Chapter 1

Introduction and Review of Literature
Introduction

*Vibrio* (term coined by Pacini in 1854) is an old genus that was first described in 1800s. Numerous researchers after Pacini conducted extensive studies on this group of heterotrophic bacteria, which are indigenous to aquatic environments such as ocean, river, inter-tidal water and ponds. *Vibrio* species are facultative anaerobic bacteria, Gram negative, straight or curved rods or spirals and motile by means of flagella (Farmer and Hickman-Brenner, 1992). They live in a wide range of aquatic environment or inside human and animal intestines (Lowrie and Borneman, 1999). *Vibrio* species are members of the family *Vibrionaceae*, which also contains other three genera including *Aeromonas*, *Plesiomonas* and *Photobacterium*. The bacteria of these four genera are all Gram negative, calatase and oxidase positive. But they can be differentiated by DNA-DNA hybridization and other distinctive characteristics.

The genus *Vibrio* contains more than 50 species that are found free-living in aquatic habitats such as marine coastal waters, estuaries, sediments, and aquaculture settings as well as in association with marine organisms such as coral, fish, mollusk, seagrass, zooplankton, and shrimp (Thompson *et al*., 2004). *Vibrios* associate with marine animal tissues as commensal microflora on fish mucosal surfaces, as symbionts in the light organs of fish and squid, and as pathogens causing disease in fish, coral, and crustaceans. *Vibrios* may also be bound as a biofilm to inanimate surfaces such as exoskeletons of crustaceans aiding survival during starvation and environmental stress. They play a role in nutrient regeneration in the aquatic milieu by taking up dissolved organic matter, producing essential polyunsaturated fatty acids needed in the aquatic food web, and degrading chitin. Some *Vibrios* have a role in biodegradation of polycyclic aromatic hydrocarbons in polluted marine sediments. Among marine bacteria, *Vibrios* are prolific producers of antimicrobials as well as they are most resistant to antimicrobials. This interspecies and intraspecies competition suggests an important role for *Vibrios* in structuring and maintaining bacterial communities within marine environments.

Coastal waters present a plethora of ecological diversity. Water properties, such as temperature, salinity, pH, sunlight, oxygen content, and nutrient availability, can change dramatically, temporally, and spatially. *Vibrios* variable response to environmental factors as well as in their associations with marine organisms, affect their occurrence and prevalence in the aquatic environment (Lipp *et al*., 2002; Tantillo *et al*., 2004). This immense variability of
Vibrios to cope with broad variations in the ecology is reflected in the diversity of their genomes.

Vibrios colonized on zooplankton or accumulated in bivalve shellfish may survive longer than free-living cells providing important bacterial reservoirs under favorable conditions (Chiavelli et al., 2001). Coastal ecosystems provide ideal niches for the proliferation of Vibrios as opposed to the open seas. Thus, aquaculture settings, which provide fish food products, are ideal reservoirs for pathogenic Vibrios to thrive and to acquire new genes via horizontal transfer increasing the plasticity of their genomes. At least 12 Vibrios cause disease in humans, some of which also cause disease in aquatic animals. Clinical symptoms of Vibrio infections are divided into three categories, gastroenteritis, primary septicemia, and wound infections. Vibrios are important waterborne pathogens and non-cholera Vibrio pathogens are considered important emerging pathogens that are transmitted by handling or consuming contaminated fish products or by exposure of an open wound to an aquatic environment (Feldhusen, 2000; Lipp et al., 2002; Tantillo et al., 2004). Over the history, much of the research on Vibrio came from clinical microbiology and was primarily related to cholera disease, the diarrhea symptom caused by Vibrio cholerae, which is one of the most infamous species in this genus. Cholera, one of the most noticeable diseases caused by V. cholerae displays a very clear seasonal pattern of epidemics.

1.1 V. cholerae and its Pathogenesis

V. cholerae is a human pathogen and an example for waterborne disease (Colwell, 2004). The hallmark of cholera is a profuse “rice water” diarrhea that is a result of cholera toxin (CT) production. CT is an A-B ADP-ribosylating toxin containing one A (enzymatic) subunit (molecular weight 28,000) and five identical B (binding) subunits (molecular weight 11,500 each) (Kaper and Srivastava, 1992). CT is the most critical virulence factor made by V. cholerae (Kaper et al., 1995) Co-regulated with CT production is the toxin-coregulated pilus (TCP), a type IV pilus essential for intestinal colonization (Kirm et al., 2000; Taylor et al., 1987). A regulatory cascade coordinates the expression of CT and TCP (Reidl and Klose, 2002). The three primary participants in the cascade system are the tox genes: toxR, toxS, and toxT. These three genes form a regulon which controls the ctx operon expression. The toxR gene product is a membrane resident transcriptional activator and DNA binding protein, functioning as inducer of the ctx operon. ToxS is a sensory periplasmic "chaperone" like membranous protein that assists in dimerization of the ToxR protein, activating it. ToxT is a cytoplasmic protein whose expression is induced by ToxR; it activates transcription of ctx
and tcp operons as needed for the expression of the structural toxin genes and toxin coregulated pilus, controlling a total of about 17 virulence factors (Lee et al., 1999).

The ctx genes, encoding CT, and the tcp gene cluster, encoding TCP, are activated directly by ToxT. toxT, in turn, is directly activated by two membrane-localized complexes, ToxR/S and TcpP/H. tcpPH is activated via synergistic binding of AphA and AphB to the tcpPH promoter. This transcriptional cascade differs in the intestinal milieu of the mouse where ctx transcription is dependent on the expression of TCP, and optimal ctx transcription requires ToxR but not TcpP (Lee et al., 1999). The bacterium first colonizes the intestinal surface utilizing TCP, and then, may receive a signal that induces full CT and TCP expression and the onset of disease. Additional factors are known to affect virulence of V. cholerae (Krukonis and DiRita, 2003; Reidl and Klose, 2002). Motility is characterized as a virulence factor but the link to virulence is not clear. OmpU and OmpT outer membrane porins protect the bacterium against the bactericidal effects of bile in the intestines.

In addition to CTX, V. cholerae also produces other enterotoxins like ZOT, ACE etc. ZOT affects the structure of the tight junctions, allow contents of the lumen to diffuse into underlying tissue and disrupt the ion balance and thereby cause diarrhea. Gene that encodes ZOT has been found to lie immediately upstream of ctxA/B operon on the CTX phage (Johnson et al., 1993). The mode of action of ACE (accessory cholera enterotoxin) in human is still not clear, although it causes fluid accumulation in rabbit ideal loop model. Interestingly enough, ace gene is also located closely linked to zot (Karaolis et al., 1998). It seems that almost all major virulence genes are located close to each other on a CTX phage, which has been "raptured" and integrated into V. cholerae genome.

Besides ZOT and ACE, another toxin RTX, has been found to reorganize host cellular actin filaments, thus leading to cytotoxicity in tissue culture cells. In addition to these toxins, Hemagglutinin protease (HA/protease) aids in bacterial detachment from the intestinal tissues after infection. Recent studies showed that HA/protease may play an important role in the inflammatory response in cholera. In cholera toxin negative non-O1 and non-O139 strains, it was found that HA/protease cause an increase in number of inflammatory cells with hemorrhagic response in rabbit ileal loop. It suggests that HAP can be major pathogenic factor of non-O1 and non-O139 V. cholerae (Ghosh et al., 2006).
1.2 Subtypes of *V. cholerae*

Classified by serological testing for O antigen determinants, *V. cholerae* can be subdivided into three major groups:

- *V. cholerae* O1
- *V. cholerae* non-O1, non-O139
- *V. cholerae* O139 Bengal.

*V. cholerae* O1 can be further categorized into three subtypes: Inaba, Ogawa and Hikojima, based on the agglutination with specific antisera. The bio-typing of *V. cholerae* O1 into Classical and El Tor is also widely used, when mentioning the etiologic agent of epidemic cholera (Kaltenthaler and Drasar, 1996). For bio-typing, sensitivity of strains for group IV (El-Tor Specific phage) and group V (Classical phage) is checked along with several other biochemical tests. Historically, *V. cholerae* was divided into subgroups and there was controversy as to the classification of *V. cholerae* O1 and *V. cholerae* non-O1. Later on, 16S rDNA, numerical taxonomy and DNA/DNA hybridization have all supported the hypothesis that *V. cholerae*, both O1 and non-O1, represents a single species. Isolates of environmental and clinical sources are identical in terms of 5S-rDNA sequence (Colwell and Spira, 1992). In the past, it was generally accepted that highly adapted *V. cholerae* O1 is able to exist for only very short period of time outside human intestine. But the accumulated evidence shows that *V. cholerae* is actually an autochthonous inhabitant of brackish water and estuarine system (Colwell and Spira, 1992).

1.3 *Vibrio cholerae* Genome

The whole genome sequence of *V. cholerae* El Tor N16961 was released by The Institute of Genomic Research in the year 2000 (Heidelberg *et al.*, 2000). *V. cholerae* genome consists of two circular chromosomes of size 2.96 Mb and 1.07 Mb that together encode 3885 open reading frames. The vast majority of recognizable genes for essential cell functions (DNA replication, transcription, translation and cell wall biosynthesis), and pathogenicity (toxins, surface antigens and adhesins) are located on the large chromosome. In contrast, the small chromosome contains a larger fraction (59%) of hypothetical genes compared with the large chromosome (42%), and also contains many more genes that appear to have origins other than the gamma-Proteobacteria (Dziejman *et al.*, 2002; Heidelberg *et al.*, 2000). The small chromosome also carries a gene capture system (the integron island) and host 'addiction' genes that are typically found on plasmids; thus, the small chromosome
may have originally been a megaplasmid that was captured by an ancestral *Vibrio* species. Interestingly genes for HA/protease and qrr sRNAs are also present on small chromosome. Qrr sRNAs genes are involved in the quorum sensing pathways of *V. cholerae*.

Very recently, the genomes of different *V. cholerae* strains responsible for the ongoing 7th cholera pandemic and earlier ones and those responsible for epidemics have been analyzed by the micro array technique (Dziejman et al., 2002). These studies reveal that the strains selected showed remarkable relatedness to the sequenced strain N16961 of the El Tor biotype. Although these strains varied in biotype, serogroup, and the year and site of collection, they differed by only 1% from strain N16961 in genetic content. It is very interesting to note that in spite of such a small variation in their total genetic make up, some of the strains were able to cause only sporadic infections while others have been able to spread from the place of its origin to other parts of the world and cause pandemics. All the first six cholera pandemics recorded were caused by classical strains, while strains of the El Tor biotype displaced the classical strains and flourished globally causing 7th pandemic, which emerged in the year 1961 and is still prevalent.

It is found that the environmental *V. cholerae* population is highly heterogeneous in cholera-endemic area. Because most of the recorded cholera pandemics originated in the Ganges Delta region, this ecological setting presumably favors extensive genetic exchange among *V. cholerae* strains and thus promotes the rare, multiple-gene transfer events needed to assemble the critical combination of genes required for pandemic spread (Faruque et al., 2004).

