact for helper T-cell activation, and antigen recognition. Cytokines are proteinaceous cell signaling hormones stimulating or inhibiting normal cellular functions. Particular cytokines are secreted causing the proliferation and release for T-cells, B-cells and other immunologically active cells aided by the CD8 protein, that recognize antigens combined with MHC-I molecules on the surfaces of microbe infected somatic cells, some tumor cells, and transplanted tissue cells. Cytotoxic T-cell activation involves antigen presentation involving MHC-I and MHC-II molecules with their release from secondary lymphatic organs and tissues. Immunologically competent lymphocytes circulate throughout this lymphatic circulatory system to filter microbes and pathological cells whose purpose is their destruction. Cytotoxic T-cells have receptors to target, bind, and release molecules that kill a specific microbe. B-cells are immunocompetent lymphocytes that develop into a clone of antibody-producing plasma cells or memory cells initiating an immune response by remaining T-cells from previous specific antigenic exposure occurring upon stimulation by a specific antigen. Specialized APCs possess HSP-receptors that specifically take-up HSP chaperoned antigenic peptide complexes eliciting specific immune responses. Heat Shock Proteins are the most abundant soluble molecules in cells present in pathogens as well as hosts. HSP-APC interaction functions in innate immunity as well as adaptive (peptide-dependent) immunity. These proteins possess unique properties that permit their use in generating specific immune responses against infectious agents such as Vibrio cholerae.

Transcription is increased in response to higher temperatures, and this increase is accompanied by a concomitant decrease in levels of *toxR* mRNA. Vibrio cholerae entering the intestine via the stomach would encounter harsh conditions like low pH, anoxia, starvation, proteases, and bile salts that would induce a stress response that would induce the htpG gene and thus inhibit *toxR* transcription. This type of temporal control may be beneficial to the organism in preventing expression of *toxR* activated genes too early in the infection process and allowing expression of *toxR* repressed genes and other phenotypes such as motility, which are regulated in a fashion opposite to that of *toxR* activated genes. *ToxR* is present in strains of either classical or El Tor biotypes acting at the transcriptional level. *ToxR* probably increases ctx transcription by encoding a regulatory protein that interacts positively with the ctx promoter region resulting in high levels of ctx expression.

ToxR is enhanced by the transmembrane protein *toxS* which assembles or stabilizes *toxR* monomers into the dimeric form. *ToxR* regulates the expression of *ctxAB* as well as that of 17 distinct genes which constitute the *toxR* regulon. In addition to its role in Ctx gene expression, *ToxR* is necessary for the production of a pilus colonization factor and expression of the outer membrane proteins genes coded *ompT, ompU,* and *ompW* as well as three other lipoproteins. *ToxR* contains the *ompR* homologous domain required for transcriptional activation. The *ompR* family of proteins activates transcription by binding DNA and then directly interacting with the alpha subunit of RNA polymerase.

The intestinal site for growth of Vibrio cholerae is a low-iron environment which triggers the expression of iron-regulated genes in vivo. Expression of certain genes in response to low iron concentration is another distinct regulatory system in VC controlling additional putative virulence genes including hemolysins and several OMPs. Thus, there are virulence genes that are regulated by iron or *toxR*. An iron regulated OMP may be important in the colonization process.

Except for the ctxAB genes, the protein regulatory factor *toxT* regulates the expression of ctx and tcp genes being present in the cytoplasmic matrix. *ToxR* induces *toxT* controlling the transcription of the *toxT* gene which encodes a member of the AraC family of bacterial transcription activators.

The resulting increase in *toxT* expression leads to the activation of other genes on the *toxR* egulon. Vibrio cholerae has *toxT* dependent and independent branches of the *toxR* regulon. *ToxS* is a sensory membrane spanning protein that activates *toxR*. *ToxR* is at the top of the regulatory cascade that controls the expression of Ctx and other important virulence factors in Vibrio cholerae. The transcriptional activators *toxR* and *toxT* regulate the expression of *ctxA* and *ctxB* from CTX prophages. The coordinate regulation of virulence genes through the ToxR regulon demonstrates that Vibrio cholerae has developed a mechanism of transducing signals across its cell membrane and responding to its environment. Expression of Ctx, TCP and other virulence factors differs between the classical and El Tor biotypes of Vibrio cholerae.The differential expression of the *toxR* regulon, which is under the control of environmental factors, in classical and El Tor biotypes may be due to biotype-specific control over *toxT* expression. Within the folds of the protein structure of *toxT*, there are features it has in common with AraC within the structure of *toxT.* The fatty acid cis-palmitoleic acid is present which reduces TCP and Ctx expression in Vibrio cholerae preventing *toxT* from binding to DNA in vitro. This inhibition of the *toxT* regulatory molecule by cis-palmitoleic acid interferes with the regulatory cascade and would result in the expression and proliferation of the Vibrio cholerae bacteria in the intestine that produces illness. The role of bile as well as toxT as inhibitors of Vibrio cholerae expression and proliferation in the gut directly link the intestinal host environment of VC and the regulation of virulence gene expression. The possibility exists that the structure of the fatty acid may be engineered to design a molecular inhibitor of *toxT* that can be used to treat and/or prevent cholera.

4.14 Vibriophage integration into the human host genome

The VGJΦ phage is believed to facilitate the transfer of the CTXΦ and RS1 satellite phage genomes by the formation of hybrid genomes. KSF-1Φ is an infectious filamentous bacterio- phage of Vibrio cholerae that supports RS1 satellite phage morphogenesis by heterologous DNA packaging and facilitates horizontal gene transfer integrating into the host genome containing an *att*-like sequence. The KSF-1Φ and VGJΦ phages participate in the transfer of RS1 satellite phage as well as CTXΦ in a manner independent of the toxin-coregulated pilus functioning as integrative filamentous phages. Site-specific co-integration and hybrid phage genome formation are involved in the horizontal transfer of CTXΦ and RS1 by the VGJΦ phage. RS1 transfer by KSF-1Φ seemingly occurs by heterlogous packaging of excised RS1 elements into viral particles. KSF-1Φ phage mediates the transfer of its genome to recipient cells by new infectious particle formation. This form of horizontal gene transfer differs from that involving RS1 by the VGJΦ phage previously mentioned. Vibrio cholerae strains carrying the *mshA* gene encoding the mannose-sensitive hemagglutinin type IV pilus are susceptible to KSFΦ infection.The KSF-1Φ filamentous phage uses the MSHA Type IV pilus as its host cell receptor. The other Vibrio cholerae filamentous phages 493, VEJΦ, VGJΦ, VJGΦ,VSK, VSKK, fs1, and fs2 also use the MSHA Type IV pilus as their host cell receptor in the infection of recipient strains. The mosaic genomic structure of vibriophages seemingly evolved as a result of extensive horizontal exchange of genetic material among these vibriophages and possibly other yet to be identified phages. The VGJ and KSF-1Φ phages play a role in the TCP dependent horizontal transfer of CTXΦ or the RS1 satellite phage involving site-specific co-integration and formation of a hybrid phage genome. Horizontal transfer of RS1 by KSF-1Φ seemingly occurs by heterologous packaging of excised RS1 into viral particles. Phage VSK is believed to be capable of integrating into the VC chromosome at an integration site yet to be determined. VGJΦ preferentially integrates into the right junction *attB* site of previously resident CTXΦ or RS1 prophages as is the case with CTXΦ. This integration does not occur at the left junction where the *att* site is almost identical. The right junction *attB* site is a substrate for chromosomal integration in the recombination system involving XerCD recombinases. It overlaps with the *dif* site of chromosome I of Vibrio cholerae which is preserved subsequent to any new integration event with either of the aforementioned phages. XerCD recombinases resolve chromosome dimers at this *dif* site. Since the left junction is not a substrate for this recombination system, VJGΦ integration into the left *att* site is precluded. RS1Φ uses TCP and MSHA as its host cell receptor. CTXΦ uses TCP as its host cell receptor. Phage 493 has a stem loop structure that may represent a transposable element. Fs2 also possesses this stem loop characteristic. Their putative functions are under further investigation. *CtxAB* genes unique to CTXΦ are not essential to bacteriophage production.

4.15 The effect of transcriptional and quorum sensing regulators on the vibrio cholerae virulence regulon

Propagation of CTXΦ may be associated with origination of novel toxigenic Vibrio cholerae strains from nontoxigenic progenitors. The Vibrio cholerae genome contains a class of integrons, which are gene expression elements that acquire open reading frames and convert them to functional genes. This permits the bacteria to entrap genes from other microorganisms constituting a mechanism for the clustering and spread of pathogenic genes as well as genes for other biochemical functions. Toxigenic VC strains can be induced to produce extracellular CTXΦ particles. The phage can be propagated in recipient VC strains in which the CTXΦ genome either integrates chromosomally at a specific site forming stable lysogens, or is maintained extrachromosomally as a replicative form (RF) of the phage DNA. The bacteriophage uses the TCP as a receptor, and hence expression of TCP by the bacterium is a prerequisite for its susceptibility to the phage. Thus, a virulence factor for the bacterium in humans also serves as a receptor for CTXΦ, resulting in a co-evolution of genetic elements mediating the transfer of virulence genes with the pathogenic bacterial species they infect.It is possible that environmental factors induce lysogenic CTXΦ in toxigenic VC, resulting in the release of extracellular CTX particles into the aquatic environment. The cell-free phage particles participate in the emergence of new toxigenic strains of VC through interactions with nontoxigenic strains which exist in the environment and in the human population that consume the environmental waters, or have been contaminated by those waters. CTXΦ uses TCP as its receptor. Thus, the phage can only infect VC expressing TCP. The TCP genes embodied in their pathogenicity island, that may be of phage origin, appear to be the initial genetic factors required for the origination of epidemic strains. Since genes responsible for the production of TCP are carried mostly by Vibrio cholerae O1 or O139, the CTX element is found mostly in O1 and O139 Vibrio cholerae. Most non-O1 Vibrio cholerae are nontoxigenic. Vibrio cholerae strains that are
TCP positive but lack the CTX element are not frequently found in the natural environment. Since such strains may only be present in very small numbers in the environment, their conversion to toxigenicity by CTXΦ may be enriched in the gastrointestinal environment and become detectable as new strains of toxigenic VC. CTXΦ infects recipient VC strains more efficiently in the intestinal environment, where virulence factors such as TCP are adequately expressed. Thus, the natural selection and persistence of such new toxigenic strains may involve both intestinal and environmental factors, the immune status of the host population, and the antigenic properties of the new pathogenic strain. The induction of CTXΦ lysogens is probably controlled by precise environmental signals such as optimum temperature, sunlight and osmotic conditions. This may account for the observed seasonal outbreaks of cholera in regions of endemic infections, as well as the temporal changes in the properties of toxigenic Vibrio cholerae and a continued emergence of new epidemic clones that often replace existing clones.

Expression of Ctx from the RF of CTXΦ is reportedly independent of *toxR*. Thus, phage induction may be another mechanism for the regulation of Ctx production.Their

increased concentrations in the environment may lead to epidemic outbreaks of cholera. Ctx is not only subject to transcriptional control. It is also regulated by the gene amplification on the core region of DNA of the ctx operon flanked by the RS1 repeat segments. The CTX genetic element comprising the core region and the RS1 sequences result in a type of site-specific transposon where the aforementioned *zot*, *ace* and *cep* play their respective roles. For Vibrio cholerae to cause infection and disease, this bacterium must express virulence factors including Ctx and colonization factors such as TCP. Ctx and TCP expression is coordinately regulated and greatly influenced by environmental stimuli. The *toxT* molecule coordinately regulates the expression of several virulence factors of Vibrio cholerae. The membrane proteins *tcpP/H* and *toxR/S* are necessary for *toxT* transcription to take place. Transcription of Ctx and TCP genes is under the control of a regulatory cascade in which *toxR* and *tcpP* control expression of *toxT*. *ToxT* is a transcriptional activator directly controlling the expression of various virulence genes.The *ToxT* regulatory protein directly activates Ctx and *tcpA* promoters. *ToxT* gene transcription depends on the *toxR/S* and *tcpP/H* regulatory proteins. The inner membrane proteins *toxR* and *tcpP c*ontain cytoplasmic DNA-binding domains. The *ToxR* and *tcpP* periplasmic domains are believed to interact with other transmembrane regulatory proteins, whose activities are stimulated by *toxS* and *tcpH* respectively. *ToxT* is a (cytosine arabinonucleoside-like) transcriptional activator that activates transcription of various genes including the ctx and tcp operons encoding Ctx and TCP respectively. The ToxR-ToxS and TcpP-TcpH signaling circuits comprise two distinct parallel signal transduction cascades that coordinately regulate Vibrio cholerae virulence. These circuits detect and respond to environmental stimuli and influence *toxT* expression.
ToxT activates the transcription of a number of virulence genes. The downregulation of TcpP mediates the effect of LuxO on the virulence regulon. *TcpP* transcription occurs in the presence of LuxO. The presence of LuxO is not necessary for the direct activation of the tcpP promoter since *HapR* is epistatic to *luxO*. LuxO indirectly controls *tcpP* expression by repressing *hapR* expression which represses *tcpP* expression. Vibrio cholerae is a human pathogen possessing a virulence regulon consisting of genes essential to colonization, the production of toxin, as well as bacterial survival within the host. The virulence regulon is controlled by a cascade of tran- scriptional regulators including TcpP, ToxR and ToxT. The quorum sensing proteins HapR and LuxO are also involved in regulating virulence gene expression in Vibrio cholerae.

4.16 The role of endogenous bicarbonate in human small intestinal vibrio cholerae colonization and virulence

Bicarbonate functions as a physiological buffer. Vibrio cholerae colonization occurs in the upper small intestine where bicarbonate is present at high concentration. Pancreatic cells produce bicarbonate and it is secreted into the small intestinal lumen to neutralize acid arriving from the stomach. A pH gradient exists in the mucus of the human duodenum. It is believed that the bicarbonate concentration is higher close to the intestinal epithelia enabling bicarbonate to function as a second messenger for the stimulation of maximal virulence induction. Bicarbonate may function as an essential chemical stimulus sensed by Vibrio cholerae inducing virulence throughout the natural course of cholera infection. Host

infection initiates a regulatory cascade resulting in the production of the ToxT protein directly activating the transcription of genes encoding cholera toxin, the toxin co-regulated pilus, as well as other virulence genes. T*oxT* activation commences due to the inner membrane proteins pairs ToxR-ToxS and TcpP- TcpH. ToxT expression can lead to further ToxT production by activating *tcpA* operon transcription which is the location of the *toxT* gene The action of bicarbonate influencing virulence gene expression is mediated downstream from *toxT* transcription. Within the lumen of the small intestine, lower pancreatic bicarbonate levels are attributable to diffusion throughout the intestine. This lower bicarbonate concentration produces low level ToxT activity, the result being low *tcpA* expression. Later in the infection cycle the bacteria entering the mucosal layer encounter a relatively higher bicarbonate concentration secreted by the small intestinal epithelia leading to enhanced ToxT activity as well as maximized *tcpA* and cholera toxin expression. Primary *tcpA* expression may allow the bacterium to colonize the epithelial lining of the small intestine inducing *ctxAB* expression in response to a bicarbonate second signal. Expression of the principal virulence factors of Vibrio cholerae are induced by bicarbonate by means of its enhancement of activity of the ToxT protein already present in the bacterium. Bicarbonate functions as an positive effector molecule for Tox T activity inducing Vibrio cholerae virulence gene expression during infection.

4.17 The acid tolerance response and tox regulon support vibrio cholerae survival in the human host and the environment

The Acid Tolerance Response (ATR) is an adaptive stress response to the pre-exposure of Vibrio cholerae to mild acid stress. The ATR induces the production of organic Acid Shock Proteins (ASPs) essential to this process. Vibrio cholerae is capable of surviving potentially lethal low stomach pH in the area of 4.5 after such pre-exposure. This capability is believed to contribute to the fitness of pathogenic Vibrio cholerae to persist in a host as well as spread in an epidemic fashion in the environment. Cross-protection involves the ability of such acid-adapted bacteria to tolerate acid stress as well as other environmental stresses including high temperature, nutrient deprivation, and oxidative stress. The inorganic low pH ATR does not involve theToxR regulator of transcription. ToxR is required for the organic low pH plus organic acid ATR response to occur in a ToxT-independent manner. The ToxR inner membrane protein in conjunction with ToxS senses environmental signals such as amino-acid concentration, osmolarity, pH, and temperature to directly and indirectly regulate transcription of various genes on the Vibrio cholerae circular chromosome in a yet to be fully understood manner. The Tox R regulon consists of the *toxT* dependent and *toxT* independent branch. ToxR and ToxT function synergistically with homologous inner membrane signaling complex TcpP and TcpH to activate ToxT transcription. *ToxT* encodes an AraC family transcriptional activator of the Vibrio cholerae pathogenicity island. The produced ToxT autoregulates its expression, cholera toxin, the toxin coregulated pilus as well as other necessary factors of Vibrio cholerae virulence. The outer membrane porins OmpU and OmpT are from the ToxT independent branch of the ToxR regulon. *OmpU* and O*mpT* transcription are induced and repressed respectively due to their differential regulation by ToxR. It is believed that normal levels of OmpU sufficiently mediate the production of

organic ATR, not existing as an ASP in and of itself, in concert with other ToxR-independent Acid Shock Proteins. The normal metabolic activities of bacteria result in the production of organic acids. Vibrio cholerae are believed to be exposed to organic acid stress within the host small intestine as well as during their growth in the environment at large. In this context, OmpU levels are maintained providing Vibrio cholerae protection against general organic acid stress enhancing its chances for survival within the host and in the environment.

4.18 Hemagglutinins and lactoferrin in the colonization of vibrio cholerae

Hemagglutination refers to the agglutination of red blood cells that may be immune, as a result of specific antibody either for red blood cell antigens or other antigens which coat the red blood cells. This process may be non-immune as in hemagglutination caused by viruses or other microbes. The hemagglutin is an antibody or other substance that causes agglutination. Antigen-antibody reactions lead to immune complex formation. Soluble and cell associated hemagglutinins have been measured in Vibrio cholerae which play a role as nonlipopolysacch- aride (non-LPS) protective antigens. Various fimbriae, OMPs and flagellar antigens are also non-LPS antigens. Antibodies against non-LPS components of the outer membranes may block the adhesion of vibrios to the intestinal mucosa, and such antibodies have been shown to protect against challenge with Vibrio cholerae of both biotypes. Colonization factors like the mannose- fructose resistant cell-associated hemagglutinin, the mannose-sensitive hemagglutinin as well as some outer-membrane proteins may play roles increasing adhesion and colonization. One putative colonization factor in Vibrio cholerae O1 that may induce anticolonization immunity is the Mannose-Sensitive Hemagglutinin (MSHA) identified on all strains of the El Tor biotype but rarely on the classical biotype. MSHA is a virulence factor encoding a type IV pilus consisting of six open reading frames expressed on the surface of El Tor but not classical Vibrio cholerae. The MSHA subunit protein is present on Vibrio cholerae of both biotypes, whereas its activity is associated with El Tor bacteria. Another Vibrio cholerae antigen that may produce colonization and induce specific immune responses are the fructose-sensitive hemagg-lutinin associated with classical strains and the mannose-fructose resistant hemagglutin present in both biotypes. The presence of MSHA can differentiate between the presence of classical or El Tor strains of Vibrio cholerae O1. Definitive MSHA association with a toxin coregulated pilus as a colonization factor for El Tor strains has not been fully elucidated. A soluble hemagglutinin has also been characterized that is a proteolytic enzyme that digests mucin (a secreted mucopoly-saccharide) as well as fibronectin (a fibrous linking protein and reticuloendothelial mediated host defense mechanism) and nicks the A subunit of Ctx. This protein may allow detachment of vibrios after colonization or activate cholera toxin. Slime layers, which are hemagglutinins, have also been described in some vibrio strains. Lectin is a highly specific carbohydrate-binding protein derived from plants and bacteria functioning in cell to cell recognition, signaling, adhesion, and intracellular targeting of newly synthesized proteins that effects agglutination, precipitation, or other phenomena resembling the action of specific antibody. It is not an antibody in that it was not evoked by an antigenic stimulus. Cholera Lectin, a protein made by Vibrio cholerae is also a hemagglutinin, a mucinase and a protease that appears to be involved in the attachment of Vibrio cholerae to the mucosal

cells, probably by altering mucin, fibronectin, or lactoferrin. Colonization of the intestinal mucosa by Vibrio cholerae seemingly involves a lectin-receptor interaction provided by colonization pili or fimbriae. Lactoferrin is an iron binding protein found in neutrophils (an immunocompetent white blood cell) that withholds ingested iron essential to the growth and development of ingested bacteria capable of exerting a bactericidal effect. The presence of Lactoferrin in a laboratory specimen is a marker for inflammatory diarrhea. Secreted antigens such as hemagglutinin, mucinases, proteases and neuraminidase may facilitate the colonization process. This protein alone is not diarrheagenic, but may be involved in splitting the A1 subunit from the the complete Ctx molecule. However, the correlation of hemagglutins and virulence has not been firmly established.

4.19 Lipopolysaccharide functions in vibrio cholerae colonization and virulence

Vibrio cholerae lipopolysaccharide is the most important antigen in affording anti-bacterial cholera immunity. Although the LPS of VC is a virulence factor and an important protective antigen, it has not been clearly identified as an adhesive factor. Production of bacterial lipopro- teins has been shown to be associated with resistance of the bactericidal effects of complement. Complement refers to a group of over thirty serum proteins produced by the liver found in circulating blood plasma and within body tissues involved in controlling inflammation, activating phagocytes, the prevention of excessive damage to body tissues, and cytolysis.

The cytolytic lysin is a specific complement-fixing antibody acting destructively on cells and tissue according to the type of antigen stimulating its production. With the exception of Factors B, D, and P, complement proteins are designated as C1-C9 being activated when split by enzymes into active fragments a and b. Complement proteins produce a cascade of reactions that successively produce more complement producing a net amplified effect. The activation of this system is by interaction with the classical immune system. This classical pathway occurs when antibodies bind to antigens. The antigen-antibody complex binding activates C1 leading to C3 activation whose fragments initiate phagocytosis, cytolysis, and inflammation.

Inflammation is a nonspecific defensive response by the body to tissue damage caused by distortions or disturbances of cells, abrasions, chemical irritations, extreme temperatures, and pathogens. The function of inflammation is to dispose of microbes, toxins, or foreign material at the site of injury, prevent their spread to other tissues, prepare the affected tissue for repair through vasodilation and increased blood vessel permeability, and movement of phagocytes from the blood into the interstitial fluid for the restoration of tissue homeostasis. The various components of the complement system stimulate the release of histamine, attract neutrophils by chemotaxis, promote phagocytosis, and destroy bacteria. There may be bactericidal activity in the intestinal mucosa that mimics the activities of complement and antibody in serum. Evasion of this complement-like activity via production of a lipoprotein resembling TcpC may be essential in the colonization of some bacterial pathogens.

4.20 Nonspecific defense mechanisms affecting vibrio cholerae toxicity and colonization

Neither cholera vibrios nor cholera toxin penetrate beyond the intestinal epithelium during infection. Protective immunity may depend on preventive mechanisms against colonization or toxin action in the intestine. A number of nonspecific defense mechanisms may act as a first line of defense to prevent Vibrio cholerae O1 from colonizing the small intestine. These factors include the acid environment in the stomach (normally around pH2) to which vibrios are very sensitive, intestinal peristalsis effective in preventing bacterial colonization, and intestinal secretions which continually wash the intestinal surface and contain proteolytic enzymes.Other mechanisms possibly include host-derived antibiotic factors, the normal intestinal flora which competes with more pathogenic microorganisms for essential metabolites, and the mucous layer which functions as a mechanical and chemical barrier.

4.21 Immunoglobulin a in cholera immunity

Locally formed secretory immunoglobulin A (sIgA) antibodies are important for providing specific intestinal antitoxic immunity. In noninvasive enteric infections such as cholera, sIgA appears to be the main protective molecule. Intestinal sIgA is quantitatively the most important humoral immune system in the body. Systemic immunity may be involved on a limited basis since antibodies may diffuse from the circulation and act on the surface mucosa of the intestine.

Such circulating antibodies are predominantly of the IgG isotype and are very sensitive to proteolytic degradation by intestinal enzymes. Serum IgA does not appear to contribute significantly to the total IgA in secretions. Since Vibrio cholerae O1 is non-invasive, remaining confined to the lumen and intestinal epithelium of the small intestine, this circulating serum antibody probably plays a minor role in protective immunity. The resistance of sIgA to intestinal proteases makes antibodies of this isotype well suited to protect intestinal mucosal surfaces. Vibrio cholerae-specific sIgA is believed to provide substantial protection in the intestinal lumen, since neither the VC bacteria nor cholera toxin penetrate the intestinal wall. Secretory IgA functions by immuno-exclusion whereby attachment of the pathogen to the receptor is prevented, rather than by classical complement fixation. Secretory IgA may have additional functions in protecting against disease. In antibody-dependent cell mediated cytotoxicity (ADCC) a cytotoxic reaction occurs in which Fc receptor-bearing killer cells recognize target cells via specific antibodies. Such antibodies have ADCC T-lymphocyte mediated cytotoxicity in enteric infections. $CD4^+$ T-lymphocytes (T-cells) function in the development of a protective primary mucosal immune response to Ctx-induced disease. Secretory IgA has also been shown to interfere with the utilization of necessary growth factors such as iron for bacterial pathogens in the intestinal milieu. This immunoglobulin plays various roles in the maintenance of mucosal defenses, through its ability to neutralize toxins and viruses, and its ability to block bacterial adherence to surface epithelium. This may be attributable to the effectiveness of sIgA to be retained within the mucous layer over the epithelial cells after its secretion into the intestinal lumen by its interaction with cysteine (an amino acid found in most proteins) residues contained in the mucins within the glycocalyx (an outer filamentous coating of carbohydrate-rich molecules on the surface of certain cells). It is this relative juxtaposition to the intestinal epithelial cell

which is responsible for its efficient interaction with intestinal antigens. Brandtzaeg as well as Nesper et al. state that anti-LPS IgA is capable of obstructing intestinal colonization through its interference with VC flagellar or lipopolysaccharide function causing increased bacterial susceptibility to bile (World Health Organization. The Immunological Basis for Immunization Series Module 14: Cholera 2010, 11). Anti-LPS sIgA is much more effective than anti-Ctx IgA in the prevention of Vibrio cholerae induced diarrheal disease. Mosley et al., Glass et al., and Clemens et al. believe that mainly anti-LPS antibodies exert vibriocidal action and that serum vibriocidal antibody levels are the best indicators of human resistance to cholera (Ibid., 10).