In the recent past, non-O1, non-O139 strains have gained significant attention because of their ability to produce new secretogenic toxin that is entirely different from the toxin produced by toxigenic *V. cholerae* O1 and O139 strains (Singh et al., 2001). Recent studies revealed that non-O1, non-O139 strains have ability to acquire pathogenic potential. For example, four strains of non-O1, non-O139 were identified with O1 antigen backbone background and Vibrio Pathogenicity Island (VPI) clusters (Li et al., 2002). Genomic characterization of non-O1, non-O139 strains revealed that presence of type III secretory system and these strains were found to be quite divergent from O1 and O139 *V. cholerae* (Dziejman et al., 2005). It is hypothesized that the TTSS present in some pathogenic strains of non-O1, non-O139 *V. cholerae* may be involved in the virulence and environmental fitness of these strains (Dziejman et al., 2005).
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Whole genome sequence analysis of non-O1, non-O139 strain NRT-36S, a non-cholera toxin containing strain showed presence of putative exotoxin, two different RTX toxin suggesting that non-O1 and non-O139 strains are having pathogenic mechanism distinct from O1 and O139 V. cholerae (Chen et al., 2007). All of these studies collectively suggested that much attention is needed to be paid towards the biology of non-O1, non-O139 strains to understand the survival strategy and pathogenic mechanism of these bacteria.

1.4 Quorum sensing — wide spread bacterial communication system

By definition, a quorum is the minimal number of members of a committee required to validate a decision (Oxford Dictionary). Extrapolated to bacterial language, this means that an activity controlled by quorum sensing is induced or repressed only when a critical cell population is reached. This bacterial decision-making system enables a given species to sense, integrate and process information from its surroundings, communicate with each other, and monitor its own population density and, as a response, activate or repress specific gene expression. This bacterial cell-density-dependent communication system is popularly known as quorum sensing (Fuqua et al., 1994). Most of the functions monitored by quorum sensing are most beneficial when they are performed as a population rather than by single cells, such as virulence factor production, biofilm formation, conjugation and bioluminescence.

To sense the surrounding bacterial population density, the bacterial quorum sensing system relies on one or more small signal molecules. The term autoinducer was coined for these molecules as these were produced and released by bacteria to act on themselves. These autoinducers accumulates in the medium as soon as bacteria start reaching high cell density. Quorum sensing was first described in the bioluminescent bacteria Vibrio fischeri (Hastings and Nealson, 1977; Nealson and Hastings, 1979). a symbiont of several marine animal hosts such as the squid Euprymna scalopes (Nealson and Hastings, 1979). This bacteria show bioluminescence only at high cell density. Light production is governed by a luciferase enzyme complex encoded in the lux operon (Engebrecht and Silverman, 1984). Initially, quorum sensing was thought to be present only in few bacterial species. Now it is recognized that most of the bacteria have cell-to-cell communication and that quorum sensing is an integral part of the global regulatory network (Withers et al., 2001).

Bacteria can communicate not only in same species, but also with other species present in the same niche. Thus quorum sensing also regulate interspecies communication system (Federle and Bassler, 2003; Surette et al., 1999). Both prokaryotic and eukaryotic
mechanisms have evolved to interfere with bacterial quorum sensing mechanisms (Zhang and Dong, 2004). Autoinducer antagonists and autoinducer degrading enzymes have been reported as quorum sensing interfering systems used by microbes or host organisms to block bacterial communication (Dong and Zhang, 2005). Several AHL-degrading enzymes identified in various bacteria have the potential to be used as quorum quenchers for example AiiA from Bacillus species, AiiD and AHL lactonase of Pseudomonas species inactivates AHL signaling (Gonzalez and Keshavan, 2006). It is possible that quorum quenching is used as a defense mechanism against antibiotic-producing bacteria in the ecological niche.

The autoinducers are grouped into various families based on their chemical structures (Hooi et al., 2004; Tateda et al., 2003). The best studied signals are acylated homoserine lactones (AHLs) found in Gram-negative bacteria, peptide-based signals used by Gram-positive bacteria and furanosyl borate diester (FBD), that have been identified in both Gram-negative and Gram-positive bacteria (Henke and Bassler, 2004b; Visick and Fuqua, 2005). These autoinducer molecules are highly diversified in nature in different bacterial species. This diversity not only allows specific intracellular signaling but also ensures that given bacterial species responds only to its own signals.

Based on signaling molecules and sensing mechanism, there are three major classes of quorum sensing systems:

1. Gram-negative LuxI/LuxR-like quorum sensing system that uses AHLs as signaling molecules (Fuqua et al., 1994).
2. Gram-negative V. harveyi-like two-component signaling circuits that recognize three different signaling molecules, AHLs, FBD and an uncharacterized CAI-1 molecule (Bassler et al., 1993; Bassler et al., 1994; Henke and Bassler, 2004c).

1.5 Quorum sensing signaling in Gram-negative bacteria

1.5.1 LuxI/LuxR-like quorum sensing system

The first LuxI/LuxR quorum sensing system was characterized in V. fischeri (Figure 1.1). This symbiont is found in a specialized light organ of marine hosts where it can grow to a very high cell density ($10^7$ cells/ml) (Nealson and Hastings, 1979). In exchange for nutrients, the bacteria produce light for the host to attract mate and prey or use light in
Figure 1.1 Quorum sensing in *Vibrio fischeri*; a LuxIR signaling circuit.

The LuxI synthesizes 3-oxo-C6 HSL at a low basal level. The concentration of the autoinducer increases only when the bacterial population increases in cell number. When a critical threshold concentration is reached, LuxR is activated upon binding of the 3-oxo-C6 HSL signaling molecule. The activated LuxR binds a lux box in a dimeric form and induces both luminescence and luxI. This positive feedback circuit (autoinduction) induces the full expression of the system in an exponential way.
antipredation strategies (Nealson and Hastings, 1979; Ruby and McFall-Ngai, 1992). Light is produced by the luciferase enzyme complex encoded in the luxCDABE operon. In V. fischeri, the lux operon is linked to the luxI gene encoding for the AHL synthase. The substrates for AHL synthesis are S-adenosylmethionine (SAM) and acylated acyl carrier protein (ACP) (More et al., 1996; Parsek et al., 1999; Schaefer et al., 1996; Val and Cronan, 1998). These are common metabolites found in most Gram-negative bacteria species (Val and Cronan, 1998). The LuxI synthase produces N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6 HSL) at a low basal level (Eberhard et al., 1981; Engebrecht and Silverman, 1984). Thus, the concentration of AHLs increases only when the bacterial cell density increases (Nealson and Hastings, 1979).

LuxR is a transcriptional regulator that responds to the AHL signal. LuxR is activated upon binding of the 3-oxo-C6 HSL signaling molecule. LuxR-like proteins bind with high specificity and affinity to the AHLs produced by the cognate LuxI synthases. Even though AHLs represent a highly related family of molecules, alterations in the acyl-side chains or in the substitution at the \( \beta \)-position greatly affect the activity of the cognate LuxR protein (Zhu et al., 1998). The AHL-activated LuxR dimerises and activates the luxICDABE operon by binding to a lux box in the promoter region, inducing therefore both luminescence and AHL production at the same time (Engebrecht and Silverman, 1984; Stevens and Greenberg, 1997). The lux box is a 20-bp conserved inverted repeat region located in the promoter region of many LuxR targeted genes (Egland and Greenberg, 1999). Thus, the LuxI/LuxR quorum sensing represents an efficient system to couple gene expression and cell density.

1.5.1.1 AHLs structure

All the AHLs identified share a common homoserine lactone ring moiety, whereas important variations that give specificity are found in the acyl side chains (Figure 1.2) (Fuqua et al., 1996). The acyl side chain can vary in length, in the substitution at the \( \beta \) position, and in the degree of saturation of the acyl chain bonds. The length of the acyl chain ranges from 4 to 14 carbons (Fuqua et al., 1996). The \( \beta \) position carries either a hydroxyl group, an oxo group, or is fully reduced (Fuqua et al., 1996). Thus, AHLs have an amphipathic structure, determined by a hydrophobic acyl-side chain and a hydrophilic homoserine lactone moiety. The amphipathic propriety likely allows AHLs to be both soluble in aqueous environments and to cross phospholipids layers of cell membranes, two important traits that signaling molecule should possess (Pearson et al., 1999).
Figure 1.2 Representative bacterial autoinducers.

The asterisk above the tryptophan in ComX represents an isoprenyl modification.
1.5.1.2 Biodiversity of LuxI/LuxR quorum sensing systems

This quorum sensing system is widespread among bacteria isolated from different niches, such as marine *Vibrios*, rhizosphere bacteria, symbionts and pathogens of both animal and plants. Even though the LuxI/LuxR quorum sensing system is conserved, the functions it regulates in various bacteria are disparate, including exoenzymes synthesis, conjugation, biofilm formation, luminescence, and antibiotic production (Fuqua et al., 1996; Miller and Bassler, 2001). Similarly, the genetic organization is variable and *luxI/luxR*-type loci are localized both on the chromosome and on plasmids. Many bacteria contain more than one set of LuxI/LuxR quorum sensing systems, which are often connected and form hierarchical signaling cascades. In general, each of them produces very diverse AHL signaling molecule giving specificity to every system (Smith and Iglewski, 2003). Interestingly, bacteria that possess several LuxI and LuxR members seem to have acquired each of them separately from two different origins or in pairs (Gray and Garey, 2001).

The opportunistic human pathogen *Pseudomonas aeruginosa* possesses two different LuxI/LuxR quorum sensing systems, the LasI/LasR and the RhlI/RhlR, that are organized in a hierarchical regulatory circuit (Gray and Garey, 2001). The two autoinducer synthases LasI and RhlI catalyse the formation of N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) respectively (Pearson et al., 1994; Pearson et al., 1995) The LasI/LasR and the RhlI/RhlR quorum sensing systems are sequentially activated during cell growth.

Several bacteria contain multiple LuxR homologs regulating different set of genes but responding to a single autoinducer (Hassett et al., 1999; Latifi et al., 1995; Pesci and Iglewski, 1997; Pesci et al., 1997). For instance, *Erwinia carotovora* LuxR family members CarR and ExpR regulate different functions in response to a single AHL signal, suggesting that these LuxR homologues were acquired independently (McGowan et al., 1995; Pirhonen et al., 1993). *Rhizobium leguminosarum* contains the linked cinI/cinR genes on its chromosome as well as an AHL synthase rhlI and three unlinked luxR homologs bisR, rhiR and triR, all located on a plasmid. Two additional loci involved in AHL synthesis, one on the chromosome and one on the plasmid, are also identified. The CinI/CinR system seems to be located at the top of the regulatory cascade of this complex quorum sensing signaling, since a deletion of this quorum sensing system greatly reduces the global AHL production in *R. leguminosarum* (Blosser-Middleton and Gray, 2001; Lithgow et al., 2000).
Therefore, the plasticity and variety of these systems suggest that acquisition of additional members of the LuxI/LuxR family of regulatory signaling systems by horizontal transfer allow the bacteria to gain new regulatory circuits and functional capabilities, increasing their arsenal of regulatory circuits involved in environmental adaptation.

1.5.2 *V. harveyi*-type quorum sensing systems

*Vibrio harveyi* is having two-component quorum sensing system. These types of quorum sensing systems are based on multi-step phosphorelay cascade where phosphate group transmit between several histidine and aspartate residues. This type of multi-step phosphorelay systems are known as unorthodox systems (Perraud et al., 1999). Sensor histidine kinases located in bacterial membrane detect the extracellular signals, autophosphorylate, and transmit the sensory information via a phosphorylation cascade to a response regulator that is activated.

1.5.2.1 The *V. harveyi* quorum sensing signaling cascade

In *V. harveyi* three types of autoinducers are functional- AHL, Al-2 (furanosyl borate diester) (Chen et al., 2002) and CAI-1 (cholerae autoinducer 1) (Henke and Bassler, 2004c). Bacteria for intraspecies communication use AHL, whereas Al-2 is used for interspecies communication (Bassler et al., 1997). CAI-1 may be used by bacteria for communication in *Vibrio* spp. (Bassler et al., 1997; Henke and Bassler, 2004c; Schauder et al., 2001; Surette et al., 1999).