A report referred to by Harris et al. states that serum-LPS and CTB-specific IgA levels are directly correlated to protection from Vibrio cholerae O1 infection, and serum anti-TCP IgA correlates with protection against VC O1 and O139 infections (Ibid., 11).

4.22 The anatomy, physiology, and immunological functions of the small intestine against invading bacteria

An important step in the pathogenesis of many microbial diseases is the penetration of the protective mucosal epithelium of the respiratory or gastrointestinal (GI) tracts. The wall of the small intestine, the site where Vibrio cholerae invades the human host, consists of the mucosa, submucosa, muscularis mucosae (smooth muscle), and serosa (visceral peritoneum surrounding the small intestine except for a part of the duodenum). These four layers compose most of the gastrointestinal tract. The epithelial layer of the small intestinal mucosa contains absorptive cells that digest and absorb nutrients in the chyme. The chyme is the semifluid mixture of partially digested food and secretions of the digestive process found in the stomach and small intestine during the digestion of food. Epithelial goblet cells secrete mucus. The deep crevices of the mucosa are lined with glandular epithelium in the form of the Crypts of Lieberkuhn that secrete the intestinal juice providing the liquid medium facilitating absorption from chyme in the small intestine along with pancreatic juice. The slight alkalinity of pancreatic juice buffers acidic gastric juice in the chyme, stops pepsin (a protein digesting enzyme) action from the stomach, as well as establishes the proper pH for digestive enzyme action in the small intestine.

Pancreatic juice enzymes consist of the starch digesting pancreatic amylase, the protein digesting enzymes trypsin, chymotrypsin, carboxypeptidase, elastase, the triglyceride (lipid) digesting enzyme pancreatic lipase in adults, as well as the nucleic acid digesting enzymes deoxyribonuclease and ribonuclease. The slightly alkaline water and mucus containing intestinal juice, in concert with pancreatic juice, provides a liquid medium aiding absorption of substances from chyme in the small intestine. Paneth cells secrete the bacterial enzyme lysozyme and are capable of phagocytosis for the regulation of the microbial population in the small intestine. The intestinal glands of the small intestine contain three kinds of enteroendocrine cells. The S cells, CCK cells, and K cells secrete the hormones secretin, cholecystokinin (CCK) and glucose-dependent insulinotropic peptide (or gastric inhibitory peptide, GIP) respectively.

Cholecystokinin stimulates gallbladder contraction. The gallbladder stores bile that emulsifies lipids prior to their digestion. Glucose-dependent insulinotropic peptide stimulates

insulin release during the digestion process. The duodenal submucosa contain Brunner's Glands that secrete an alkaline mucus for the neutralization of gastric acid in the chyme. Plicae circulars are circular folds of the mucosa and submucosa that enhance absorption by increasing surface area causing spiraling of the chime during its passage through the small intestine. Villi are finger-like projections of the mucosa increasing the surface area of the intestinal epithelium available for absorption and digestion. The lamina propria of the epithelium of the villi contain an arteriole, venule, a blood capillary network and a lacteal (lymphatic capillary). Absorbed nutrients pass through the capillary wall or a lacteal to enter the blood or lymph respectively. Microvilli are projections of the absorptive cells of the small intestine.They come together forming a brush border increasing surface area so that greater amounts of digested nutrients can diffuse into the absorptive cells over time. The brush border also contains carbohydrate, protein, and nucleotide digestive enzymes. Many organisms are able to penetrate the epithelial barrier through their selective uptake by the epithelial M cell. M cells process antigen. Peyer's Patches (Aggregated Lymphatic Follicles) are clusters of lymph nodules in the small intestines being most numerous in the ileum. A lymphocyte is a type of white blood cell functioning in cell-mediated and antibody-mediated immune responses found in blood and lymphatic tissues.

This lymph fluid flows through the system of lymphatic nodules and vessels eventually returning to the blood. The Mucosa-Associated Lymphoid Tissue (MALT) is the most extensively found human lymphoid tissue contained along the mucosal linings of the human body providing protection against numerous antigens consisting of solitary lymphatic nodules and Peyer's Patches. In the gut, MALT is referred to as Gut Associated Lymphoid Tissue (GALT) found in the tonsils, lamina propria of the gastrointestinal tract, Peyer's Patches, and appendix. Peyer's Patches are elevated oval patches of closely aggregated lymph follicles of GALT that respond to antigens that have penetrated the surface of the mucosal barrier of the small intestine. Soluble IgA can facilitate antigen uptake by Peyer's Patches. In addition to particles and organisms, some soluble antigens, such as Ctx require uptake into the Peyer's Patch in order to induce a local intestinal immune response. The uptake of an antigen into the underlying Peyer's Patch or lymphoid aggregate, has been shown to result in the stimulation of IgA secreting precursor plasma cells being mature differentiated B lymphocytes involved in antibody synthesis and secretion, which upon stimulation, migrate into the blood circulation via the mesenteric lymph nodes and lymphatics. The peritoneum is the largest serous membrane lining the abdominal cavity covering the viscera contained therein. The mesentery is the peritoneal fold attaching the small intestine to the posterior abdominal wall. During this migration these cells mature and seed to the lamina propria of a variety of mucosal surfaces where they secrete specific IgA. According to Harris et al., large numbers of IgA and IgG memory B-cells against Vibrio cholerae protein antigens such as TcpA and CTB persist in the Gut-Associated Lymphoid Tissue after one year of natural infection. By this time, the LPS-specific memory B-cell population has waned. Since the immune response against protein antigens depends on T-cells, they may participate in a more durable memory B-cell response against these antigens (Ibid., 9). Immune system responses against cholera toxin target the B-subunit (CTB). Thus, cholera toxin neutralization is provided mainly by anti-CTB. Lycke states that the cholera toxin subunit A possessses multifactorial immunomodulatory properties such as

increased antigen uptake by intestinal epithelial cells, enhanced antigen presentation by macrophages, IgA isotype switching in B-cells, increased antigen-specific CD4 T-cell priming, as well as regulatory T-cell induction chiefly contributed by its ADP-ribosylating properties (Ibid., 8).

Local intestinal immunity to cholera is believed to be essential to the prevention of disease. This may not apply to Vibrio cholerae O139 where invasive disease has been reported, probably mediated through its possession of a polysaccharide capsule. This capsule may afford Vibrio cholerae O139 the ability to bind to epithelial M Cells. Thus, it is possible that anti-capsular antibody may provide protection against systemic disease caused by Vibrio cholerae O139. Qadri et al. as well as Asaduzzaman et al. state that systemic and mucosal immune responses occur in patients infected with Vibrio cholerae O1 El Tor and O139 that are comparable in degree and prevalence to such responses observed in the presence of lipopolysaccharide and the mannose sensitive hemagglutinin (Ibid., 7)

4.23 Immunoglobulins, small intestinal defense mechanisms, and antigenic memory in vibrio cholerae

Immunoglobulins are bifunctional molecules. One region of the molecule binds to antigen. The other region possesses effector functions including binding of the immunoglobulin to host tissues, to various cells of the immune system, to some phagocytic cells, and to the first complement of the classical complement system. The complement system consists of at least twenty distinct serum proteins, their cellular receptors, and related regulatory proteins functioning as the effector of immune cytolysis as well as other biological functions including anaphylaxis, phagocytosis, opsonization, and hemolysis. IgA is the predominant immuno- globulin in seromucous secretions such as saliva, colostrum, milk, tracheobronchial and genitourinary secretions. Specific sIgA antibodies against some enteropathogens are usually found in the milk of lactating mothers. A protective effect of antitoxic as well as antibacterial antibodies in such milk against cholera is believed to occur. Symptomatic cholera has been seen in significantly lower numbers in children who drank milk that contained high levels of anti-Ctx or anti-bacterial sIgA antibodies or both than in children who drank milk with low levels of such antibodies. However, children between two and nine years of age have a relatively higher risk of Vibrio cholerae infection in endemic areas according to Glass et al. (Ibid., 5). Secretory IgA plays a role in local intestinal immunity. IgD may play a role in antigen triggered lymphocyte differentiation. IgE is found on the surface membrane of basophils and mast cells.

It also sensitizes cells on mucosal surfaces. IgE may play a role in immunity to Helminthic (intestinal permiform) parasites and is associated with allergic diseases. IgG is the major Ig innormal human serum and the major antibody of secondary immune responses and the exclusive antitoxin class. IgM is largely confined to the intravascular pool and is the predominant early antibody frequently seen in the immune response to antigenically complex infectious organisms. Specific anti-cholera antibody levels in the jejunal fluid of cholera patients consist of IgG as the predominant intestinal immunoglobulin during the early stage of the infection. Qadri et al. state that the order of response of the cholera toxin-specific

antibody secreting cells of the IgA and IgG subclasses in the circulation of convalescent patients is IgG1>IgA1>IgG2>IgA2 with
low levels of IgG3 and IgG4 ASCs. In addition, lipopolysaccharide as well as cholera toxin are immunogenic eliciting mucosal and systemic immune responses according to Svennerholm et al. (Ibid., 13). Qadri et al. further state that a comparison of Vibrio cholerae O1 and O139 immune responses demonstrated that despite the existence of a polysaccharide capsule in VC O139, comparable systemic vibriocidal and antitoxin antibodies, as well as gut-derived IgM and IgA antibody secreting cells in the peripheral blood are generated when natural infection occurs (Idid., 5). After recovery, IgA is the predominant immunoglobulin in the small intestinal secretions after cholera. No significant increase in specific serum IgA occurs. Specific intestinal IgG and IgM is believed to be a transudate from serum postrecovery. Anti-Vibrio cholerae immunity may be rendered more efficient by increased antigen presentation by various mucosal cells including enterocytes and enhanced production of sIgA according to Quiding et al. (Ibid., 9). Although the intestinal sIgA response to antigen exposure is of relatively short duration, lasting only a few weeks to a few months, this system can exhibit a potent long-lasting immunologic memory for more than a year that could rapidly and efficiently be stimulated by repeated antigen exposure. Intestinal mucosal memory cells are present. Anti-Ctx antibody secreting cells (ASCs) may exist in the peripheral blood. IgA and IgM isotypes predominate. T-lymphocyte control of anti-Ctx ASC is believed to occur. Immunological memory is seemingly induced in convalescent patients following infection or an episode of cholera contributing to long-term protection against the disease.

Gut defense mechanisms as well as serum immune responses against Vibrio cholerae infection occur as a result of immunity acquired from natural infection and cholera vaccines.
The acid barrier of the stomach can be vibriocidal to a certain extent; as well as secretory sIgA and cationic anti-microbial peptides produced by mucosal epithelial cells of the small intestine within the intestinal lumen are anti-Vibrio cholerae gut defense mechanisms. The nature of serum immune responses to Vibrio cholerae infection are the anti-CTB IgA (surrogate marker for VC O1), anti-LPS IgA surrogate marker for VCO1), anti-LPS IgM (surrogate marker for protection), anti-CTB IgG, anti-LPS IgG, anti-TcpA IgA (surrogate marker for VC O1/O139), as well as gut-derived IgM and IgA antibody secreting cells (Ibid., 6).

4.24 The immunological properties of the cytokines interleukin and interferon in cholera immunity

Cytokines are soluble molecules that mediate interactions between cells. The interleukins (IL-1 through IL-15) are a family of multifunctional cytokines produced by lymphoid and nonlymphoid cells having some effect on the lymphopoietic system involved in signaling between cells of the immune system stimulating white blood cell formation. IL-1 was mentioned previously in the context of Vibrio cholerae RTX toxin that is encoded within a cluster of genes linked to the VC prophage functioning in the host inflammatory response in innate immunity resulting in mononuclear phagocyte and endothelial cell synthesis of leukocyte-activating chemokines. T_H cells help to generate cytotoxic T-

lymphocytes and cooperate with B-lympho- cytes in the production of antibody responses. Helper cells recognize antigen in association with Class II Major Histocompatibility Complex molecules, whose products function in signaling between lymphocytes and cells expressing antigen. Interferons (IFNs) are also a group of cytokine molecules involved in signaling between cells of the immune system. Interferons stimulated by bacteria and endotoxins perform immunoregulatory functions such as inhibition of B-cell activation and antibody production, enhancement of T-cell and natural killer cell activity, as well as inhibiting the growth of non-viral parasites. Cell-mediated immune reactions such as MHC-restricted cellular cytotoxicity, natural killer cell activity, and antibody-dependent cell-mediated cytotoxicity probably play a small role in protective immunity and are of little importance in the prevention of a noninvasive infection such as cholera. However, IFN gamma (IFNγ) producing T-cells may also play a role in intestinal immune defense.

These cells are present in large numbers in the human duodenal (small intestinal) mucosa.There they may undergo a significant increase in numbers following intestinal antigenic exposure. INFγ may contribute to protection against cholera by enhancing IgA production, increasing the MHC antigen as well as secretory component receptors on the enterocyte surface, and probably allowing antigen presentation by different mucosal cells including enterocytes. It may also interfere with tight junction permeability between intestinal epithelial cells as well as with active electrolyte secretion by enterocytes, thereby preventing enterotoxin fluid secretion. Quiding et al. state that the number of gamma interferon-producing T-cells in the intestinal mucosa may increase substantially upon antigenic exposure to function in defense of the host (Ibid., 9).

4.25 The physiology of cholera diarrhea in the small intestine, Vibrio cholerae pathogenicity, virulence, and immuogenicity

Diarrhea results from a disturbance in the normal balance between absorption and secretion in the intestine. Absorption normally predominates. Diarrhea may occur when the balance is shifted towards secretion by inhibition of absorption or by stimulation of secretion. In the jejunum, salt and water absorption is stimulated by the absorption of nutrients which is largely a passive process. In the ileum (small intestine) and colon (large intestine), active transport of Na^+ and Cl^- ions is mainly responsible for dehydrating the luminal contents. Impaired absorption leading to diarrhea may occur because of the presence of non-absorbed solutes in the lumen (osmotic diarrhea), reduced jejunal permeability, impaired active transport processes, or increased intestinal motility. Diarrhea may also be produced when intestinal secretion is stimulated. Secretion results from an increase in apical membrane permeability for Cl^- ions and occurs when one or more of three intracellular second messengers such as cAMP, cyclic guanylic acid, cGMP, a major component of ribonucleic acids, and free calcium are generated in increased amounts. Each of these may be increased by a number of secretagogues, including bacterial toxins, mediators of inflammatory responses and various neuropeptides. The small intestine is the site of the most active fluid exchanges occurring in the intestine. Normally about ten to eleven liters of fluid enter the upper intestine during each twenty-four hour period, consisting of ingested fluid from the stomach, fluid secreted by the pancreas, liver, and biliary tree, as well as fluid

excreted by the small intestine itself. Nearly all of this fluid is reabsorbed in the small intestine, with only a relatively small volume reaching the large intestine,
where the remainder is absorbed. Normally about two hundred milliliters per day are excreted in fecal material. During cholera, this balanced system of fluid movement is severely disrupted, and large volumes of diarrheic fluid are excreted. Fluid loss occurs from all segments of the small intestine, mostly from the upper part. There appears to be little contribution from the salivary glands and gastric mucosa. The small intestine in human beings is normally sparsely colonized with bacteria, which are mostly of the respiratory commensal type (gram-positive cocci such as streptococci and staphylococci). Specifically lacking are gram-negative enteric organisms (such as cholera) and anaerobes (microorganisms that live and grow in the absence of free oxygen). The total concentration of organisms in the small intestine is less that ten to the power of five per milliliter of intestinal fluid or per gram of intestine. Thus, vibrios have little microbial competition preventing colonization and infection of the upper small intestine.

Vibrios surviving the passage through the stomach must colonize the small intestine in order to establish infection and disease in an infective dose greater than ten to the power of five vibrios. Vibrios possess a single polar-sheathed flagellum that facilitates their movement to the surface of enterocytes. The Vibrio cholerae flagellum is sodium driven. The aforementioned Na^+ pumping NQR enzyme complex creates a sodium motive force (smf) across the bacterial membrane that may energize flagellar rotation via translocation of sodium ions. Their darting rapid motility is not believed to be important in pathogenesis. It has been postulated that their motility may be of more importance to the survival of these organisms in the environment, where they survive well attached to water plants and shellfish. The polar flagellum of Vibrio cholerae functions in pathogen- icity as well as immunogenicity .The Vibrio cholerae polar flagellum is believed to be a virulence factor. Thus, antiflagellar antibodies may serve a protective function by preventing Vibrio cholerae colonization by a mechanism yet to be fully identified and understood according to Yancey et al. and Sinha et al. (Ibid., 7). Sodium regulation is believed to play a role in the intestine and external water sources which are the two major environments in which Vibrio cholerae may reside.

The expression of virulence factors and motility of Vibrio cholerae are believed to be linked by a mechanism that has not been fully characterized to date. Vibrio cholerae attach themselves to mucosal cells of the small intestine and proliferate to large concentrations of ten to the seventh or eighth power per milliliter. While actively colonizing the mucosa, and closely approximating themselves to enterocytes, they produce the enterotoxin responsible for the alteration of fluid transport in the small intestine. Vibrio cholera colonizes the mucosal surface of the small intestine and does not colonize enteric epithelial cells. Protection against infection by Vibrio cholerae is believed to be mediated mostly by antibodies reaching the mucosal surface. Vibrio cholerae encounters a high-viscosity environment in the intestinal mucosa during infection. The sensing of this viscosity change by Vibrio cholerae may constitute one of the biosignals that converts this bacterium from its environment to its pathogenic phase.

4.26 Vibrio cholerae viability in the gastric acid environment of the stomach

Vibrio cholerae O1 must survive the transit through the gastric acid of the stomach to colonize the intestine. Gastric acid acts as a nonspecific defense mechanism against enteric infections.Vibrio cholerae is vulnerable to the low pH of the human stomach. A highly infectious ~10^8 amount of Vibrio cholerae must be present for the onset of severe cholera. The infectious doseof Vibrio cholerae for the onset of severe infection in individuals who produce less stomach acid such as young children, the elderly, as well as consumers of antacids is ~10^4.

In a study of normochlorhydric adults, doses of up to ten to the power of eleven pathogenic Vibrio cholerae O1 given without buffer or food did not reliably cause illness. Food may protect vibrios by buffering gastric acid or by sequestering the organisms inside the ingested mass of food. It has been shown that Vibrio cholerae taken with a large volume of water may be protected from destruction by gastric acid as well. Vibrio cholerae are extremely sensitive to an acidic environment and are killed in minutes in gastric juice with pH <2.4. Hypochlorhydria (an abnomally small amount of hydrochloric acid in the stomach), which has been found in cholera patients, apparently predisposes to the development of the illness, rather than resulting from cholera. Stomach acid production may be relatively lower in normal infants than adults further predisposing them to infection. Medications that reduce acid secretion or neutralize acid such as antacids put individuals at a higher risk of enteric diseases such as cholera. The regular heavy use of cannabis is associated with lower mean basal and histamine-induced levels of gastric acid production that can result in an increased risk of cholera infection and more severe diarrhea in affected individuals. An increased risk of infection was attributed to such use in the Ganges Delta, where cholera is endemic and cannabis use is common among segments of the population. An inverse relationship has been observed in the basal levels of gastric acid and the development of clinical cholera. Gastric colonization with gram-negative organisms including Vibrio cholerae have been associated with hypochlorhydria.

4.27 Abberant electrolyte secretion mechanisms of the small intestine attributable to vibrio cholerae infection

The small intestine is the primary site of infection with Vibrio cholerae and is the source of the secretory diarrhea that is the major pathological disturbance of cholera. The major effects of Vibrio cholerae are to increase active chloride and bicarbonate secretion into the intestinal lumen by crypt cells and to decrease villous absorption of sodium chloride. Both of these effects are mediated by cholera toxin, the B subunit of which binds to receptors on the mucosal surface of the intestinal epithelium that contain the glycolipid ganglioside, and the A subunit of which enzymatically activates adenylate cyclase (enzyme acting on ATP to form cyclic AMP) and increases intracellular concentrations of cAMP. Cyclic AMP then acts as an intracellular second messenger to inhibit active sodium chloride absorption and increase chloride and bicarbonate secretion (high or low bicarbonate ion values indicate metabolic alkalosis or acidosis respectively.)

Prostaglandins (PG) are physiologically active substances that act as vasodepressors, stimulators of intestinal smooth muscle, antagonists to hormones influencing lipid metabolism and influence uterine stimulation. Naturally occurring alpha (α) prostaglandins act in the cells in which their synthesis occurs as well as in surrounding cells. Their actions and effects differ depending on their cell type, concentration and hormonal environment. They are found in intestinal fluid secretions and are in the stools of cholera patients. The increase in stool prostaglandins may be a primary event or occur in response to the dehydration and hypovolemia (a deficiency in the amount of blood in the body) seen in cholera. Since the stool concentrations of sodium and chloride are roughly proportional to their concentrations in the blood, their concentrations in plasma are usually normal or slightly decreased in the untreated cholera patient. A factor in secretory diarrhea is the Ctx induced release of amines and peptides activating the enteric neurotransmitter VIP which stimulates fluid secretion, as well as the intestinal fluid producing role of cAMP previously discussed. The increased chloride and bicarbonate secretion in crypt cells during cholera is associated with an increase in the passive transfer of water across the mucosal surface into the intestinal lumen. Water absorption, which normally occurs when NaCl is absorbed by villus cells, is also inhibited by the action of Ctx. These changes markedly increase the amounts of water and electrolytes in the lumen of the small intestine. A-B subunit cholera exotoxin causes Cl^- secretion by the crypt cells and decreased NaCl absorption by villus cells. Water exits the epithelial cells across an osmotic gradient into the lumen of the small intestine resulting in diarrhea. The profuse watery diarrhea, or “rice water stools” of cholera result. The stool, whose consistency resembles the water in which uncooked rice has been washed, has a characteristic fishy odor and may contain flecks of mucus and occassional polymorphonuclear leucocytes (white blood cells) but no red blood cells. Watery yellowish or loose stool may also occur. The rice water stool seen of patients may represent a natural form of intestinal lavage.

4.28 The large intestinal dysfunction, dehydration, hypotension, hyperglycemia , and hypoglycemia of vibrio cholerae infection

The transport functions of the colon (large intestine) vary somewhat from that of the small intestine. Unlike the small intestine, the colon does not actively absorb glucose and amino acid, nor is there any glucose and amino acid stimulated sodium absorption (except during the neonatal period). The healthy human colon absorbs water, Na^+ and Cl^- and secretes K^+ and HCO_3^-. The mechanism of colonic dysfunction in human cholera may be the result of Ctx causing an elevation of cAMP in the colonic mucosa. Cholera patients who are severely dehydrated are usually hyperglycemic. This condition is caused by elevations in the concen- trations of epinephrine (the simpathomimetic neurohormone adrenaline whose effects include vasoconstriction, vasodilation and relaxation of intestinal smooth muscle), glucagon (the polypeptide hormone whose actions include the decrease in gastric motility and gastric and pancreatic secretions as well as the urinary secretion of potassium and nitrogen), and cortisol (the hydrocortisone steroid glucocorticoid hormone) in serum. Production of these compounds is stimulated by the profound hypotension occurring in patients with severe dehydration.

These hormones have an anti-insulin effect. Children with cholera and hypoglycemia have usually been sick for twelve or more hours prior to medical treatment and usually have not eaten during that time. These children have exhausted their glycogen stores and are dependent on gluconeogenesis (glycogen formation from noncarbohydrates such as protein or fat) for maintaining their blood glucose concentrations. Neither hyper- nor hypoglycemia is specific to cholera diarrhea.

4.29 Small and large intestine dysfunction and metabolic acidosis of vibrio cholerae infection

The concentrations of potassium and bicarbonate in cholera stool are two and four fold higher, respectively, than they are in plasma as a result of the active secretion by the ileum (small intestine) and colon of K^+ and bicarbonate during cholera. An abnormally high HCO_3^- value indicates metabolic alkalosis. An abnormally low HCO_3^- value indicates metabolic acidosis. Their secretion by the colon has been attributed to the direct effect of Ctx on the colonic epithelial cells and to an increase in circulating concentrations of aldosterone by hypovolemia.This steroid hormone acts directly on colonic epithelial cells to increase bicarbonate and potassium secretion as well as sodium absorption. Despite large K^+ losses in the stool, plasma K^+ concentrations are usually normal or modestly elevated when cholera patients first present for care. This results from the accompanying acidosis, since in a homeostatic response to the acidosis, extracellular hydrogen ions are exchanged for intracellular K^+ ions. When the acidosis is corrected by the administration of bicarbonate, plasma potassium ions move back into the cells and plasma potassium ion concentrations can fall precipitously resulting in the ileal and electrocardiogram changes to be mentioned later. The most important electrolyte derangement in cholera patients is the metabolic acidosis that results from the loss of bicarbonate in the stool. This is exacerbated by lactic acid accumulation secondary to tissue hypoperfusion and anaerobic glycolysis. An increase in the relative concentrations of anionic plasma proteins and phosphate also contribute to the metabolic acidosis.

Serum calcium and magnesium concentrations are elevated in dehydrated patients with cholera. The increased concentrations of these two cations which are bound to serum proteins result from the hemoconcentration and the increase in serum protein concentration found in the dehydrated cholera patient. When the volume depletion and acidosis of severely dehydrated cholera patients are corrected, the concentration of the physiologically important ionized calcium component usually decreases, sometimes to abnormally low levels. The detection and measure-ment of the aforementioned electrolyte derangements are indicative of the severity of the particular case of cholera and can lead to the identification of other complications associated with this infection.

The metabolic acidosis of cholera results from a combination of factors including lactic acidemia, hyperphospatemia, hyperproteinemia and transient renal failure. The action of Ctx promotes secretion by the small intestinal mucosal cells of a bicarbonate-rich, protein free isotonic fluid from the extracellular body water. Thus, hyperproteinemia results from hemoconcentration of serum protein after the loss of plasma water. Lactic acidemia results

from decreased perfusion of tissues during hypovolemic shock. Hyperphosphatemia probably results from a combination of hydrolysis of cellular phosphate esters during acidosis, a shift of this organic phosphate into the extracellular fluid, and the failure of the kidney to excrete this added phosphate load. Vomiting which takes place with most cholera patients would be expected to ameliorate the metabolic acidosis through the loss of hydrogen ions. The vomit commonly associated with cholera is a clear watery alkaline fluid from the small intestine. There is little nausea and usually no blood or bile in the vomit. Severe cholera causes acidosis with relatively little change in serum chloride but an increased serum anion gap. The acidosis is more pronounced than would be expected as a result of stool losses of bicarbonate due to super- imposed lactic acidemia and renal failure.