*V. harveyi* possesses three quorum-sensing systems that work in parallel and converge to a common transcriptional activator via a phospho-transfer mechanism (Figure 1.3). System I is composed of the AHL synthase LuxM and the cognate sensor LuxN (Bassler et al., 1997). LuxM synthase produces \( N-(3\text{-hydroxybutanoyl})\text{-L-homoserine lactone} \) (3-hydroxy-C4-HSL) (Cao and Meighen, 1989). LuxN is a hybrid sensor kinase, containing both a sensor histidine phosphotransfer domain (HPt) and a response regulator phosphoaspartate domain (Bassler et al., 1997; Goudreau and Stock, 1998). System II is composed of the autoinducer synthase LuxS, which synthesizes a Furanosyl Borate Diester (FBD) (Chen et al., 2002), also termed Al-2, and the cognate sensor complex composed of the two proteins LuxP and LuxQ (Bassler et al., 1994). LuxP is a periplasmic protein that binds Al-2 and forms a complex with the perisplasmic domain of LuxQ at the surface of the inner membrane (Bassler et al., 1994; Neiditch et al., 2005). System III is composed of the autoinducer synthase CqsA (**cholerae** quorum sensing autoinducer) and the cognate sensor CqsS (**cholera
Figure 1.3 *Vibrio harveyi* Quorum Sensing Systems

*V. harveyi* produces and responds to three distinct autoinducers. The sensory information is fed into a shared two-component response regulatory pathway. The arrows indicate the direction of phosphate flow in the low-cell-density state. CAI-1, HAI-1, and AI-2 are respectively represented by green circles, red triangles, and blue double pentagons. OM-outer membrane; IM- inner membrane.
quorum sensing sensor) (Henke and Bassler, 2004b; Miller et al., 2002). CqsA is required for the production of an undetermined CAI-1 autoinducer, which binds the sensor CqsS. CAI-1 is likely not an AHL and represents a novel class of autoinducer molecules (Miller et al., 2002). CqsS is homologous to LuxN but has a different sensory domain, indicating that they sense different signal molecules (Miller et al., 2002).

\( \text{V. cholerae} \) mirrors the quorum sensing signaling pathways of \( \text{V. harveyi} \) (Figure 1.4). In \( \text{V. cholerae} \) AHL mediated signaling cascade is absent but recent studies suggest presence of third sensory system. The idea behind the possible existence of the third quorum sensing circuit in \( \text{V. cholerae} \) stems from the fact that strains lacking the two known autoinducers detectors (CqsA and LuxPQ) or lacking LuxU activity retain density dependent gene expression while the LuxO mutants are incapable of doing so (Miller et al., 2002). Recently Bassler and colleagues have identified the third sensor kinase-response regulator pair known as VarS/VarA phosphorelay system which acts in parallel to the CAI-1/CqsS and AI-2/LuxPQ circuits of this bacterium (Lenz et al., 2005). VarS/VarA homologues have also been identified in a spectrum of Gram negative bacteria, including \( \text{E. coli} \) (BarA/UvrY), \( \text{Salmonella typhimurium} \) (BarA/SirA), members of genus \( \text{Pseudomonas} \) (GacS/GacA) and \( \text{Legionella pneumophila} \) (LetS/LetA). According to recent studies, BarA/UvrY, the VarS/VarA homologue of \( \text{E. coli} \), activate transcription of genes encoding the sRNA CsrA and CsrC, which antagonize the activity of CsrA protein (Suzuki et al., 2002; Weilbacher et al., 2003). In \( \text{E. coli} \), CsrA (carbon storage regulator), a RNA binding protein has been shown to regulate diverse cellular process including glycolysis, gluconeogenesis, glycogen metabolism and biofilm development (Romeo, 1998; Wei et al., 2000). Owing to have its RNA binding ability, CsrA protein interacts with the leaders of target mRNAs and alter their translation as well as stability. Two noncoding sRNA CsrB and CsrC act to antagonize CsrA, thereby tightly controlling the active pool of this protein.

In case of \( \text{V. cholerae} \), activation of VarS/VarA results in the expression of three sRNAs, known as CsrB, CsrC and CsrD, which further inhibit the function of CsrA protein (Lenz et al., 2005). Upon inactivation of VarS/VarA, CsrBCD are not made, which left the CsrA remains active to feed the sensory input on LuxO (Figure 1.4). In quorum sensing network of \( \text{V. cholerae} \), CsrA seems to intersect on LuxO through an unidentified mediator (Lenz et al., 2005). Taken together, the VarS/VarA-CsrA/BCD regulatory components relay information through CsrA, in a LuxU independent but LuxO dependent manner, to control the expression of the genes encoding the four Qrr sRNAs.
Figure 1.4 *Vibrio cholerae* quorum sensing Cascade

*V. cholerae* quorum sensing cascade is consist of three signaling pathways. The VarS/VarA-CsrA/BCD system functions with CAI-1-CqsS and AI-2-LuxPQ systems. Pentagons and triangles denote CAI-1 and AI-2 respectively. The arrows indicate the direction of phosphate flow in the low-cell-density state. Flow is reversed at high-cell density. Dotted lines denote hypothetical interactions. OM-outer membrane; IM-inner membrane.
Genetic analysis indicates that sensory information from all systems converges at the central molecule LuxO by a shared phosphorelay protein, LuxU (Lilley and Bassler, 2000). At low cell density (i.e., in the absence of autoinducers), LuxU activates LuxO through phosphorylation. This phosphorylated LuxO (LuxO-P) together with σ^{54} turns on the expression of a downstream repressor. In a series of elegant genetic experiments, it was demonstrated that the repressor is the sRNA chaperone Hfq and five sRNAs (Lenz et al., 2004). In *V. cholerae* four of these sRNAs are present. Mechanistically, the LuxO-P–σ^{54} complex drives the expression of the loci encoding all sRNA and repression occurs via Hfq-sRNA-mediated destabilization of the *luxR* mRNA transcript (Figure 1.3, 1.4).

On the other hand, at high cell density (i.e., in the presence of autoinducers), LuxO is dephosphorylated and becomes inactive. Inactive dephospho-LuxO permits LuxR expression. LuxR can then regulate the expression of various targeted genes involved in diverse functions including bioluminescence (Lilley and Bassler, 2000; Mok et al., 2003).

The *V. harveyi* quorum sensing regulates physiological processes such as light production, siderophores, colony morphology, metalloprotease production, type III secretion system and several genes of unknown function (Bassler et al., 1993; Bassler et al., 1994; Henke and Bassler, 2004c; Lilley and Bassler, 2000; Mok et al., 2003). Similarly in *V. cholerae*, quorum sensing positively regulates the Hap metalloprotease but negatively regulates the expression of the virulence factors Toxin co-regulated pili and Cholerae Toxin (Kaper et al., 1995; Kovacikova and Skorupski, 2002; Kovacikova et al., 2003; Taylor et al., 1987; Zhu et al., 2002). In *V. cholerae*, quorum sensing regulates these functions by regulating the expression of the *V. harveyi* LuxR homolog HapR (Lenz et al., 2004). HapR is an essential activator of the metalloprotease HapA (Jobling and Holmes, 1997) and also down regulates the expression of tcpPH, by repressing the transcription of aphA (Kovacikova and Skorupski, 2002; Kovacikova et al., 2003).

The quorum sensing components are quite similar in many *Vibrios*, but the regulatory mechanisms vary in complexity and regulate different set of functions. All *V. harveyi*-like quorum sensing systems analyzed converge to LuxO and indirectly to LuxR homologues. However, an important variation in the type and the number of converging signaling systems is observed. *V. harveyi* and *V. cholerae* both possess three parallel circuits converging to LuxO. In *V. harveyi* three parallel cascades are there whereas only one circuit operates in *V. vulnificus* (AI-2). *V. fischeri* contains an additional LuxI/LuxR quorum sensing system linked via LuxO and LitR to the two *V. harveyi*-like quorum sensing circuits (AHL, AI-2). This
diversity implies that the signaling phosphorelay circuits may respond differently to the presence of the various signals in *Vibrios* spp.

### 1.5.2.2 AinS/LuxM family of AHLs synthase

In *V. fischeri* AinS catalyzes the formation of C8- HSL and LuxM of *V. harveyi* direct the synthesis of 3-hydroxy-C4-HSL (Bassler et al., 1993; Gilson et al., 1995). Although AinS and LuxI are not homologues, they use similar precursors, SAM and acyl-ACP, for AHL synthesis (Hanzelka et al., 1999). However, AinS can accept either Acyl-ACP or Acyl-CoA in a relatively efficient manner as an acyl group donor (Hanzelka et al., 1999), whereas members of the LuxI family can only accept Acyl-ACP. This flexibility might be advantageous under certain physiological condition that causes an imbalance in the pool of precursors. Interestingly, RhlI inhibitors such as S-adenosylhomocysteine and holo-ACP also inhibit AHL synthesis by AinS (Hanzelka et al., 1999), suggesting that the two families of AHLs synthases likely share a common enzymatic mechanisms.

### 1.5.2.3 Furanosyl borate diester (AI-2) and LuxS family

LuxS belongs to a new family of proteins responsible for autoinducer production. This family is widespread both in Gram-negative and Gram-positive bacteria (Bassler et al., 1997; Surette et al., 1999). Since the AI-2 signaling molecules are produced and detected by many bacteria, it is suggested that this signal is involved in interspecies communication (Bassler et al., 1997; Surette et al., 1999; Xavier and Bassler, 2003). Crystallographic studies of the AI-2 receptor LuxP of *V. harveyi* showed that the protein is complexed with a furanosyl-borate-diester (3A-methyl-5,6-dihydro-furo (2,3-D) (1,3,2) dioxaborole-2,2,6,6A-tetraol; S-THMF-borate) (Chen et al., 2002). FBD is a by-product of the activated methyl cycle, involved in the recycling of SAM (Chen et al., 2002; Vendeville et al., 2005).

SAM is the major methyl donor in both prokaryotes and eukaryotes. When used as a methyl donor, SAM is converted to S-adenosyl-L-homocysteine (SAH), a toxic compound. SAH is detoxified by the PfS enzyme (also called 5'-methyl thio adenosine/S-adenosyl homocysteine nucleosidase) to S-ribosyl homocysteine (SRH), the substrate of LuxS. LuxS acts on SRH to make homocysteine and DPD (4,5-dihydroxy-2,3-pentanedione). DPD is the precursor of FBD. DPD spontaneously undergoes cyclisation followed by reaction with borate to form a relative stable molecule of furanosyl borate diester (S-THMF-borate) (Chen et al., 2002), the *V. harveyi* AI-2 autoinducer. The boric acid molecule required for this reaction is widely available, for instance in sea water. However, boron is not always
associated with AI-2. In *Salmonella typhimurium*, the AI-2 binding protein LsrB interacts with a AI-2 signal lacking boron (R-THMF) (Miller *et al.*, 2004). Moreover, S-THMF-borate and R-THMF have a capacity of inter-conversion, with an equilibrium influenced by the level of boric acid (Miller *et al.*, 2004). Thus two different AI-2 molecules formed from the same precursor have been characterized. The different active forms may depend on the availability of boron found in the different bacterial niches.

As previously mentioned, LuxS has an alternative role in addition to AI-2 synthesis in the cell. LuxS is an integral component of the activated methyl cycle (Winzer *et al.*, 2003). In fact, AI-2 has been shown to be more tightly linked to the physiological and metabolic state of the bacteria than to cell density (DeLisa *et al.*, 2001a, b; Kim *et al.*, 2003; Winzer *et al.*, 2002). The fact that LuxS has an important role in central metabolism could explain the widespread conservation of the *luxS* gene among bacteria. An alternative hypothesis suggests that AI-2 could be used as an indicator of the metabolic state of the cell to monitor the growth potential of the population instead of being a true quorum sensing signaling molecule (Hardie *et al.*, 2003; Vendeville *et al.*, 2005; Xavier and Bassler, 2003). In addition, the AI-2 molecule could be a by-product of the SAH detoxification pathway rather than a signaling autoinducer (Winzer *et al.*, 2002).

### 1.5.2.4 CAI-1

CAI-1 is an autoinducer produced by CqsA and recognized by the sensor CqsS. This quorum sensing system was identified in *V. cholerae* and is relatively similar to the LuxM/LuxN and LuxS/LuxPQ systems of *V. harveyi*. However, several significant differences have been identified suggesting that CAI-1 is not an AHL or a FBD (Miller *et al.*, 2002). No AHL activity has been detected from *V. cholerae* strains by using different AHL biosensors designed to detect a variety of AHLs (McClean *et al.*, 1997; Ravn *et al.*, 2001). Moreover, mixing culture supernatants containing CAI-1 with a *Bacillus* strain that produces the homoserine lactonase AiiA (Dong *et al.*, 2000; Dong *et al.*, 2001), an enzyme that inactivates AHL by hydrolysing the lactone bond of homoserine lactones, has no effect on CAI-1 activity (Henke and Bassler, 2004c). AI-2 biosynthesis can be achieved in vitro by adding SAM to a bacterial cell lysate from an *E. coli* strain that expresses an ectopic LuxS (Schauder *et al.*, 2001). A similar experiment performed with an ectopic expression of CqsA in *E. coli* did not produce CAI-1 activity, suggesting that CAI-1 biosynthesis requires different or additional precursors than SAM (Miller *et al.*, 2002). Together, these data strongly suggest that the biosynthesis and the structure of CAI-1 are different to previously
described autoinducers. In addition, the sensory N-terminus of CqsS presents no homology to
the sensory domain of LuxN, implying that CqsS recognizes a different type of signal
molecule (Miller et al., 2002). Since V. cholerae CAI-1 containing supernatant can modulate
V. harveyi gene expression and vice versa, CAI-1 activity is not restricted to one bacterial
species and is likely involved in interspecies communication (Henke and Bassler, 2004c). In
contrast to the LuxS/AI-2 system, the synthase CqsS and its product CAI-1 are not
widespread among many bacterial species and have only been identified in closely related
marine Vibrio species (Henke and Bassler, 2004b). Hypothetically, CAI-1 interspecies
signals may only be used by Vibrio species that cohabit in the same environmental niche
(Henke and Bassler, 2004b). The bacterial species that have been identified to produce CAI-1
activity are V. cholerae, V. harveyi, V. anguillarum, V. alginolyticus, V. parahaemolyticus
and V. furnissii (Henke and Bassler, 2004b).

1.5.2.5 The phosphotransferase LuxU

LuxU has a unique central role in the V. harveyi quorum sensing cascade as a
phosphotransferase. The three signaling systems converge on this protein funneling the signal
to the downstream response regulator LuxO (Henke and Bassler, 2004c). LuxU belongs to a
phosphotransferase protein family that possesses little sequence homology, except in the
region localized to the conserved histidine (His-58) phosphotransferase (HPt) domain (Figure
1.5). Examples of phosphotransferases within this family are Ypd1 from Saccharomyces
cerevisiae (Posas et al., 1996). HPt domain of ArcB from E. coli (Matsushika and Mizuno,
1998; Tsuzuki et al., 1995), and SpoOB from Bacillus subtilis (Burbulys et al., 1991). One
advantage of introducing phosphorelay signaling intermediates in an unorthodox two­
component system is to allow other regulatory circuits to feed into the phosphorelay to
modulate the output of the signaling cascade (Perraud et al., 1999). One example is the
sporulation control system of B. subtilis which contains two phosphorelay signaling
intermediates (SpoOF and SpoOB) intercalated between the sensor histidine kinases (KinA
and KinB) and the response regulator (SpoOA). The two sensor histidine kinases, KinA and
KinB, function as phosphodonor for the intermediate receiver domain of SpoOF. SpoOF
transmit the phosphoryl group to the Hpt domain of Spo0B. Finally Spo0B is the
phosphodonor for the receiver domain of the transcriptional activator Spo0A (Burbulys et al.,
1991). The RapA and RapB phosphatases have been shown to dephosphorylate the
intermediate receiver domain of Spo0F, counteracting the activity of the two sensor kinases
KinB and KinA and thereby interfering with the phosphorylation cascade (Perego et al.,
**Figure 1.5 LuxU homologs of Vibrios and other species**

Sequence alignment for LuxU from *V. harveyi* (VH), *V. cholerae* (VC) and two known histidine phosphorelay proteins ArcB-HPt and Ypd1. The degree of homology is indicated by color shading ranging from blue (no homology) to dark orange (sequence identity). The conserved active-site histidine residues are enclosed in a box.
This kinase-phosphatase competition is an example of the possibility of integration of multiple signals via phosphotransfer proteins of a signaling pathway, adding supplementary control along the signaling pathway that ensures the bacteria to respond optimally to different environmental signals. Similarly, in E. coli, the SixA phosphatase dephosphorylates the HPt domain of ArcB (Ogino et al., 1998). Interestingly, the ArcB HPt domain can phosphorylate non-cognate effector proteins, such as CheY and OmpR, in addition to the cognate ArcA protein. ArcB is thus classified as a promiscuous phosphodonor that can be used as a multi-signal transducer in cross-communication (Ishige et al., 1994; Tsuzuki et al., 1995; Yaku et al., 1997), allowing one signal to be transmitted to several response regulators involved in various functions. Therefore, it is possible that LuxU might be the target for additional regulatory signaling than just quorum sensing. LuxU may also transfer the phosphate to additional response regulators other than LuxO, thereby transmitting the signals to regulons that are independent of LuxO regulation.

1.5.2.6 The sigm54-dependent regulator LuxO

LuxO is a member of the NtrC family (bacterial enhancer-binding protein family) of response regulators that activates gene expression together with the alternative sigma factor σ54 (Lilley and Bassler, 2000). This family is mainly characterized by a highly conserved central region that contains the site involved in σ54 interaction, a NTP binding motif, and a region with NTPase activity (Rombel et al., 1998). These proteins are usually composed of a C-terminal H-T-H DNA binding domain and a N-terminal sensor domain that regulates activation upon binding of various environmental signals (Shingler, 1996). Many of these σ54-dependent regulators belong to the family of response regulators which are activated upon phosphorylation of an aspartate motif located in their N-terminal sensor domain (Perraud et al., 1999). Interaction of the σ54-dependent transcriptional activator with σ54-RNA polymerase followed by NTP hydrolyses activate the conversion of the σ54-holoenzyme closed complex to the open transcriptional complex, thereby initiating transcription (Rombel et al., 1998).

The active phosphorylated LuxO induces the expression of four small regulatory RNA (qrr1-qrr4; for quorum regulatory RNA) in V. cholerae and probably five in V. harveyi, V. parahaemolyticus and V. vulnificus (qrr1-qrr5). As described previously, these sRNAs in conjunction with the RNA chaperone Hfq (host factor required for phage Qβ RNA replication, also known as host factor 1) inhibit the expression of LuxR and homologs by binding and destabilizing the corresponding mRNA (Lenz et al., 2004).
1.5.2.7 Small regulatory RNAs (sRNAs)

More than 200 putative sRNAs have been identified in *E. coli* and at least 60 are known to be expressed and likely functional (Majdalani *et al.*, 2005). Many of the known sRNAs are an integral part of the global regulatory network of bacteria. For instance, OxyS, an sRNA induced in response to oxidative stress, has been shown to regulate the expression of at least 40 genes, among them the global regulators *rpoS* and *fhlA* (Altuvia *et al.*, 1998). The characterized sRNAs are regulated by various signaling pathways. However, most of identified and studied sRNAs appear to be induced under stress conditions such as oxidative stress, temperature and starvation (Storz *et al.*, 2004).

sRNAs have been classed in two major groups based on their mechanism of regulation. The first group is composed of sRNAs that act by base pairing with targeted mRNA. This interaction requires an RNA chaperone to promote sRNA-mRNA base pairing and/or to protect sRNAs from RNases cleavage (Folichon *et al.*, 2003; Majdalani *et al.*, 2005; Moll *et al.*, 2003; Moller *et al.*, 2002; Storz *et al.*, 2004). sRNAs can repress or activate translation by blocking or promoting ribosome binding to the targeted mRNA (Altuvia *et al.*, 1998; Majdalani *et al.*, 1998; Zhang *et al.*, 1998) or sRNAs can modulate the stabilisation of mRNA by regulating the mRNA accessibility to ribonucleases (Masse and Gottesman, 2002; Storz *et al.*, 2004). The identified *qrr1-qrr5* of *V. harveyi* belongs to this class of sRNAs (Lenz *et al.*, 2004).

The second group of sRNAs act by binding and modulating the activities of targeted protein. The best studied example of this class are CsrB/CsrC that bind and inhibit the activity of the global translational regulator CsrA (Majdalani *et al.*, 2005; Romeo, 1998; Storz *et al.*, 2004).

The different sRNAs involved in *V. harveyi*-like quorum sensing regulation may be differentially regulated by various environmental conditions. This would allow additional regulatory mechanisms to control the targeted genes in addition to quorum sensing. Indeed, in addition to the LuxO binding site, putative binding consensus sequences of some regulatory factors have been identified in the promoter region of the sRNAs *qrr2* and *qrr3* (Lenz *et al.*, 2004).

1.5.2.8 The family of *V. harveyi* LuxR homologues are members of the TetR family

The *V. harveyi* LuxR-type subfamily belongs to the TetR family of transcriptional regulators (Ramos *et al.*, 2005). The members of the family are either repressors or activators,
or both. The TetR family of proteins has a very conserved DNA binding domain composed of a H-T-H motif. Most of the well characterized members of the family form homodimers that bind palindromic sequences in the promoter region of targeted genes (Ramos et al., 2005), which are most often involved in the physiological adaptation of bacterial cell to environmental changes (Ramos et al., 2005). In general, proteins belonging to the TetR family have unconserved regulatory domains that interact with diverse inducers. Phosphorylation of the regulatory domain or binding of a signal molecule induces a conformational change in the conserved DNA binding domain, promoting activation or derepression of transcription (Ramos et al., 2005).

*V. harveyi* LuxR-type proteins have been identified in many marine *Vibrios*. In addition to the conserved DNA binding H-T-H domain, the LuxR homologs are highly homologous throughout the protein sequence (McDougald et al., 2000). The *V. vulnificus smcR* shares 90%, 80.3%, and 70% identity with the *V. harveyi luxR*, the *V. parahaemolyticus opaR* and the *V. cholerae hapR*, respectively (McDougald et al., 2000). The genetic organization of the loci of the different luxR homologs and phylogenetic analysis showed that they form a new subfamily of transcriptional regulators that are ubiquitously present among most of the *Vibrios* and that the genes have been acquired by vertical transmission from a common ancestor (McDougald et al., 2000). Even though the proteins share a highly conserved amino acid sequence, they have been shown to regulate both common and different phenotypes (Fidopiastis et al., 2002; Jobling and Holmes, 1997; McCarter, 1998; McDougald et al., 2000, 2001; Miyamoto et al., 1994; Miyamoto et al., 1998; Shao and Hor, 2001). Examples of commonly regulated functions involve protease activity, motility and exopolysaccharide production (opacity). The genes involved in these phenotypes may belong to the ancient LuxR regulon inherited from a common ancestor. However, LuxR homologs are also integrated in diverse regulatory circuits and functions that may have evolved during adaptation of the various *Vibrio* spp. to their respective niches in combination with the possible acquisition of transposable elements into their genome. All members of the LuxR family control phenotypes that could be involved in colonization and survival, two events associated with bacterial environmental adaptation, a hallmark of the TetR family of transcriptional regulators (Ramos et al., 2005).