4.30 Excessive fluid and potassium loss , hypokalemia, and renal failure attributable to dehydrating intestinal infections such as cholera

Cholera patients with severe dehydration and volume depletion have a marked decrease in renal perfusion and glomerular filtration, with a resulting decrease in or absence of urine production. The glomerulus is a loop of capillaries at the beginning of each uriniferous tubule in the kidney. The diminished glomerular filtration further exacerbates electrolyte and acid-base disturbances, as the kidney is unable to excrete excess hydrogen ion. This prerenal failure also results in increased concentrations of blood urea (the end product of nitrogen metabolism) nitrogen in serum and creatinine in urine which are normally filtered and excreted by the kidney.

Creatine is an amino acid-like molecule synthesized by the liver, kidney, and pancreas transported to muscle fibers that when relaxed produce more ATP than needed for resting metabolism. This excess ATP synthesizes energy rich creatine phosphate found exclusively in muscle fibers. The creatine phosphate level is three to six times more than that of ATP in relaxed muscle fiber. Creatine kinase catalyzes the transfer of a high energy phosphate group from ATP to creatine resulting in the formation of creatine phosphate and ADP.

Muscular contraction causes an increase in the level of ADP causing creatine kinase to catalyze the transfer of a high energy phosphate group from creatine phosphate back to ADP causing a direct phosphorylation reaction and the regeneration of new ATP molecules to energize muscular contraction. What remains of this metabolic activity of muscle is creatinine, a cyclic anhydride of creatine which is the final product of phosphocreatine decomposition, which is excreted as a component of urine. The measurement of creatinine excretion rates are diagnostic indicators of kidney function Renal failure in patients has been attributed to acute tubular necrosis caused by hypotension (abnormally low blood pressure), renal hypoperfusion (decreased blood perfusion through an organ), and to hypokalemic nephropathy which is an abnormally low blood potassium concentration attributable to factors including excessive loss of potassium by the gastrointestinal or renal route.

4.31 Pulmonary edema attributable to inadequate oral rehydration therapy in cholera patients

Pulmonary edema has been reported in cholera patients with severe acidosis who received fluids lacking bicarbonate. The persistent acidosis in those patients may have resulted in sustained peripheral vasoconstriction. Because of the peripheral vasoconstriction, a disproportionate share of administered fluid remained in the large vessels, with a resultant increase in pulmonary blood flow and central venous pressure. Pulmonary edema can also occur if the volume of fluids administered exceeds requirements. It can occur even in patients with normal cardiac reserve if the rapid rates of fluid infusion used to rehydrate cholera patients are continued after rehydration is complete. This further obviates the importance of replacement of the appropriate amounts of fluid and electrolytes upon clinical assessment of cholera within the medically indicated time frame to prevent further morbidity and mortality.

The World Health Organization introduced oral rehydration therapy globally as a cornerstone of programs for the control of diarrheal diseases in 1979. The Organization states that oral rehydration salt therapy alone can successfully treat cases of cholera occurring at the rate of eighty percent presenting with mild to moderate acute watery diarrhea. Table 3 outlines proper oral rehydration therapy for such patients.

Table 3: Principles of Fluid Therapy in Cholera Patients

1. Fluid therapy is divided into two components: rehydration of dehydrated patients and maintenance of continuing stool losses.
2. All severely dehydrated patients should be rehydrated with intravenous fluids.
3. The intravenous fluid used should have a composition similar to that of cholera stool.
4. Rehydration can be accomplished rapidly within 2 to 4 h of admission.
5. Oral rehydration solution should be used for maintenance fluid therapy in all patients except those with continued high purging rates (>10ml/kg/h) or persistent vomiting. ORT can also be used to rehydrate patients with mild or moderate dehydration.
6. Oral rehydration solution should be administered in a volume sufficient to match ongoing stool losses and insensible losses. Stool losses should be monitored, and a record should be kept of stool losses and fluids provided.
7. Patients can be discharged from the hospital (or clinic) once the rate of stool volume has moderated and they have demonstrated that they can drink sufficient oral rehydration solution to keep pace with stool water loss. For most severely dehydrated patients, this will require a minimum of 24 h from the time of admission and will rarely require more than 72 h. (Wachmuth et al. 1994, 240)

4.32 Oral rehydration and intravenous fluid replacement therapy for cholera patients

The electrolyte content of the most commonly used oral rehydration solution recommended by the World Health Organization and used in most countries contains glucose, sodium chloride, potassium chloride (KCl), bicarbonate or equivalent base such as trisodium citrate per liter of sanitary potable water. A quantity of glucose, sucrose or a cereal such as that derived from rice may also be a constituent of ORT preparations. Cereal based ORT has been shown to provide a limited number of additional calories and contribute to the decrease in the duration and volume of diarrhea stool. Home-made preparations containing

inappropriate or omitted amounts of essential electrolytes can exacerbate the clinical manifestations of cholera. Given their inadequacy for the treatment of the severe dehydration resulting from cholera, home-made preparations are not recommended by the WHO. It is important to provide nutrition appropriate to the given patient. Withholding food in order not to stress the bowel is not indicated. The volume of oral replacement solution ingested during the maintenance of therapy should be sufficient to replace normal insensible fluid losses plus continuing losses from diarrhea and vomiting. The output of stool, urine and vomit should be collected and measured in separate calibrated vessels as much as possible.

The surface of a cholera cot has a strategically placed void through which the patient's stool falls into a calibrated vessel for the clinical measurement of fluid loss for the assessment of patient dehydration. Insensible fluid loss based on body weight is calculated as being 100ml/kg for the first 10kg of body weight, 50 ml/kg of body weight for the next 10Kg, and 10ml/kg for every kg of body weight over 20kg. One kilogram equals 2.2 pounds. Proper clinical assessment of the cholera patient involves a variety of symptoms and laboratory findings.

TABLE 4: Clinical Assessment of Dehydration and Fluid Deficit (Bennish, 1994)

Clinical index	Sign/symptom/laboratory finding in:		
	Mild/no dehydration	Moderate dehydration	Severe dehydration
Cerebral status	Alert	Restless/lethargic	Apprehensive/lethargic/ Stuporous/comatose
Thirst	Present	Present	Marked
Radial pulse rate and character	Normal	Rapid	Rapid and feeble/ impalpable
Respiratory rate and character	Normal	Tachypnoeic	Tachynopeic, deep, laboured
Elasticity of subcutaneous tissues (determined by skin pinch)	Immediate retraction	Slow retraction	Very slow retraction
Eyes	Normal	Sunken	Dramatically sunken
Urine flow	Normal	Scant/dark	Scant/absent
Serum specific gravity*	<1.027	1.028-1.034	>1.034
Approximate fluid deficit (mg/kg body weight)		[illegible]	>90 [illegible]
Preferred method of rehydration	ORT	ORT or IV, depending on presence of	IV

	vomiting and rate of continued faecal loss

*Patients with malnutrition often have a lower baseline specific gravity; therefore, the listed values may not be applicable. ORT, oral rehydration therapy, IV, intravenous.
(Drasar and Forest 1996, 82)

Twenty percent of patients present with severely dehydrating cases of cholera requiring rehydration with an intravenous solution.. The polyelectrolyte intravenous (IV) solution manufactured to the standard of the WHO has the identical constituents as that of its recommended oral rehydration solution. Intravenous Ringer's Lactate, or in its absence normal saline, as well as oral rehydration salts for cases of severe cholera dehydration may be used. After IV therapy, the administration of oral rehydration therapy (ORT) is used as long as clinically indicated. "The adequacy of fluid replacement can be readily assessed clinically (Nalin and Morris, 1991, Mahalanabis et al, 1992 Greenough; 1995):

(a) return of the rapid pulse to normal strength and rate; <90/min;
(b) return of skin turgor to normal; if this normalizes while a rapid pulse rate continues, other causes of shock, e.g. sepsis and cardiac infarction, should be sought;
(c) the patient feels comfortable and cyanosis, muscular cramps, nausea and vomiting have resolved; children who are initially drowsy or stuporous may not become fully alert for 12-18 hours, despite adequate hydration;
(d) return of normal 'fullness' of the jugular vein(s);
(e) satisfactory weight gain; a severely dehydrated patient should gain 8-10% body weight following rehydration; and
(f) return of urinary output; in a severe case, this usually occurs within 12-20 hours after initiating rehydration". (Drasar and Forest 1996, 83)

Although not essential to successful cholera therapy, antibiotics are capable of reducing disease duration, the volume of required rehydration fluids, as well as the shedding of Vibrio cholerae into the environment by patients. Antibiotic therapy will be discussed further in a subsequent section of this text.

4.33 Cardiac dysfunction attributable to the hypokalemia, hypoglycemia, and acidosis of severe cholera

Severe cholera can lead to abnormal and arrhythmic cardiac function. Abnormalities may occur due to excess loss of potassium and bicarbonate ions in the stool resulting in hypokalemia and acidosis. Sinus tachycardia (rapid heart beat) may be present. Hypokalemic arrhythmia and focal myocarditis (inflammation of the muscular walls of the heart) may develop and lead to sudden death during dehydration. Patients with severe uncorrected hypoglycemia and acidosis may develop fatal cardiac arrhythmias. Timely and proper fluid and electrolyte replacement therapy can prevent these complications.

4.34 Paralytic ileus attributable to cholera sicca

Paralytic ileus (Adynamic Ileus) is the nonmechanical obstruction of the small intestine from the jejunum to the caecum caused by the inhibition of intestinal motility affecting the intestinal wall attributable to a variety of causes including cholera. In the rare condition of Cholera Sicca (Dry Cholera), the intestinal secretions induced by Vibrio cholerae obstruct the markedly distended colon due to the pooling of fluid in this portion of the large intestine. This "dry" form of cholera produces no diarrhea. These conditions can occur if the loss of potassium ions in the stool is not replaced in a timely and appropriate fashion. Adequate treatment results in a resolution of the abdominal distension and no residual effects have been observed.

4.35 Grand mal seizures attributable to hypoglycemia resulting from cholera dehydration

Grand mal type seizures of unknown cause may occur in children before or during therapy, rarely occurring in adults. The seizures are usually associated with hypoglycemia and is treated with IV glucose and low doses of Diazepam or Phenobarbitone (sedatives). Seizures are a poor prognostic sign, sometimes leading to coma and death.

4.36 Maternal and fetal morbidity and mortality attributable to cholera

Pregnant mothers with cholera run a fifty percent risk of fetal death of unknown cause during the third trimester, even in woman who receive adequate rehydration. Most fetal deaths usually occur within twenty-four hours of the disease. Placental ischemia (local anemia due to mechanical obstruction of the blood supply) and hypoxia resulting from poor visceral perfusion may play a role. Placental retention after abortion is also a complication of severe cholera during pregnancy. Although pregnant women have historically had a much higher mortality rate from cholera than women who were not pregnant, current fluid replacement therapy has reduced mortality in pregnant women to approximately the same level as that of the general population affected by cholera.

4.37 Bacteremia attributable to intravenous rehydration therapy

Vibrio cholerae O1 bacteremia is rare. When it does occur, it is often the result of nosocomial infection caused by bacterial contamination of the intravenous infusate or colonization of the catheter used for infusion. The responsible organisms are the usual cause of IV-infusion related sepsis, including non-glucose fermenting gram-negative rods and Staphylococcus Aureus. Sepsis can be prevented if a given portion of intravenous solution is only used for a single patient and if IV catheters are removed as soon as they are no longer needed. In an effort to conserve supplies and limit expenditures, personnel in treatment centers that are inadequately funded and equipped may unwisely save partially used IV solutions and reuse IV tubes, needles, and catheters that may not be adequately sterilized. Such practices are not recommended since sepsis can result.

CHAPTER FIVE: ANTIMICROBIAL THERAPY FOR VIBRIO CHOLERAE INFECTION

5.1 The effect of antimicrobial therapy on the duration and severity of cholera symptoms, oral and intravenous rehydration therapy in the context of antimicrobial therapy, the antimicrobial therapies of choice for adults and children, supplemental zinc therapy for seroconversion of vibriocidal antibody in children and the reduction of duration and occurance of symptomatic cholera diarrhea, mass chemoprophylaxis and routine antibiotic treatment of cholera patients

The purpose of antibiotic usage in the treatment of clinical cholera is to decrease the volume and duration of diarrhea, since these agents are not in themselves life-saving. Antimicrobial therapy can reduce the volume of cholera stool purged during illness by half, as well as shorten the duration of symptoms and the duration of excretion of Vibrio cholerae by the same amount. Cholera patients who do not receive this therapy will usually have diarrhea that continues from four to six days. Diarrhea persists for two to three days for patients who have received antimicrobial therapy. If an inexpensive agent is used, antimicrobial treatment of cholera patients is extremely cost-effective, as it reduces the duration of hospitalization in addition to reducing the volume of IV and ORT fluids required for rehydration. These are important considerations because cholera often occurs in locations where adequately staffed and supplied medical treatment facilities do not exist. Antibiotics should be administered orally, after completing the initial phase of the rehydration regimen, approximately four to six hours after the initiation of therapy in a severely dehydrated patient. Three hundred milligrams of Doxycycline administered orally as a single adult dose is the antibiotic of choice in most cases.