### 1.5.2.9 Functions of *V. harveyi* LuxR homologues

The *V. harveyi* LuxR positively regulates bioluminescence, synthesis of polyhydroxybutyrate, metalloprotease and negatively regulates type III secretion and rugose
colony morphology (Henke and Bassler, 2004a; Lilley and Bassler, 2000; Miyamoto et al., 1998; Mok et al., 2003; Showalter et al., 1990). LuxR shares significant identity with V. vulnificus SmcR, V. parahaemolyticus OpaR, V. cholerae HapR, V. fischeri LitR and V. anguillarum VanT (Figure 1.6). The V. fisheri LitR has been shown to regulate motility, opacity, a putative exopolysaccharide locus and bioluminescence by activation of LuxR, the transcriptional activator of the V. fisheri LuxI/LuxR-type quorum sensing system (Fidopiastis et al., 2002; Lupp et al., 2003; Lupp and Ruby, 2004, 2005). The V. vulnificus SmcR plays an important role in starvation adaptation. Inhibition of quorum sensing signaling in V. vulnificus by using signal antagonists affects starvation survival, demonstrating that SmcR dependent starvation regulation is controlled by quorum sensing signaling (McDougald et al., 2001). In addition, SmcR regulates both positively and negatively several virulence factors. It activates the metalloprotease vvp and the elastase vvpE but represses indirectly the cytolsyn vvhA, motility, fimbriae production and biofilm formation (Jeong et al., 2003; McDougald et al., 2001; Shao and Hor, 2001). Finally, SmcR positively regulates opacity, a phenotype usually associated with capsular or exopolysaccharide production. Capsular polysaccharide has been shown to be a major determinant of virulence in V. vulnificus and many other bacteria species (Shao and Hor, 2001).

Similarly, the V. parahaemolyticus OpaR controls swarming motility and colony opacity, a phenotype associated to capsular polysaccharide production and to other OpaR regulated unidentified determinants (Guvener and McCarter, 2003). Disparity in V. parahaemolyticus colony morphology is regulated by phase variation within the opaR promoter region (Enos-Berlage et al., 2005; Guvener and McCarter, 2003). Interestingly, both the opaque and translucent cell types form stable biofilm structures, however, the biofilm architectures and attachment characteristics to diverse surfaces differs, indicating that OpaR may play a central role in the adaptation of biofilm formation under different conditions.

The V. cholerae HapR activates the metalloprotease HapA but represses both virulence and biofilm formation (Jobling and Holmes, 1997; Kovacikova and Skorupski, 2002; Kovacikova et al., 2003; Zhu et al., 2002). HapR indirectly represses the synthesis of the toxin-co-regulated pilus and cholera toxin by inhibiting the expression of the AphA regulator located at the top of the regulatory cascade (Kovacikova and Skorupski, 2002; Kovacikova et al., 2003). HapR represses biofilm formation by inhibiting the expression of the vps operon, a locus required for exopolysaccharide biosynthesis and biofilm formation.
Figure 1.6 LuxR homologs of *Vibrios* species

Sequence alignment for LuxR homologs from *V. harveyi* (luxR), *V. cholerae* (hapR) *V. vulnificus* (SmcR), *V. parahaemolyticus* (OpaR), *V. fischeri* (LitR) and *V. anguillarum* (VanT). 100% sequence identity is highlighted with grey colour.
A model of quorum sensing regulation via HapR during the *V. cholerae* infectious cycle has been proposed (Figure 1.7) (Zhu et al., 2002).

At least two of the LuxR homologs, LuxR and HapR, bind their own promoter and repress their transcription (Chatterjee et al., 1996; Lin et al., 2005). This autorepressor activity allows these proteins to tightly control their action by modulating their intracellular levels. Precise regulation of several *V. harveyi* LuxR homologs is important since overexpression of these regulators in certain strains is cytotoxic or at least inhibit growth, suggesting that essential unknown cellular functions could be controlled by these family of regulators (Chatterjee et al., 1996; Fidopiastis et al., 2002).

The high homology shared by the members of the *V. harveyi* LuxR family allows in some cases functional interchangeable ability, with respect to the regulation of the *V. harveyi lux* operon (Jobling and Holmes, 1997; McCarter, 1998; Shao and Hor, 2001). HapR, OpaR and SmcR carried in trans could activate the expression of the *V. harveyi lux* operon in an *E. coli* background, but the expression of LitR in trans failed to restore bioluminescence production (Fidopiastis et al., 2002). Even though the H-T-H DNA binding domain is extremely conserved among the *V harveyi* LuxR homologs, no LuxR binding consensus sequence has been identified among the various targeted genes, suggesting that these regulators likely recognizes diverse promoter sequences (McDougald et al., 2000).

### 1.6 Quorum sensing signaling in Gram-positive bacteria

In addition to the widespread interspecies *V. harveyi*-like AI-2 signals, Gram-positive bacteria use secreted peptides as autoinducers for specific interspecies communication. These extracellular peptides, often called pheromones, are synthesized first as precursors inside the bacterial cell and are then processed and secreted via an ATP-binding cassette transporter (Kleerebezem et al., 1997; Miller and Bassler, 2001). The described pheromone peptides vary in length and many of them are modified oligopeptides. Examples of modifications comprise hydrophobic modifications on particular amino acids or cyclization by formation of a thiolactone ring ((Lazazzera et al., 1997; Magnuson et al., 1994; Mayville et al., 1999). Two mechanisms of signal recognition have been described in Gram-positive bacteria. The first mechanism is based on two-component signaling systems (Perraud et al., 1999), and is therefore similar to the *V. harveyi* quorum sensing signaling system in that, a phosphorelay transmits the signal to regulate gene expression (Bassler et al., 1994; Freeman and Bassler,
Figure 1.7 Quorum Sensing and its Role in pathogenesis of V. cholerae

After ingestion, quorum sensing signaling activates HapR expression and HapR represses exopolysaccharide production and therefore biofilm formation. Bacterial cells detach from the biofilm and become planktonic cells. HapR expression is repressed at low cell density in planktonic bacteria, allowing the expression of virulence factors and colonization of the intestinal epithelium. Bacteria can multiply and reach high cell densities in the intestinal epithelium, causing activation of HapR. HapR inhibits virulence genes expression and activates the metalloprotease HapA. Induction of HapA production promotes bacterial detachment from the epithelium and releases of the bacteria into the environment via the intense diarrhea.
1999a, b; Freeman et al., 2000; Miller et al., 2002). The pheromone acts by binding to a
cognate sensor histidine kinase in the bacterial membrane. The binding of the autoinducer to
the sensor domain triggers the transmission of the signal via auto-phosphorylation or
dephosphorylation of the sensor histidine kinase domain. The phosphate group is then
transferred to the aspartate domain of the cognate response regulator which becomes
activated, allowing it to regulate transcription of various targeted genes (Lina et al., 1998;
Pestova et al., 1996; Solomon et al., 1995). The second mechanism of signal recognition in
Gram-positive bacteria is based on the direct recognition of the pheromone by an intracellular
receptor. The peptide pheromones are transported into the cell by an oligopeptide permease
that belongs to the large family of ATP-binding cassette transporters (Lazazzera et al., 1997;
Solomon et al., 1995; Solomon et al., 1996).

Two interesting examples of pheromone regulation in *B. subtilis* and *Staphylococcus
aureus* show the similitudes and differences characterizing quorum sensing in Gram-negative
and Gram-positive bacteria.

### 1.6.1 Bacillus subtilis: Quorum Sensing Signals

*B. subtilis* possesses at least two different pheromones, ComX, a ten amino acid
modified oligopeptide (Figure 1.2) that acts by activating a two-component signaling
cascade, and CSF (competence and sporulation factor), a linear pentapeptide that interacts
with an intracellular receptor (Magnuson et al., 1994; Solomon et al., 1996). These two
quorum sensing systems have been shown to regulate both competence and sporulation in *B.
subtilis* (Figure 1.8) (Grossman, 1995).

Accumulation and binding of ComX to its cognate sensor kinase ComP triggers
autophosphorylation of ComP, and subsequent transfer of the phosphate group to the
response regulator ComA. The activated ComA induces the expression of ComS, an essential
regulator required for the development of the competent state. CSF, when present at a low
internal cellular concentration, promotes competence by inhibiting the activity of the RapC
phosphatase. RapC is an inhibitor of competence that deactivates ComA by
dephosphorylating the active phospho-ComA. However, when present at a high intracellular
concentration, CSF inhibits competence by repressing ComS expression, maybe by inhibiting
the activity of the sensor ComP. At the same time, CSF promotes sporulation by inhibiting
the activity of the RapB phosphatase, a repressor of the sporulation signaling cascade
(Lazazzera et al., 1997; Lazazzera and Grossman, 1998; Miller and Bassler, 2001; Perego et
Figure 1.8 *Bacillus subtilis* Quorum Sensing Cascades

*Bacillus subtilis* produces two autoinducing peptides that regulate two different developmental pathways: competence and sporulation. ComX is represented as a chain of purple ovals and CSF is shown as a chain of red ovals.
In summary, \textit{B. subtilis} possesses two quorum sensing systems, which (1) converge on the same response regulator acting synergistically at a low to moderate cell density and which (2) regulate two divergent functions at high cell density, with one of the signals (CSF) becoming an antagonist of the second (ComX). Even though two different quorum sensing signaling systems converge to the same integrator like in \textit{V. harveyi}, in \textit{B. subtilis} the functional output (agonism or antagonism) of this converging signaling depends much more on the autoinducers concentration than simply on their simultaneous presence as suggested for \textit{V. harveyi}. Thus, the concentration of CSF is the determinant for how a \textit{B. subtilis} population chooses between two mutually exclusive types of physiological strategies.

### 1.6.2 Staphylococcus aureus and the ultra specificity of the signal

\textit{S. aureus} is a pathogen that causes many human illnesses, such as food poisoning, skin infection and toxic shock syndrome. The peptide quorum sensing system regulates many of the virulence determinants produced by \textit{S. aureus} strains (Novick and Muir, 1999). The signaling pheromones, called AIP for autoinducing peptide, is synthesized by the AgrD and AgrB machinery and act via a two-component signaling system comprising the AgrC sensor kinase and the AgrA response regulator (Lina \textit{et al.}, 1998; Novick and Muir, 1999; Peng \textit{et al.}, 1988). The particularity of the AIPs peptide signaling system in \textit{S. aureus} resides in the important variation of AIP that exists among different strains. All the AIPs have a common thiolactone ring and a conserved DEPEVP motif, but have different amino acid sequences and peptide lengths (7-9 amino acids) (Dufour \textit{et al.}, 2002). Therefore, \textit{S. aureus} strains have been divided in four different groups (I-IV) which class strains by production and recognition the same AIP. The genes, \textit{agrBCD}, involved in synthesis and recognition of AIP, are also highly variable among \textit{S. aureus} strains (Ji \textit{et al.}, 1997; Lyon and Novick, 2004; Mayville \textit{et al.}, 1999). Interestingly, each different AIP initiates an \textit{agr} virulence cascade in strains that produce similar AIP but inhibits the \textit{agr} systems in other \textit{S. aureus} groups that produce a different AIP (Ji \textit{et al.}, 1997). This inhibition is a form of bacterial interference that presumably gives a selective advantage for the strain that produces the most abundant and most potent AIP. For instance, the first established strain in a given niche could use its signal pheromone to out-compete secondary invading strains. This is a good example showing that cell-cell signaling in bacteria has evolved to strengthen the degree of specificity above the species barrier. Bacteria that grow in communities such as biofilms in the natural
environment may require multiple forms of intra- and inter-species communication to adapt optimally to environmental variation.