Doxycycline is the first-line drug of choice recommended by the World Health Organization. It is as effective as longer courses of therapy in reducing stool volume in cholera patients. Erythromycin is the WHO recommended drug of choice for children. Orally administered Tetracycline is administered as a second choice in children. Tetracycline is contraindicated in children less than eight years old because it can cause permanent staining of the teeth; although this would probably not occur during the commonly short course of treatment for cholera. Supplementary administration of Zinc has been found to be effective in reducing the duration of cholera diarrhea as well as the number of successive occurrences of diarrhea in children.

The daily oral administration of ten milligrams of Zinc for two weeks is recommended for affected children below the age of six months. The recommended administration of Zinc for children between the ages of six months and twelve years is twenty milligrams daily for two weeks as long as the cholera diarrhea occurs. (Prevention and Control of Cholera Outbreaks: WHO Policy and Recommendations 2008, 3, and Haiti Training Manual: a Full Course for Healthcare Providers 2011, 16). Zinc supplementation enhances seroconversion to vibriocidal antibody in children to which oral cholera vaccine has been administered. Although Vitamin A is also believed to decrease the duration and severity of cholera diarrhea in this manner as a subject of further study, it does not have the same

effect as Zinc in the context of oral cholera vaccination in children. Such micronutrients contribute to immunocompetence. Short-course antibiotic regimens have consistently been less efficacious than three or five day courses of therapy in eradicating Vibrio cholerae O1 from the stool. During an outbreak, representative patient samples should be tested for antibiotic sensitivity to enable the selection of the most appropriate antibiotic agent based on actual local sensitivity patterns for treatment purposes. An example of this concept is that in the event of outbreaks attributable to Tetracycline resistant strains, other clinically effective antibiotics such as Ciprofloxacin, Azithromycin, Erythromycin, Cotrimoxazole, Chloramphenicol, and Furazolidone are alternative choices for treatment. Depending on the type of patient involved, the antibiotic treatment guidelines for cholera published by the different international health organizations will vary. Studies comparing treatment outcomes for cholera patients administered IV fluids and antibiotics with those receiving solely IV fluids have shown that antibiotics reduced stool output by 8-92%, diarrhea duration by 50-56%, and duration of positive VC bacterial culture by 26-83% (Centers for Disease control and Prevention. Recommendations for the Use of Antibiotics for the Treatment of Cholera 2013, 92). When compared to three to five day treatment regimens, the lower cost and greater logistical ease of administration of short-course antimicrobial regimens outweigh the disadvantage of slower eradication of Vibrio cholerae from the stool. Although providing this therapy to community contacts of patients with cholera has been shown to reduce its incidence, simply reducing the duration of excretion of VC in patients who come to medical facilities for care is unlikely to have much impact on transmission of the disease within the community. Even if all patients with cholera who came to a medical facility were treated with an antimicrobial agent, there would be a large environmental reservoir of Vibrio cholerae as well as many asymptomatic or mildly symptomatic people who are excreting the organism and never seek medical attention. Although it is uncommon, some infected individuals continue to shed VC for months without symptoms. Shortening the duration of excretion of Vibrio cholerae in hospital patients may affect transmission within hospitals since nosocomial outbreaks have been reported. Mass chemoprophylaxis has been found to have no effect on the spread of cholera. Routine antibiotic treatment of individuals en mass can increase antimicrobial resistance and result in a false sense of security within susceptible communities.

5.2 The genetics and mechanisms of antimicrobial resistance in vibrio cholerae

Antimicrobial therapy of cholera is complicated by the increase in multiply antibiotic resistant strains of all of the first-line antimicrobial agents. The monitoring of antibiotic susceptibility by means of regular testing of sample VC isolates from various geographic areas during cholera outbreaks or epidemics should occur. Although Vibrio cholerae O1 and O139 do not have a tendency to accrue resistance to antibiotics, they exhibit spatial and temporal fluctuations. Periods of antibiotic resistance fluctuate with periods of sensitivity reflective of the overuse of antibiotics in a given region. Since the risk of cholera in an endemic area extends over a prolonged period of time, antibiotic chemoprophylaxis is impracticable and not cost- effective resulting in the emergence of antibiotic resistant strains. Strategies of control that put intense selective pressure on Vibrio cholerae during an ongoing

epidemic may lead to genetic changes to the microorganism. Widespread antibiotic resistance in Vibrio cholerae was not commonly known prior to 1977. Resistance has been observed to be mediated via conjugative plasmids (an extra-chromosomal element that can effect its own intercellular transfer by exchanging genetic material). Conjugated plasmids and transposons as well as Integrons (a group of chromosomal elements incorporating open reading frames or gene cassettes converting them to functional genes) are vehicles of acquisition of antimicrobial resistance genes enabling the intracellular movement of genetic determinants of resistance to antibiotic agents. Although integrons are inherently mobile, they are found within conjugative plasmids which assure their mobility. Glass et al. described a 1979 outbreak of cholera in Matlab, Bangladesh caused by a VC strain carrying a multiple-drug-resistant plasmid transferable via conjugation with other bacteria that conferred resistance to various antibiotics (Kitaoka 2011, 343).

This multiple drug-resistant Vibrio cholerae strain disappeared from this area within a decade according to Faruque et al. (Ibid.). The spread of antibiotic-resistant Vibrio cholerae strains involves horizontal gene transfer by means of self-transmissible mobile genetic elements such as the SXT mobile DNA element from the class of Integrative Conjugating Elements (ICEs). ICE exchange between two bacteria involves conjugation. ICEs integrate into the chromosome for propagation to occur because they are not capable of replicating autonomously. Such horizontal dissemination of SXT-encoded antibiotic resistance genes involves regulation by the bacterial SOS response. Stress alleviates the SetR Sxt-encoded repressor activating excision and conjugation of the element. Antimicrobials are capable of promoting the proliferation of antibiotic resistance genes playing a role in the spread of SXT elements in the Vibrio cholerae population.

5.3 The emergence of antibiotic resistance in vibrio cholerae: genetics and mechanisms of dissemination

The SOS response is a cellular stress response to DNA damage involving stoppage of cell replication at its fork, mutagenesis, and DNA repair. The response is activated by bacteria significantly exposed to agents of DNA damage. The SOS response is a regulon controlling the expression of various genes simultaneously distributed throughout the genome. This SOS regulon is primarily controlled by the gene product of *LexA* which regulates its own expression as well as that of the other proteins comprising the regulon. The *LexA* repressor enzyme and the *RecA* inducer are essential proteins in this response. The *RecA* inducer protein mediates recombination between DNA molecules. When a bacterium becomes stressed, the *RecA* protein changes its activity becoming a specific protease. The *LexA* repressor protein is the primary target of the proteolytic *RecA* protein.

The *LexA* protein represses gene expression. *LexA* cleavage removes the repression allowing gene expression. The *RecA* protein cleaves the λ phage repressor protein resulting in Promoter (P) function allowing for gene transcription. λ phages occur in the gram negative Escherichia coli enterobacterium having some similarity to the gram negative Vibrio cholerae enterobac- terium. Normal growth involves *LexA* binding to the promoter gene sequence of the SOS box preventing SOS gene expression. A cell can sense when DNA damage occurs causing the *LexA* repressor gene to self-cleave depressing the

prevention of SOS gene expression. The *RecA* filament binds single-stranded DNA promoting *LexA* cleavage inducing the SOS response. Upon completion of DNA repair, the inducing signal will no longer occur allowing *LexA* to accumulate repressing the SOS genes once again since the DNA damage has been remediated allowing for cell growth at the bacterial replication fork. Thus, *RecA* participates in recombination and repair and stimulates the auto-proteolysis of the gene product of *LexA. LexA* cleaves itself in the presence of bound *RecA* causing cellular levels of *LexA* to decrease resulting in the induction or coordinate derepression of the SOS regulon genes. As the damage is repaired *RecA* releases DNA in an unbound form no longer causing *LexA* auto-proteolysis. Cellular *LexA* levels increase to normal once again shutting down the expression of SOS regulon genes. This is referred to as Error-prone Translation DNA Synthesis because there can be a notable degree of DNA repair inaccuracy and some mutation inherent in this process. The CONSTIN (SXT Element) is an acronym for a Conjugal, Self-Transmissible, Integrating Element. Waldor et al. state that the SXT element was initially noticed in Vibrio cholerae O139 given present genes conferring host bacterial resistance to sulfamethazole, trimethoprim, and streptomycin (Kitaoka 2011, 392). Hochhut et al. as well as Burrus et al. state that many Vibrio cholerae O1 and O139 strains isolated throughout the world have acquired SXT elements due to natural spread (Ibid., 392). The conjugal SXT element encodes an integrase similar to the lamboid (λ) family of site-specific recombinases of lamboid phages. Its recombinative excision and integration mechanisms involving the Xis directionality factor and the Int integrase bear more resemblance to lamboid phages than to those of transposons. The SXT constin forms an extrachromosomal circular element. Recombination takes place between local identity attachment sites on the *attP* and the *attB* element during integration as well as between *attL* and *attR* during excision in a process independent of homologous recombination. Such excision and integration requires a functional SXT-encoded *int* gene. Integrons possess the "*intl*" integrase gene, the "*attl*" attachment site into which resistance genes are inserted, as well as a promoter sequence allowing resistance gene expression by means of cassette-associated genes not having promoters. Insertion of a gene or reading frame is a site-specific recombination mediated by the enzyme integrase encoded by the recipient. The integrase mediates integration and excision of the SXT element. It is necessary for the SXT element encoded *int* gene to be expressed in the donor as well as in the recipient for conjugal transfer of this element in a process in which excision occurs prior to conjugal transfer. The SXT element *int* is believed to not be transferred during conjugation with the SXT element DNA, being expressed in recipient cells mediating integration of the element into the chromosome. Hochhut and Waldor state that SXT elements integrate into the 5' end of *prfC* which is a chromosomal gene encoding Peptide Chain Release Factor 3, and that site-specific recombination between the circular ICE *attP* and the almost identical *attB* chromosomal sequence occurs via the SXT-encoded integrase family tyrosine recombinase Int (Ibid., 342). The SXT integrated element replicates with the host chromosome. Subsequent conjugation that occurs with other bacteria occurs by means of interbacterial translocation encoded by SXT element genes capable of mediating gene transfer to various bacterial species. Daccord et al. as well as Hochhut et al.state that in addition to the conferral of antibiotic resistance, SXT elements are capable of mobilizing

conjugative plasmids and genomic islands s*in trans* resulting in alternative antibiotic resistance gene transfer mechanisms (Ibid., 342).

Antibiotic resistance gene dissemination also occurs if Vibrio cholerae share mobile integrons with other bacterial cells. Beaber et al. as well as Guerin et al. state that the recombination of integrons such as SXT elements is stimulated by the bacterial SOS response triggered by a variety of antibiotics (Ibid., 343). Class I integrons as well as SXT constins have been associated with the spread of antibiotic resistance genes. Integrons function in genetic assembly incorporating open reading frames such as antibiotic resistance gene cassettes by means of site-specific recombination proximal to a promoter driving their expression. Integron-infused gene cassettes possess a central reading frame flanked on each side by imperfect repeat elements. The boundaries of integrated gene cassettes are genetic sequences referred to as core sites displaying the amino acid sequence Glycine, Threonine, Threonine, Arginine, Arginine, Tyrosine(GTTRRY) and are targets of recombination. Super Integrons (SIs) consist of large islands of gene cassettes in many species of bacteria including the Vibrio cholerae genome. All SIs possess the same integrase gene (*int*), *attI* site, and numerous gene cassettes. The integrase gene at the 5' end of the SI oppositely orientated to the gene cassettes possesses a potent promoter for their expression. Novel gene cassettes are integrated into the SI at the *attI* site found between the *int* gene and the first gene cassette. Gene cassettes have a gene with an imperfect 3' end direct repeat. Direct repeats are 59-base elements containing the GTTRRY core site which is an *attC* recognition site for SI integrase. Subsequent to integration into the SI the gene cassette, by imperfect direct repeats, has a core site on either side of the gene. Recombination occurs at the core sites. Gene cassettes are initially added to the SI at the *attI* site and can be excised for any location at available *attC* sites. Excised gene cassettes can be reinserted at the *attI* site changing their place in the gene array and the likelihood the gene may be expressed. Vibrio cholerae repeat sequences referred to as VCR clusters, present in a broad range of VC serotypes from geographically and temporally distinct isolates as an ubiquitous element, consist of integrase genes similar in sequence to integrons associated with drug resistance. This is suggestive of gene acquisition prior to Vibrio cholerae speciation. Non-pathogenic Vibrio cholerae serotypes also possess this integron.

Chromosomal integrons may act as large gene pools for horizontal gene transfer among members of the Vibrio genus. This chromosomal super integron of Vibrio cholerae may provide for the acquisition of new genes facilitating the adaptation of the VC bacterium to novel environments as well as its evolution and proliferation as a pathogen. Genome sequencing of Vibrio cholerae has confirmed the presence of integrons. All Vibrio cholerae isolates possess large chromosomal integrons for the quick transfer of gene cassettes having antibiotic resistance genes. Vibrio cholerae in the clinical and environmental setting may also possess mobile integrons consisting of smaller gene cassettes in mobile elements such as conjugative plasmids and transposons capable of horizontal gene transfer.

The Vibrio cholerae genome was found to be bi-chromosomal consisting of one larger and another smaller chromosomal replicon. The larger Chromosome I consists of most of the genes of cell division, transcription, and translation. The major elements associated with pathogenicity such as the VPI and CTX element are located on the larger chromosome. The integron island islocated on the smaller Chromosome II, also consisting of genes associated

with plasmids. The smaller chromosome also contains most of the hypothetical genes currently under investigation whose functions are not yet thoroughly understood. Over one hundred reading frames have duplicate copies in the larger and smaller chromosomes. There are more larger than smaller replicons in the bacterial genome. The Vibrio cholerae genome possesses a large varietyof genes encoding proteins that have a wide range of substrate specificities. Their existence may confer an evolutionary advantage upon the Vibrio cholerae bacterium given the different environments of the human intestines and estuarine habitats in which it resides. Each replicon contains unique genes. All representative Vibrio cholerae serotypes contain both replicons. VC O139 strains emerging in 1992 carried a conjugative self-transmissible chromosomally integrating SXT (constin) element that encodes resistance to Sulphamethoxazole, Trimethoprin, Chloramphenicol and Streptomycin. The antibiotic-resistance genes are in the SXT family of constins. So called empty (group D) SXT constins can insert antibiotic resistance genes as well as other Vibrio cholerae virulence factors transferable to other strains by conjugation. Multiple-antibiotic resistance occurs in Vibrio cholerae upon its acquisition of R plasmids belonging to conjugative group C. SXT constins as well as class I integrons have been associated with the spread of genetic determinants of resistance to antimicrobial agents. Nine distinct classes of integrons have been identified according to the sequences of their integrases most commonly detected in integrase class I clinical isolates. Integrons are found within conjugative plasmids that assure their mobility since they are not inherently mobile. Vibrio cholerae O1 as a Multiple Antibiotic Resistant Virus (MARV) became a major concern first in Tanzania and subsequently in Bangladesh. Strains resistant to antibiotics including Tetracycline, Ampicillin, Kanamycin, Streptomycin, Sulphonamides, Trimethoprin, and Gentamycin have been observed in several cholera endemic countries in the past twenty years. Fluroquinolone resistant strains of VC have been reported in India. However, most isolates of VC O139 remain susceptible to these agents. Resistant strains invariably disappear with time and are replaced with sensitive strains. In practice, suspicion of antimicrobial resistance usually occurs based on observations indicating an impaired clinical response. An episome is an accessory extrachromosomal replicative genetic element existing autonomously or integrated with the bacterial chromosome functioning as a plasmid. The development of episomally propagated antibiotic resistance has occurred in various Vibrio cholerae strains. Given the potential for environmental VC strains to develop resistance to therapeutic antibiotics attributable to overuse, such therapy is best reserved for the treatment of severe cases of cholera in an attempt to limit the antibiotic drug content of cholera diarrhea discharged into the environment resulting in antimicrobial resistance.

Efflux pumps are transport proteins functioning in the extrusion of toxic substances including antibiotics from cells found in gram positive and gram negative bacteria as well as eukaryotes. Multidrug efflux pumps are utilized by Vibrio cholerae to export a broad array of chemically and structurally diverse antibiotics. The ABC family of Vibrio cholerae efflux pumps involves ATP hydrolysis as an energy source. The other Vibrio cholerae group of efflux pumps involves an energy source referred to as the Proton Motive Force (PMF) of transmembrane H^+ or Na^+ gradients. PMF pump systems include Multidrug and Toxic Compound Extrusion (MATE), Resistance Nodulation Cell Division (RND), as well as the Major Facilitator Superfamily (MFS).

VcaM is a Vibrio cholerae bacteria ATP-driven ABC family(ATP-binding cassette) multidrug resistance efflux pump conferring antimicrobial resistance to structurally diverse antibiotics. Vibrio cholerae utilizes the MATE-family efflux systems VcmA, VcmB, VcmD, VcmH, VcmN, and VcrM described by Begum et al., and Huda et al. (as cited in Kitaoka, 2011, p. 400). Colmer at al. and Wooley et al. state that Vibrio cholerae MFS transporters consist of VC efflux
system substances such as those conferring resistance to bile, antibiotics, and certain proton gradient-uncoupling agents (Ibid., 402). Van Bambeke et al. noted that Vibrio cholerae RND efflux systems are encoded by the *vexRAB, vexCD, vexEF, vexGH,* vexIJK, and *vexLM* operons exhibiting substrate specificity (as cited in Kitaoka, 2011, p.401). Vibrio cholerae RND systems function in the efflux of various compounds including bile salts and certain antibiotics as well as in bacterial colonization. Bacterial efflux pumps participate in antibiotic drug resistance as well as in the expression of essential Vibrio cholerae virulence genes. It is believed that bacterial efflux pumps evolved as a mechanism for the expulsion of noxious substances such as antibiotics from bacteria in order to enhance their capability to survive. The intrinsic resistance of many gram negative bacteria to certain antibiotics is attributable to the corresponding efflux pump. VceCAB is a multiple-drug resistance (MDR) efflux operon containing *vceC* which codes for the Vibrio cholerae VceAB MDR efflux pump. *VceR* codes for the transcriptional autoregulatory protein that negatively regulates expression of the *VceCAB* operon and is modulated by MDR efflux pump substrates.

The discovery of substances that would inhibit the efflux of antibiotics from bacterial cells while not diminishing their efficacy would diminish antibiotic resistance and be of therapeutic value to infected patients. Although efflux pump encoding genes occur on plasmids, the presence of these genes on the chromosome provides bacteria with a mechanism to intrinsically survive in the presence of antibiotics enabling mutant bacteria over-expressing such genes to be selected absent of the acquisition of new genetic material. Research into spontaneous mutations of genes encoding antibiotic target site proteins may result in the discovery of efflux pump inhibitors usable in association with substrate antibiotics capable of sufficiently mitigating resistance to antibiotics used for cholera therapy.

5.4 Conceptual and actual drug mechanisms for the inhibition of vibrio cholerae pathogenesis

The discovery of an effective drug for the inhibition of the aforementioned Cystic Fibrosis Transmembrane Conductance Regulator in bacterial epithelial cells lining the small intestine, which is the molecular target of cholera toxin, would serve as a possible treatment option for the massive fluid efflux into the intestinal lumen resulting in the rapid fluid loss characteristic of cholera diarrhea. Hung et al. stated that the development of an effective drug that would inhibit Vibrio cholerae virulence mechanisms such as the transcriptional activators for cholera toxin synthesis ToxR, ToxT, and TcpP, as well as the toxin coregulated pilus would serve as another treatment for cholera (Ibid., 404).

Inherent in the aforementioned antibiotics for which resistance has developed in various Vibrio cholerae strains there is a characteristic antimicrobial mode of action that

interferes with their maturation and pathogenicity according to Katzung et al in the examples discussed in this paragraph (Ibid., 399). Ampicillin is a β-Lactam antibiotic that inhibits bacterial cell growth. Tetracycline inhibits protein synthesis in Vibrio cholerae cells. Sulfonamide and Trimethoprim inhibit the folic acid metabolism of Vibrio cholerae cells. Fluroquinolones inhibit DNA replication.

5.5 Pharmacologically active agents in the treatment of cholera

No antisecretory or antidiarrheal agent has proved efficacious in the clinical management of cholera. This applies to Kaolin, Pectin, Bismuth Subsalicylate and charcoal (which can inhibit the effects of Tetracycline). Opiate and opiate-like compounds have been shown to be useless and may harm children. Antiperistaltic agents such as Imodium or Lomotil are not clinically useful. Anti-inflammatory agents such as Asprin, Indomethacin, Chlorpromazine, and Nicotinic

Acid have shown no appreciable quantifiable benefit. The anti-inflammatory agent Berberine, a plant alkaloid made from extracts of the Berberry bush (Berberis Aristata), is used in Ayurvedic medicine in India as well as traditional Chinese medicine to treat diarrhea. Alkaloids are nitrogenous basic substances in plants; many of which are pharmacologically active. Berberine, which is marketed as a pharmaceutical agent in India, has been found to specifically inhibit the action of Vibrio cholerae enterotoxin in animal models upon previous or simultaneous administration. Berberine reportedly inhibited the secretory response induced by Vibrio cholerae even when administered after Ctx had bound to the intestinal mucosa. (Sack and Froehlich 1982, 471).

The intestinal secretory response induced by Vibrio cholerae enterotoxin acts through the stimulation of adenylate cyclase. The inhibition of secretion by Berberine occurs before or after Ctx is bound to the intestinal mucosa. No structural damage to the intestinal mucosa by Ctx was observed. Theoretically, Berberine may act at a biochemical point after adenylate cyclase activation or it may function nonspecifically to enhance intestinal absorption. Berberine has been observed to function beneficially to reduce the severity of cholera diarrhea somewhat without any reported side effects in humans. According to Rabbani et al., Berberine and Clonidine did not result in a significant reduction in fluid secretion; the same result applies to Aspirin and Somatostatin as stated respectively by Islam et al. and Molla et al. Van Loon et al. as well as Rabbani et al. who observed that Indomethacin and Nicotinic Acid reduced intestinal fluid secretion (Kitaoka 2011, 404). The exact mechanism of action of Berberine as well as its actual effect as an antidiarrheal medication has yet to be fully quantified.

The antihypertensive agents Clonidine and Somatostatin have not proven to be useful in routine cholera therapy

Chapter six:Vaccination and immunization

6.1 Vaccination and the protective anamnestic immune response

Vaccination is the injection of a live attenuated variant strain of a pathogenic virus modified to excite the production of protective antibodies yet not producing the specific disease, or a killed culture of a specific bacteria, virus or ricketsiae as a means of prophylaxis or cure of the disease caused by that microoganism. Vaccinations should be safe to administer, induce the right type of adaptive immunity, and be affordable and accessible to the targeted population. The stimulation of antibacterial and antitoxic immunity are goals in vaccine development. A synergistic effect resulting from the interplay of antibacterial and antitoxic immune responses may occur.

The antigen(s) of a vaccine must induce clonal expansion in specific T and/or B lymphocytes, leaving behind a population of memory cells. Memory cells must enable the next encounter with the same antigen to induce a secondary anamnestic (memory) response which is more rapid and effective than the normal primary response. Antigen-specific memory involves a more rapid response and enhanced antigen titers upon secondary antigenic exposure. The primary immune response often occurs too slowly to prevent serious disease. Responses by memory T-cells develop at least seven days subsequent to Vibrio cholerae infection in human beings at a time before and concurrent with the development of B-cell responses. Thus, T-cell responses to Vibrio cholerae antigens are believed to be essential for generating and stabilizing memory B-cell responses. Maruyama et al. state that protective memory against Vibrio cholerae is dictated by follicular B-cells in a process requiring help by T-cells (World Health Organization.The Immunological Basis of Cholera Module 14: Cholera, 2010, 12). The main purpose ofvaccination is to generate long-term immunological memory.

6.2 Orally administered cholera vaccines: mechanisms of action and protective immunity

Since Vibrio cholerae infects the intestinal mucosa, orally administered cholera vaccines are believed to result in more protective mucosal immune responses against cholera. Oral immunization provides for the most effective induction of immunity in immunologically naïve populations. One commercially available orally administered cholera vaccine is the killed whole cell-cholera toxin recombinant B subunit vaccine (WC-rBS). Vaccines prepared by recombinant DNA techniques are deleted of genes encoding the A subunit of cholera toxin. Dukoral® is such a vaccine stimulating antibacterial and anti-toxic local immunity used principally as a travel vaccine. Dukoral® acts locally in the gastrointestinal tract inducing an IgA antitoxic, antibacterial, and anamnestic response comparable to that induced by cholera.

Locally produced intestinal sIgA antibodies are believed to mediate protective immunity.The protection provided is biotype and serotype specific to the content of the vaccine.O-antigens and the B subunit induce immunity. The vaccine induces intestinal

antitoxin IgA in 70-100% of vaccinated individuals. Serum vibriocidal and antitoxic antibodies have been detected in vaccinated subjects. Booster doses elicited anamnestic responses (Crucell Sweden AB. Product Monograph Dukoral®, p.17). The vaccine neither diminishes the fecal spread of cholera bacteria nor reduces its transmission. Dukoral® consists of heat inactivated classic strain Vibrio cholerae O1 Inaba vibrios, formalin inactivated El Tor strain VC O1 vibrios, heat inactivated classic strain VC O1 Ogawa vibrios, formalin inactivated classic strain VC O1 Ogawa vibrios, and the non-toxic recombinant cholera toxin B (rCTB) subunit contained in a vaccine vial. A separate sachet contains the clinically relevant non-medicinal ingredients sodium hydrogen carbonate, saccharin sodium, and raspberry flavor in effervescent granules functioning as a buffer solution. The buffer solution protects the vaccine from inactivation due to the effects of stomach acid. The contents of the buffer sachet and the vaccine vial are added and mixed in cool water as indicated in the packaging instructions corresponding to recommended pediatric and adult dosages. The adult dose consists of five ounces of cool water as well as the contents of the buffer sachet and vaccine vial. For the pediatric dose corresponding to children two years of age and older, half of the dissolved buffer solution should be discarded prior to mixture with the contents of the vaccine vial. This vaccine is not recommended for children less than two years old. Primary vaccination for adults and children six years of age and older consists of two oral doses taken at least one week and up to six weeks apart. The second dose should be taken at least one week after the first dose and one week prior to traveling to an area affected by cholera. Protection against cholera begins about one week after the final scheduled dose lasting about two years. Should more than six weeks elapse between the first and second doses, the vaccination regimen must be initiated anew with a first dose. A single booster dose will renew protection against cholera when administered between two and five years after the final dose of the prior vaccination. The primary vaccination course should be initiated if more than five years have elapsed since the final dose of a previous vaccination. Primary vaccination for children two to six years of age consists of three oral doses administered at least one week and up to six weeks apart and culminating at least one week prior to traveling to an area affected by cholera.