1.7 Bacterial cross-talk

Cell-cell communication is often required for a bacterial population to develop into complex organized communities such as surface adherent biofilms or to coordinate synergistic colonization and establishment into a host (Davies et al., 1998; Fidopiastis et al., 2002; Lupp et al., 2003; Lupp and Ruby, 2005; Parsek and Greenberg, 1999, 2005). Examples of interactions or colonization of a eukaryotic host by a single bacterial species are more an exception than a general occurring event. Most of the symbiotic and pathogenic interactions are characterized by the coexistence of multiple microorganisms located in the same environment. It is likely that multispecies communities are composed of a majority of bacteria that use cell-cell signaling as a mechanism for environmental adaptation. Therefore, interspecies bacterial cross-talk may play an important role in the dynamics of these microbial communities by allowing bacterial species to act in concert or to out compete other species during their establishment in various environmental niches. Multispecies synergistic behavior that allows a synchronization of various important functions may enhance the survival of the entire community, like the formation of mature multispecies biofilm structures.

*P. aeruginosa* colonizes the lungs of cystic fibrosis (CF) patients where it uses quorum sensing signaling to form mature biofilms (Davies et al., 1998; Parsek and Greenberg, 1999). *Burkholderia cepacia* is another opportunistic pathogen found in the lungs of CF patients that is often associated with *P. aeruginosa* (Eberl and Tummler, 2004; Taylor et al., 1993). Both bacteria species possess a *V. fischeri* LuxI/LuxR-type quorum sensing system and produce AHLs. Each of these bacteria activates their respective quorum sensing regulon in response to the AHLs produce by the other bacterial species (Lewenza et al., 2002). Moreover, the addition of the *P. aeruginosa* 3-oxo-C12-HSL to *B. cepacia* cultures increases its production of quorum sensing-regulated virulence factors (McKenney et al., 1995). Therefore, a mixed population of *B. cepacia* and *P. aeruginosa* co-coordinately regulates their virulence factors to improve their capacity to colonize the lung of CF patients.

*Streptococcus gordonii* and *Porphyromonas gingivalis* are two members of the biofilms communities of dental plaque (McNab et al., 2003). The interspecies AI-2 autoinducer, produced by both species, is essential for the establishment of a mixed species
biofilm using both strains but is not required to form a single species mature biofilm. The *S. gordonii luxS* null mutant forms biofilm on polystyrene surfaces but is impaired in formation of a mixed species biofilm with a *P. gingivalis luxS* knock out mutant. Complementation of the *luxS* mutation in one of the two species restores their aptitude to form a biofilm community (McNab *et al.*, 2003).

### 1.8 Biofilm- a complicated well maintained process

In nature, especially in natural aquatic environment, bacteria are predominantly present as multi species communities attached to submerged surfaces not as free floating stage (O'Toole *et al.*, 2000; Stoodley *et al.*, 2002). These surface-attached (sessile) specialized communities are known as biofilm. Biofilm development have three distinct stage of development (i) a free-swimming (termed planktonic) stage, (ii) a transient monolayer stage comprised of an association of single cells that become immobilized on a solid surface, and (iii) mature biofilm, a highly organized, three-dimensional cellular network that may facilitate nutrient availability to certain members of its population (O'Toole *et al.*, 2000). This cellular network is made up of self-produced matrix of extra cellular polymeric substances (EPS). Biofilm act as survival strategy for bacteria in adverse condition as bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming (planktonic) state (Costerton *et al.*, 1999; Donlan and Costerton, 2002; Dunne, 2002). This resistant nature also helps pathogenic bacteria for persistent infections in the human body as well as to troublesome biofilms in industrial processes. Biofilms including pathogenic bacteria growing inside the human body, e.g. in lungs or on implant surfaces (Costerton *et al.*, 1999; Dunne, 2002) can threaten human health (Szewzyk *et al.*, 2000). In industrial processes biofilms cause malfunction of equipments, lower the efficiency of heat exchangers, and lower the end-product quality or safety in food industry (Carpentier and Cerf, 1993; Flemming, 2002). To eradicate biofilms biocides are used intensively, however, it can create resistant bacteria and most importantly, has limited effects, as the biocides are generally more effective against planktonic cells than against bacteria in biofilms.

Bacteria in biofilm produce EPS which work as sticky matrix for bacterial interaction with each other and with surfaced on which they are adhered (Flemming, 2002; Neu *et al.*, 2001). These EPS are generally composed of polysaccharides, but may also contain proteins, nucleic acids and polymeric lipophilic compounds. Till early Nineties, biofilm were considered as surface-attached microbes embedded in their EPS in unorganized way.
But current opinion about biofilm is changed as the whole process of biofilm is found to much more complicated and well-controlled (Figure 1.9). Bacterial growth mode and nature of cells as biofilm, is found to be profoundly different for planktonic cells of same species (Kuchma and O'Toole, 2000; Stoodley et al., 2002). (Stoodley et al., 2002) reviewed that biofilm formation can occur by at least three mechanisms: by the redistribution of surface-attached but motile cells, by the multiplication of attached cells, and by recruiting cells from the bulk fluid. The relative contribution of these mechanisms depends on the organisms involved, on the substratum, and on the environmental conditions. The maturation of a biofilm, resulting in the complex architecture with water channels, is influenced by a number of biological factors and by hydrodynamic features (Stoodley et al., 2002). The biological factors include cell-to-cell signaling between the biofilm bacteria, growth rates of the bacteria, extent of EPS production, motility of the biofilm bacteria as well as possible competition or cooperation between the bacteria.

A mature biofilm with its complex architecture provides niches with distinct physicochemical conditions, differing e.g. in oxygen availability, in concentration of diffusible substrates and metabolic side products, in pH, and in the cell density. Consequently, cells in different regions of a biofilm can exhibit different patterns of gene expression (Costerton et al., 1999). Mixed-species biofilms can contain niches with distinct groups of bacteria having metabolic cooperation (Kuchma and O'Toole, 2000). The EPS matrix of a biofilm community can also contain microzones with different charge and hydrophobicity (Wolfaardt et al., 1998). Watnick and Kolter (2000) summarized that a mixed species biofilm is a dynamic community harboring bacteria that stay and leave with purpose, compete and cooperate, share their genetic material, and fill distinct niches within the biofilm. They stated, “The natural biofilm is a complex, highly differentiated, multicultural community much like our own city”.

1.8.1 Biofilm mode of growth

Bacteria in aquatic environment sense the surface (O'Toole et al., 2000), After coming in contact with suitable surface, bacteria initiate complex differentiation program result in secretion of product which are require for EPS production. For example genes essential for aginate synthesis in Pseudomonas aeruginosa start up-regulating within 15 min after attachment (Dunne, 2002). After that, numerous changes in gene regulation differentiate biofilm cells with their planktonic counterparts (Kuchma and O'Toole, 2000; Stoodley et al., 2002; Watnick and Kolter, 2000; Whiteley et al., 2001). This difference has been
Individual planktonic (free-swimming) cells can reach the surface by active or passive means. The initial cell-to-surface contact is often reversible, but if environmental cues and possible signaling molecules favor surface-attached growth, then the cells attach irreversibly with the aid of excreted EPS. Division of cells and growth of the population, while keeping cell-to-cell contacts, result in the formation of microcolonies. Mature biofilms often possess a hallmark architecture where microcolonies are surrounded by a network of water channels allowing the flow of nutrients into the interior of the biofilm. Some cells can be released to a planktonic lifestyle ensuring the occupation of new niches.
persuasively shown for e.g. *Escherichia coli*, *P. aeruginosa*, *Pseudomonas putida*, and *Bacillus cereus* ([O'Toole et al., 2000; Oosthuizen et al., 2002; Stoodley et al., 2002]). Whiteley et al., (2001) demonstrated by DNA microarray technology that in mature biofilms of *P. aeruginosa* genes e.g. for synthesis of flagella and the sigma factor RpoS were repressed, whereas genes encoding proteins for e.g. temperate bacteriophage, urea metabolism, membrane transport, translation and gene regulation were expressed.

### 1.8.2 Initial adhesion of bacteria

On non-living surfaces bacteria adhere initially by non-specific interactions, but on living organism such adhesion is mediated by molecular docking mechanisms (Dunne, 2002). Later on, during the biofilm growth, also on non-living surfaces cell-to-cell adhesion can be mediated by specific adhesions such as the polysaccharide intercellular adhesin (PIA) of *Staphylococcus epidermidis* (Dunne, 2002; Rupp et al., 2001). An important phenomenon in the initial adhesion of bacteria to non-living surfaces, e.g. to stainless steel, is surface conditioning (Carpentier and Cerf, 1993). It means that when the surface will be modified by the adsorption of inorganic salts, proteins, glycoproteins, and humic compounds etc., depending on the environment, this conditioning affect the adhesion of bacteria with such surfaces (Korber et al., 1995). In practice, most bacteria moving from the bulk aqueous phase towards a surface have their primary contact with a conditioned surface. Once a surface has been conditioned, its properties such as hydrophobicity are often permanently altered, so that the affinity of an organism for a native and a conditioned surface can be quite different (Carpentier and Cerf, 1993; Dunne, 2002). The interaction between the cell and the conditioned surface is dictated by physicochemical variables, which are explained by two different theories (Korber et al., 1995). The "wetting" or the "surface free energy/hydrophobicity" theory is based on surface thermodynamics. If the total free energy of the system is reduced by cell contact with a surface, then adsorption will occur. This theory relies on determining critical surface tension of the bacteria and substratum, and is not taking electrostatic interactions into account (Carpentier and Cerf, 1993). The extended DLVO theory equates repulsive and attractive forces acting on an adhering particle (Busscher et al., 1998).

Microbial adhesion is described as a balance between attractive Van der Waals forces, electrostatic forces (often repulsive as most bacteria and conditioned surfaces are negatively charged), short range Lewis acid-base interactions and Brownian motion forces. Magnitudes of these forces are affected by the distance of the bacterium from the surface.
(long-range and short-range forces) and by ionic strength. The theory predicts that there are two regions where attraction may occur (primary and secondary minimum, <1 nm and 5-10 nm from the substratum). Between these two positions, there is a repulsive energy barrier. Generally it is assumed that bacteria adhere reversibly to the "secondary minimum" and irreversibly to the "primary minimum" with the aid of cell surface appendages or EPS that can pierce the repulsive energy barrier. Poortinga et al., (2001) showed that bacteria forced to adhere in the primary minimum by application of a high positive electrode potential could hardly be stimulated to desorbs, indicating strong irreversible adhesion.

1.8.3 Bacterial behavior on surfaces

After the initial adherence due to change in gene expression of different genes, bacteria change its behavior (Korber et al., 1995). This attachment behavior can be of several types. For e.g. Motile attachment behavior of *P. fluorescens* allows the flagellated cells to move along surfaces in a semi-attached condition within the hydrodynamic boundary layer, independent of the flow direction (Korber et al., 1995). “Near- surface swimming” through which *E. coli* reversibly adhere on surface (Vigeant and Ford, 1997) and “Irreversible attachment” when microbe can no longer move away from surface (Busscher et al., 1998). Most of microbes initially use reversible attachment and later get irreversibly adhered on surfaces for further development of biofilm (Korber et al., 1995). *Vibrio cholerae* and *E. coli* first utilize the flagella to spread across the surface, and then anchor onto the surface with pili and possibly outer membrane proteins (O'Toole et al., 2000). *P. aeruginosa* requires type IV pili for twitching motion on a surface and for the subsequent build-up of stagnant microcolonies (O'Toole et al., 2000). Microbes can attach irreversibly, while retaining active motility by mechanisms known as gliding, swarming, twitching, swimming, darting and sliding. Uropathogenic *E. coli* cells were shown to attach irreversibly and yet actively migrate along solid surfaces (Harkes et al., 1992). It was generally assumed that the biofilm structures in mature biofilms are rigidly “cemented” to their place on a surface until they age and detach. This view was challenged when time-lapse microscopy showed downstream migration of ripple-like biofilm structures of mixed species growing in turbulent flow (Stoodley et al., 1999). Tolker-Nielsen et al., (2000) showed that cells of *Pseudomonas* sp. move actively by their flagella inside and between the microcolonies of a developing biofilm.
1.8.4 Effect of surfaces on attachment

In attachment of bacteria, surfaces, their ionic strength, flow and nutrient condition greatly affect the attachment process. It is found that bacteria can attach various surfaces with different level of such interaction, some surfaces used to be very adhesive while with others bacteria show low adhesion tendency. In 1995 Costerton and Lappin-Scott made an apt remark: "For the past 15 years the corridors of Academe have echoed to the exuberance of scientists who are confident that they have discovered a material whose surface is inherently resistant to bacterial colonization. Laboratory tests have been uniformly encouraging and practical tests have been equally uniformly disappointing, and we believe that we can now conclude that bacteria will eventually adhere to and colonize the surface of any man-made material." Reason for such remark is adaptability nature of bacteria on different surfaces and changing environment. Bacteria can changes its surface property from generally occurring hydrophobic surface to hydrophilic nature through hydrophilic appendages. They can change net surface charge from negative to positive according to conditions e.g. *Stenotrophomonas maltophilia* has positive surface charge at physiological pH (Dunne, 2002). Poortinga et al., (2000) showed that in a field of direct current the rod-shaped cells of *Actinomyces naeslundii* adhered flat on the indium tin oxide surface, and in an alternating current field the cells adhered parallel to the field. Adhesiveness of bacteria also varies with different growth phases (Flint et al., 2001; Peng et al., 2001). Moreover, the surface properties of any novel "colonization-resistant" surface will be gradually altered by adsorption of salts, proteins, glycoproteins and other molecules from the environment.

1.8.5 Eukaryotic organisms in biofilms

In addition to bacteria, other multicellular organism can also be the part of biofilm as the part of same biofilm or can make biofilm like structures themselves. *Legionella* spp (Szewzyk et al., 2000) and *V. cholerae* O139 (Abd et al., 2005) may be enclosed in cysts of fresh water amoeba and therefore is protected from any environmental challenge. The dimorphic yeast *Candida albicans* is commonly present in medical devices (catheters, heart valves and voice prostheses) (Kumamoto, 2002). It forms biofilms composed of a layer of cells in the yeast form adhering to the substratum and, above this, a layer of cells in the hyphal form surrounded by an extensive EPS matrix. The most notable phenotypic attribute of the fungal cells within a biofilm is their increased resistance to antibiotics, strikingly similar to the biofilm phenotype of bacteria. The resistance may arise from contact-dependent expression of drug efflux genes not expressed in planktonic cells (Kumamoto,
Biofouling of industrial processes is frequently caused by mixed-species biofilms. Reports exist on the presence of fungi in these biofilms, but only a few detailed studies have been performed despite the fact that fungi are excellent colonizers of surfaces (Jones 1995).

1.8.6 Cell-to-cell signaling in bacterial biofilms

Davies et al., (1998) published the first study that showed a role for quorum sensing in the formation of biofilms, and launched a period of active research of cell-to-cell signaling in biofilms. Davies et al., (1998) showed that last- mutant cells of P. aeruginosa that were unable to synthesize 3OC12-HSL (3-oxododecanoylhomoserine lactone) were able to attach and initiate the biofilm formation similar to the wild type cells, but the mature biofilms were continuous sheets lacking the differentiated architecture with microcolonies and water channels. The biofilms were also sensitive to SDS in contrast to the wild type biofilms. When 3OC12-HSL was added the last- mutant cells formed biofilms that resisted the detergent wash and had architecture similar to the wild type biofilms. Similarly, biofilm development of Aeromonas hydrophila and Burkholderia cepacia involved AHL-mediated signaling (Conway et al., 2002; Lynch et al., 2002). It has been shown that addition of 7, 8-cis-tetradecenoyl-HSL to aggregates of Rhodobacter sphaeroides mutant cells caused cells to disperse and to grow as individual cells in suspension (Greenberg, 1999). Similarly, AHLs and/or another factor present in stationary-phase culture supernatants mediated a reduction of P. fluorescens biofilm and loss of EPS (O'Toole et al., 2000). These studies suggest that AHL signals may be involved in biofilm dispersal as well. In mixed-species biofilms, AHLs have been shown to mediate interspecies communication (Riedel et al., 2001). The Australian macroalga Delisea pulchra produces halogenated furanone compounds that interfere with the AHL-mediated quorum sensing, and in this way protect the macroalga from bacterial fouling (Hentzer et al., 2002). Addition of a synthetic furanone compound made P. aeruginosa biofilms thinner and less virulent, and enhanced bacterial detachment. The furanones are attractive candidates for biofilm control in the future.

1.8.7 Resistance of biofilms to antimicrobials

It is established now that bacteria in biofilm show far more resistance to antimicrobial substances (biocides and antibiotics) then planktonic counterparts. Many factors are responsible for such property of bacteria. These multiple factors are regulated by different mechanism in different bacterial species (Costerton et al., 1999; Donlan and Costerton, 2002; Dunne, 2002; Stoodley et al., 2002; Watnick and Kolter, 2000; Whiteley et al., 2001). One of
the major reasons for such resistance is presence of EPS matrix this EPS matrix can work as permeability barrier or make complexes with antimicrobials thus interfering with the antimicrobial action. Reactive oxidants may be deactivated in the outer layers of EPS faster than they diffuse. Extracellular enzymatic activity inside the biofilm may be high enough to destroy the antimicrobials. Different microenvironments existing at the deepest biofilm layers with altered pH, pCO2, pO2, cation concentrations etc., may affect the activity of antimicrobials. Bacterial cells of the biofilm phenotype may have reduced susceptibility because of altered cellular permeability, metabolism or growth rate. (Das et al., 1998) reported changes in susceptibility that were greater than twofold and occurred immediately on attachment, and in the presence of biocide concentrations, which exceeded the minimum inhibitory concentrations for the planktonic cells. Grobe et al., (2002) quantified the degree of biofilm resistance by calculating a resistance factor that compared killing times for biofilm and planktonic cells in response to the same concentration of biocides. The resistance factors averaged 29 for DBNPA, 34 for glutaraldehyde, 120 for chlorine and 1900 for a quaternary ammonium compound. This indicates that data on antimicrobial efficacy obtained with planktonic bacteria are not reliable indicators of biocide performance against biofilms. A part of the biofilm resistance could be attributed to incomplete penetration of chlorine, glutaraldehyde and DBNPA due to neutralizing reactions with EPS. The results also implicated presence of a tolerant subpopulation for the quaternary ammonium compound.

1.9 Biofilm Formation in V. cholerae

Wai et al., (1998) discovered that nutrient starvation condition lead to switching of the smooth variant to rugose variant of V. cholerae O1 strain. This rugosity is found to be responsible for their resistance to biocides, acids, osmotic stress and oxidative stresses as well as their ability to form biofilms differ greatly. The exopolysaccharide produced by V. cholerae TSI-4/R was found to have a composition of N-acetyl-D-glucosamine, D-mannose, 6-deoxy-D-galactose, and D-galactose (7.4:10.2:2.4:3.0). The expression of an amorphous exopolysaccharide promotes biofilm development under static culture conditions (Wai et al., 1998). In clinical strains of V. cholerae, different conditions stimulate switching of this phenotype from smooth to rugose with high frequency. Rugose EPS and high-frequency rugose EPS production (HFRP) provide an evolutionary and adaptive advantage to specific epidemic V. cholerae strains for increased persistence in the environment (Ali et al., 2002).

Similar phenomenon was discovered in O139 strain of V. cholerae having common epitope to O1 strain for exopolysaccharide antigen (Mizunoe et al., 1999).
mutagenesis of *V. cholerae* El-Tor revealed that three type of major gene clusters are involved in process of biofilm development in *V. cholerae* (i) those with insertions in genes involved in type IV pili biogenesis and function; (ii) those with insertions in genes involved in flagellar motility; and (iii) those with insertions in genes involved in EPS synthesis. Flagellar motility allows the bacterium to swim through a repulsive potential towards the abiotic surface. As the bacterium reach nears the surface, the MSHA pilus is able to tether the bacterium and pull it onto the surface, where attractive interactions with the surface lead to attachment. Movement along the surface again involves the generation of force by flagella, because the attraction between the bacterium and the surface must be overcome in order to move. Finally, EPS is required to stabilize cell to cell interactions that are integral to the formation of a three-dimensional biofilm (Watnick and Kolter, 1999).

It is revealed that in El Tor strains mannose sensitive heamagglutinin pilus (MSHA) play important role in adherence to abiotic surface while toxin related pili (TCP) is not used by bacteria to form biofilm on abiotic surface. In comparison to abiotic surface, on biotic surfaces like chitin bacteria does not use MSHA for adherence suggesting that bacteria use divergent pathways for adherence in response to biotic and abiotic surfaces (Watnick et al., 1999). The exopolysaccharide secretion in O1 El tor strain depends upon gene clusters designated as *vps* gene clusters (Yildiz and Schoolnik, 1999). Most of the *vps* genes are clustered on the large chromosome of the *V. cholerae* O1 El Tor strain and organized into the *vps* I (*vpsA–K*) and *vps*II (*vpsL–Q*) regions (Casper-Lindley and Yildiz, 2004; Yildiz and Schoolnik, 1999). Two positive transcriptional regulators, VpsR and VpsT, both of which exhibit homology to two-component response regulator were found to be the important regulator of biofilm formation in *V. cholerae*. Disruption of *vpsR* gene lead to switching of rough variant to smooth variant and greatly effect exopolysaccharide secretion (Yildiz et al., 2001).

In O1 strains, exopolysaccharide production depends upon synthesis of UDP-galactose via UDP-glucose. Important genes involved in the process are *galU* and *galE*. These genes are required for biofilm development in phage resistant rugose variant of *V. cholerae* (Nesper et al., 2001). Bacteria defective in *galU* were found to be more sensitive to antimicrobial substances, bile salts and hydrophobic substances due to their effect on working of bacterial outer membrane as effective barrier against these molecules (Nesper et al., 2001).

It was also observed that *V. cholerae* O139 forms biofilms more quickly than *V. cholerae* O1 El Tor (Watnick and Kolter, 2000; Watnick and Kolter, 1999). In *V. cholerae*
O139, flagellum regulates exopolysaccharide secretion. Defect in *flaA* gene lead to secretion of more exopolysaccharide in colonies, in planktonic bacteria and within the infant mouse intestine. This exopolysaccharide, although required for the development of a three-dimensional biofilm, interferes with virulence in an infant mouse model of cholera. Thus, precise regulation of exopolysaccharide is likely to be of critical importance to the survival of *V. cholerae* O139 in a variety of environments (Watnick *et al*., 2001).