The first dose is indicated at least three weeks prior to the trip, the second dose at least one week after the first dose, and the third dose at lease one week after the prior dose. Protection commences about one week after the final dose lasting for about six months. Primary dosing must be initiated once more with the first dose if more than six weeks has elapsed between any of the doses. A single booster dose will renew protection if the child took the last dose previously between six months and five years ago. The primary course of vaccination is indicated if more than five years has elapsed since the last dose of a previous vaccination. The vaccine mixture should be at room temperature and consumed within two hours of mixture. Dukoral® is acid labile. Ingestion of food and/or liquid increases production of acid in the stomach which may impair the effect of the vaccine. Eating, drinking or consumption of other medication one hour prior to and one hour subsequent to vaccination is prohibited. Preservative cold-chain refrigeration transport and storage at between 35 to 46 degrees Fahrenheit must be maintained and the vaccine should not be frozen. It can be stored at room temperature for up to two weeks just once. The antibacterial intestinal antibodies produced prevent Vibrio cholerae bacterial attachment to the intestinal

epithelium impeding VC O1 colonization. The antitoxin intestinal antibodies prevent cholera toxin binding to the intestinal mucosa preventing toxin-mediated diarrheal symptoms.

mORC-VAX™ is a killed whole cell-only oral cholera vaccine devoid of a cholera toxin B subunit produced and licensed in Vietnam. Its whole cell components are formaldehyde or heat inactivated. The vaccine should be stored in a refrigerator at a temperature of 35 to 46 degrees Fahrenheit. Its shelf-life is two years from the date of manufacture. It is administered as a two dose regimen in an initial 1.5 millliliter dosage for adults and children followed-up by another such dose two weeks later. It is a bivalent Vibrio cholerae O1 and O139 vaccine consisting of a VC O1 classical Ogawa, VC O1 classical Inaba, VC O1 classical El Tor, and the new VC O139 biotypes. A booster dose is recommended two years after primary immunization. Since the World Health Organization does not presently recognize the national regulatory authority of Vietnam, the vaccine is not available for world-wide use. As requirements such as globally recognized Good Manufacturing Practices standards, WHO production guidelines, more detailed product information, technology transfer agreements, and consensus within the WHO and the mainstream scientific community are completely satisfied, mORC-VAX™ is expected to be WHO-prequalified for more widespread use by 2015 (International Vaccine Institute. Currently Available Cholera Vaccines 2012, 2).

Shancol™ is a bivalent killed whole cell-only vaccine reformulated by modification of mORC-VAX™ which altered the toxic strain in a manner conforming to global Good Maunfacturing Practices standards and WHO production guidelines. The vaccine consists of a mixture of the ingredients VC O1 Inaba El Tor formaldehyde killed, VC O1 Ogawa classical heat killed, VC O1 Ogawa classical formaldehyde killed, VC O1 Inaba classical heat killed, and VC O139 formaldehyde killed strains. Excipients are esssentially inert substances added to a prescribed substance to confer an appropriate consistency or form to the therapeutic agent. Shanchol™ contains not more than 0.02% of Thimerosal which is an organomercurial preservative and q.s. (sufficient quantity) to 1.5 milliliters of buffer as excipients formulated together with the active medicinal ingredients. Primary immunization consists of oral administration in two doses of 1.5 millliters two weeks apart to a patient one year of age or above. The onset of protection occurs between seven to ten days after completion of primary immunization. Shanchol™ acts locally in the gastrointestinal tract inducing an IgA antibody response that includes memory as that induced by cholera. Antibacterial intestinal antibodies prevent Vibrio cholerae O1 and O139 bacterial colonization of the intestinal epithelium provid- ing biotype and serotype specific protection from cholera. A booster dose is recommended after two years of primary immunization. Preservative cold-chain refrigeration transport and storage at a temperature of 35 to 46 degrees Fahrenheit is required. As with all medication, thevaccine should be administered prior to the expiration date stipulated on the product packaging. Shancol is licensed by the government of India where it is manufactured and prequalified for use by the World Health Organization. Herd immunity refers to the resistance of a group to a disease attributable to the immunity of a large population of group members and the resulting decreased likelihood of an affected individual contacting a susceptible individual within the group.Induction of herd immunity has been observed with Dukoral® and is believed to be a possibility for Shanchol™ and mORC/VAX™.

Orochol is another vaccine consisting of an avirulent mutant of VC strain CVD103-HgR (HgR is a mercury resistance gene permitting identification of the vaccine strain) administered orally as a single-dose lyophilized (freeze dried) vaccine. Protection results within a week of vaccination. Production of this recombinant live oral vaccine ceased in 2004. PaxVax Incorporated is developing the PXVX0200 single-dose, oral, rapid onset, live, attenuated cholera vaccine using the same CVD103-HgR strain previously marketed in six countries under the Orochol brand name. This strain is considered to have a low development risk since it has thus far been well tolerated exhibiting generally infrequent and mild adverse events.Its expected finished product shelf life is two years; three years for frozen bulk quantity stored vaccine. Once completion of clinical trials and field studies as well as fulfillment of requirements such as globally recognized Good Manufacturing Practices standards, WHO production guidelines, United States Food and Drug Administration licensing requirements, more detailed product information, technology transfer agreements, and consensus within the WHO and the mainstream scientific community are completely satisfied, the PXVX0200 vaccine is expected to be licensed in the United States and WHO-prequalified for widespread use.

6.3 Parenteral cholera vaccination

Parenteral (via injection) vaccines were first developed at the end of the nineteenth century.Parenteral cholera vaccines were initially developed and tested in the 1960's. The various types of parenteral cholera vaccines consist of live attenuated vaccines, killed whole cell vaccines, a purified lipopolysaccharide vaccine, individual killed whole cell vaccines each containing a given adjuvant, as well as a polysaccharide-cholera toxin conjugate vaccine. Adjuvant is a nonspecific stimulator of an immune response. An adjuvant compound that combines with a soluble antigen forms a precipitate. Upon vaccination, slow release of the antigen from the precipitate results in a potent and durable antibody response. Following mucosal or systemic vaccination, Vibrio cholerae toxin is a strong immunogen capable of acting as an adjuvant potentiating local and systemic immune responses co-administered with various antigens bya mechanism yet to be fully elucidated according to Williams et al and references therein (The Immunological Basis for Immunization Series Module 14: Cholera, 2010, 17). Certain aluminum containing compounds such as alum or aluminum hydroxide can function as vaccine adjuvants. Pal et al. state that the duration of protection conferred by parenteral vaccines may be increased by adjuvants, particularly those involving alum-absorption (Ibid., 18). The more common yet infrequently used parenteral cholera vaccine consists of phenol-killed VC organisms of the classical, El Tor, Inaba and Ogawa serotypes. The vaccine is ~50% effective for three to six months against Vibrio cholerae O1 and is considered to be of use to confer short-term protection. Parenteral cholera vaccines have a high adverse event profile after intramuscular or subcutaneous innoculation and may result in localized pain, erythema, induration, fever, malaise and headache in some individuals. This often precludes its administration to pregnant women. The parenteral cholera vaccine may be administered to individuals at five years of age and above. Infants less than six months of age should not be inoculated. If subsequent ongoing protection is indicated, a booster dose is recommended every six months. There is a scarcity of data

available concerning the efficacy of this type of vaccine among immunologically naïve individuals. Parenteral cholera vaccines exhibit low immuno- genicity and high reactogenicity. The parenteral cholera vaccine has not proven to be widely effective during cholera outbreaks or capable of interrupting transmission of Vibrio cholerae organisms in a community. The World Health Organization never recommended the use of the parenteral cholera vaccine since the level of protection afforded was deemed to be insufficient.

During a natural infection, secreted Vibrio cholerae toxin is key to the induction of mucosal immunity in humans making cholera toxin subunit B an essential component of killed or live oral vaccines. As is the case with all vaccines, they should not be administered to anyone having a known hypersensitivity to any of its components or who has exhibited such a reaction after previous administration of the vaccine. Vaccines can lower the risk of illness with regular appropriate use. They will not protect against all cholera cases since local intestinal immunity can be mitigated by a high inoculum. Effective suitable vaccines provide substantial protection without notable side-effects.

Conclusion

A comprehensive understanding of the pathogenesis, virulence, and host colonization of Vibrio cholerae involves Environmental Biology, Microbial and Human Biology, Genetics, Immunology, Physiology and Pathology, Serology, Biochemistry, Bacteriology, Virology, Antimicrobial Pharmacology, Chemoprophylaxis, Vaccination, Epidemiology, as well as Clinical and Preventive Medicine. Cholera is an infectious disease of environmental origin occurring primarily in poorly capitalized developing countries lacking the necessary infrastructure and services for sewage treatment, public sanitation, and the distribution of clean potable water to the community at large.

The healthcare infrastructures are also inadequate and insufficiently accessible for the provision of timely preventive and urgent local community healthcare. There also exists a lack of general public health education. The income levels of the population are such that simple commodities like soap, water purification supplies such as fuel and vessels to boil water, chlorine tablets, acidic citrus juices, and covered water containers are unattainable to a large segment of the susceptible community. Their low income also precludes them from seeking timely healthcare in private for profit settings as well as purchasing prescribed therapeutic agents. They are dependent upon whatever free healthcare may be available from governmental or charitable organizations Civil instability present in some developing countries can also interfere with the ability of people to reach a healthcare facility or for relief workers and their supplies to arrive in affected areas on a sufficiently timely basis. Continued biomedical research as well as the establishment of accessible and adequate preventive and clinical healthcare infrastructures are possible through the material support and cooperation of concerned external non-governmental and governmental organizations as well as their counterparts in the affected countries. The construction and maintenance of infrastructural facilities for the delivery of clean potable water, sewage treatment, and garbage removal are also essential. The organizational commitments must be all encompassing and long-term in nature. There are constantly evolving environmental and genetic variables that enable Vibrio

cholerae to adapt, proliferate and survive as pathogenic entities capable of host colonization and infection as well as antibiotic resistance.

There are no currently available curative agents, vaccines that confer durable long-term immunity, or sufficiently effective chemoprophylactic agents against cholera infection. Some dispute exists within the scientific community as to whether the world is still in the midst of the seventh cholera pandemic, or if the eighth pandemic has indeed begun. A continually evolving understanding of the Science of Cholera in Biology, Medicine, and Public Health is the basis upon which research and clinical discovery will result in novel therapeutic treatment and prevention modalities effective in further reducing the morbidity and mortality associated with cholera.

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Cholera is an inverted movement of everything in the whole body to the stomach, to the belly, and to the intestines-a very sharp malady. For the matters collected in the stomach escape by vomiting, and the fluid matters in the belly and intestines run through by the lower passage. What is first vomited is like water, but what passes by stools stercoraceous fluid and of ill odour. For continued bad digestion has been the cause of this. But what is washed away is first like phlegm, afterwards like bile.

At the beginning the disease is free from pain, but after that, there are tension of the stomach and tormina of the belly; but if the disease increases, the tormina are augmented, there is syncope, the limbs are unknit, there is helplessness, loathing of food; and if they swallow anything, yellow bile rushes out unceasingly by vomiting with sickness, and the dejections are like. There are spasms, and drawing together of the muscles of the calves and legs and of the arms. The fingers are twisted; there is vertigo and hiccup; and the nails are livid; there is cold refrigeration of the extremities, and the whole body becomes rigid; but if the malady runs on to its end, then the man is covered with perspiration; black bile bursts out upwards and downwards. There is retention of urine from spasm of the bladder; but indeed, much water is not collected in it, owing to the pouring out of the fluids into the intestines. There is loss of voice; the pulse becomes very small and frequent, as in syncope; there are constant fruitless attempts at vomiting, desire to evacuate with tenesmus, but dry and without fluid; death, full of pains and miserable, with spasms and suffocation, and fruitless vomiting…But if he rejects everything by vomiting, and a perpetual perspiration flows, and the patient becomes cold and ash-coloured, and the pulse approaches extinction, and the patient becomes speechless, it is well, under such circumstances, (for physician) to make a graceful (becoming) retreat.

Arataeus of Cappadocia, 2nd century A.D.

Joseph Berberena earned a Bachelor of Arts Degree in Education from Queens College of the City University of New York and a Master of Science Degree in Education from the C. W. Post Center of Long Island University. His doctoral level studies to date are in the areas of Human Anatomy, Embryology, Biomedical Ethics, Computers in the Health Sciences, Epidemiology of Infectious Diseases, Histology, Immunology, Medical Terminology, Microbiology, and Physiology. His professorial career began in 1980 teaching English Composition. He continues to serve as a Visiting Professor of Anatomy and Physiology as well as Medical Terminology depending upon assignment each semester in various colleges and universities in New York City.

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