It was found in *V. cholerae* that increase concentration of cytidine leads to increase exopolysaccharide secretion. CytR a catabolic repressor of nucleoside uptake and metabolism was found to negatively regulate exopolysaccharide secretion and biofilm formation in *V. cholerae*. It is suggested that CytR involvement is not in initial surface attachment process as *cytR* mutants showed similar surface attachment phenotype as wild type but these mutant form biofilms more rapidly than wild type (Haugo and Watnick, 2002).

Biofilm formation in *V. cholerae* is also affected by pH as it is found that decrease in pH from physiological range to 6 lead to increased susceptibility to hydrophobic drugs, a loss of motility and a reduction in the ability to form a biofilm in cells (Hommasis *et al*., 2002).

To maintain the proper highly organized structure of biofilm, *V. cholerae* require *mbaA* (maintenance of biofilm architecture) gene. This gene does not affect initial adherence but regulate the synthesis of some unknown components of biofilm structures. *mbaA* mutants were found to be defective in biofilm structure with excessive amount of extracellular material (Bomchil *et al*., 2003). Recently, a novel protein RbmA was identified which is required for the maintenance of rugose colony morphology and development of wild type biofilm architecture (Fong *et al*., 2006). It is predicted that this protein may be involved in binding with carbohydrates present in EPS, thus giving rise to the tightly packed and organized biofilm structure (Fong *et al*., 2006).

### 1.9.1 Environmental determinants of biofilm development in *V. cholerae*:

In biofilm development of bacteria, the surface of adherence and other environmental factors play important role. Several organic and inorganic molecules were found to effect the exopolysaccharide secretion as well as initial adherence with the surface. Cations play important role in biofilm development in several bacterial species. Na⁺ and Ca²⁺ in growth media enhance biofilm formation in diverse bacteria (Kim *et al*., 1999; Knobloch *et al*., 2001; Rose and Turner, 1998; Sheikh *et al*., 2001). Except these cations, carbohydrates composition of the growth medium also influences EPS production in many bacterial species.
EPS play crucial role in biofilm community and it is found that both exopolysaccharide dependent and independent biofilm formation occurs in several bacterial species. When quorum sensing is eliminated in \textit{P. aeruginosa} by mutation of the \textit{lasI} gene, however, a flat, densely packed biofilm is formed. This biofilm exhibits increased susceptibility to detergents (Davies \textit{et al.}, 1998). In \textit{E. coli}, a similar flat biofilm is observed when genes for synthesis of the exopolysaccharide colanic acid are disrupted (Danese \textit{et al.}, 2000).

In O1 and O139 \textit{V. cholerae} both \textit{vps}-dependent and independent biofilm formation take place and environmental factors play crucial role in it. In fresh water only \textit{vps}-dependent biofilm formation can take place, while in sea water both biofilm development take place through both pathways (Kierek and Watnick, 2003a). These biofilms also differ in their architecture, \textit{vps}-dependent biofilm contains taller pillars with more water channels in comparison to \textit{vps}-independent biofilms (Kierek and Watnick, 2003a). Further analysis revealed that monosaccharides- glucose and mannose if supplemented with 10 mM NaCl and CAA leads to increase in thickness and biomass of biofilm. These monosaccharides not only provide building blocks for exopolysaccharide production but also activate the transcription of \textit{vpsL} gene. It suggest that carbohydrate component of growth medium may be require for \textit{vps}-dependent biofilm development (Kierek and Watnick, 2003a). In artificial seawater medium, transcription of \textit{vpsL} gene was found to be low, but exopolysaccharide composition of EPS matrix remains similar to \textit{vps}-dependent biofilm. It suggest that \textit{V. cholerae} does not secrete any alternative \textit{vps}-independent exopolysaccharide matrix in \textit{vps}-independent biofilms (Kierek and Watnick, 2003a).

It was found that Ca\textsuperscript{2+} is important component of defined seawater, which is, required for biofilm growth in defined seawater. Further results revealed that Ca\textsuperscript{2+} can interact with capsular polysaccharide and O-antigen of bacterial lipopolysaccharide (LPS) of O139 \textit{V. cholerae}. This interaction may be because of negatively charged phosphate group of O-antigen moiety of LPS with Ca\textsuperscript{2+} ions (Kierek and Watnick, 2003b). Nevertheless, neither Mg\textsuperscript{2+} nor Mn\textsuperscript{2+} can restore biofilm formation in DSW, suggesting that Ca\textsuperscript{2+} is integral part of biofilm matrix. Even unencapsulated strain of O139 strain was found to be dependent on Ca\textsuperscript{2+} ions in defined seawater medium. Many other \textit{Vibrio sp.} and other clinical and environmental...
strains of *V. cholerae* showed calcium dependency of biofilm formation (Kierek and Watnick, 2003b).

Iron also plays important role in regulation of biofilm formation in *V. cholerae* El Tor strain N16961. Addition of iron chelators in growth medium adversely affects both EPS secretion in rugose switching of N16961. A small RNA *ryhB* was found to effect biofilm formation in N16961 strain. The N16961 *ryhB* mutants failed to form biofilms unless excess iron was provided (Mey *et al.*, 2005). *ryhB* mutants also showed decreased chemotaxis in low iron medium. RyhB is necessary to regulate normal cellular iron metabolism thus affecting biofilm formation and chemotaxis in iron limitation. Addition of succinate to the growth medium restores biofilm formation in *ryhB* mutants (Mey *et al.*, 2005). It has been proposed that succinate may be a component of the EPS produced by *V. cholerae* growing in biofilms (Yildiz *et al.*, 2004). The potential target of RyhB may be succinate dehydrogenase operon. RyhB also play crucial role in motility and chemotaxis pathways and *ryhB* mutants were found to show reduced expression of flagellar gene. Reduced expression of flagellar gene can affect flagellum-dependent signaling pathway that regulates EPS production, but still all these pathways are unclear (Mey *et al.*, 2005).

1.9.2 Role of quorum sensing in Biofilm formation in *Vibrio cholerae*

Quorum sensing plays important role in lifecycle of *V. cholerae*. Along with regulation of various pathogenic factors, it also regulates production of biofilm by regulating genes required for EPS production (Hammer and Bassler, 2003). It was found that quorum sensing regulator HapR negatively regulate the expression of *vps* genes, hence affect *vps*-dependent biofilm formation in *V. cholerae* (Hammer and Bassler, 2003; Zhu and Mekalanos, 2003). It is shown that ΔhapR mutant that is 'locked' in the regulatory state characteristic of low cell density has an enhanced biofilm phenotype, ΔhapR mutants were found to show huge pellicle like structure. ΔluxO mutant that is 'locked' in the regulatory state characteristic of high cell density is impaired for biofilm formation (Zhu *et al.*, 2002). Previous studies revealed that reason for enhanced biofilm by ΔhapR mutants is due to induction of *vpsA-K* and *vpsL-Q* EPS operons (Hammer and Bassler, 2003). Similarly deletion of *luxO* and *luxU* gene activates HapR expression at low cell density thus negatively affect biofilm formation (Hammer and Bassler, 2003). It was suggested that, at low cell density, phospho-LuxO represses *hapR* expression. The absence of HapR results in expression of the *vpsB* and *vpsL* operons and, in turn, biofilm formation. At high cell density, inactivation of LuxO results in production of HapR. HapR represses the expression of the *vpsB* and *vpsL* operons, resulting
in no biofilm formation. It is not yet clear whether HapR-mediated repression of the vps loci is direct or indirect (Hammer and Bassler, 2003). Interestingly, it was found that deletion of the AI-2 synthase gene *luxS* does not affect biofilm formation, while deletion of the CAI-1 synthase gene *cqsA* results in the formation of thicker biofilms. These data suggested that AI-2 signals are largely dispensable, while CAI-1 signaling is important for regulating biofilm formation (Zhu and Mekalanos, 2003). Different mutation in LuxO gene like D47E (aspartate to glutamate), F94W (phenylalanine to tryptophan) (Freeman and Bassler, 1999a) or L104R (lysine to aspargine) (Vance et al., 2003) change confirmation of LuxO protein in such a way that LuxO show molecular mimicry with activated LuxO without any phosphorylation. This dephosphorylated LuxO remain active at even high cell density that’s why named as Constitute active LuxO or con-LuxO. Con-LuxO mutants show great enhancement in biofilm formation and make pellicle like structure of water air interface (Freeman and Bassler, 1999a; Vance et al., 2003).

Recently it was revealed that autoinducer CAI-1 accumulate more in biofilm associated cells in comparison to planktonic cells, hence induction of quorum sensing occurs earlier in biofilm associated cells. Induction of quorum sensing induces HapR expression thus negatively regulate EPS secretion and biofilm formation through *vps* operon. This early induction of hapR in biofilm associated cells temporally regulates the thickness and biomass of biofilms. This action results in the formation of normal biofilm structures from which bacteria can rapidly disperse in order to efficiently colonize the host when necessary. This process could be critical since *V. cholerae* may enter hosts from environmental reservoirs in the form of biofilms (Liu et al., 2006).

### 1.10 Significance of the present study

*V. cholerae* strains belonging to the O1 and O139 serogroup are responsible for epidemic spread of cholera across the world. Strains belonging to serogroup other than O1 and O139 collectively known as non-O1 and non-O139 are capable of causing sporadic diarrhea (Thungapatra et al., 2002). During, the span of 1997-1998, an episode of upsurge in the abundance of drug-resistant non-O1, non-O139 *V. cholerae* occurred in Calcutta, India. Although, these strains lacked *ctx* filamentous phage (CTXΦ) and other known virulence genes (Thungapatra et al., 2002), they caused diarrhea which was clinically indistinguishable from that associated with cholera. Because of their hitherto unknown virulence mechanism, these strains were categorized as “enteropathogenic *V. cholerae*” (EPVC). In the months of
July and August 1998, the EPVC constituted one-third of the *V. cholerae* strains isolated from hospitalized patients.

As discussed earlier, genome level analysis of non-O1 and non-O139 strains showed the presence of putative exotoxin genes, type III secretory system and different RXT toxin genes. Genome analysis also revealed that there is 6-16% genome difference in non-O1 and non-O139 strains in comparison to O1 El Tor strain N16961 (Chen et al., 2007; Dziejman et al., 2005). These analyses suggest the presence of different pathogenic mechanism by these strains in comparison to O1 and O139 *V. cholerae*. Interestingly, these strains are present in environment in abundance. Their presence in clinical and environmental samples during outbreak of cholera along with O1 and O139 *V. cholerae* strains suggest important role of these strains in *V. cholerae* pathogenicity and/or environmental survival of pathogenic *V. cholerae*.

It is also possible that aquatic environment is working as hub of horizontal gene transfer and emergence of new drug resistance traits is taking place during aquatic phase life cycle of *V. cholerae*. Quorum sensing and biofilm forming ability of O1 and O139 *V. cholerae* is studied by several group. Both of these phenomena play important role in survival and pathogenicity of O1 and O139 *V. cholerae*. Our knowledge of pathogenic mechanism and biofilm formation in non-O1, non-O139 strains is scanty. It is proposed that these strains may be acquiring new traits during their environmental stay in aquatic water. In the present studies, we want to address several unanswered questions that whether non-O1 and non-O139 *V. cholerae* strains form biofilm or not, what are the environmental factors which affects biofilm formation by these strains. Does the quorum sensing mechanism and/or biofilm formation play a crucial role in the pathogenesis/survival of these strains either in host or environmental cycles? The comparative study will broaden our understanding towards the biology and pathogenic mechanism amongst various serogroup of *V. cholerae*. 