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Chapter 4
Quorum Quenching and Biofilm Inhibition: Alternative Imminent Strategies to Control the Disease Cholera

Lekshmi Narendrakumar, Bhaskar Das, Balasubramanian Paramasivan, Jayabalan Rasu, and Sabu Thomas

Abstract *Vibrio cholerae*, the causative agent of the disease cholera still threatens a large proportion of world's population and is considered as a top priority enteric pathogen. Role of biofilm in *V. cholerae* pathogenesis is well established as it provides the bacterium with enhanced transmission ability during epidemics and also enhanced tolerance to antimicrobial agents. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug resistant *V. cholerae.* The rapidly increasing number of cholera outbreaks in several developing countries and emergence of multidrug resistant *V. cholerae* necessitates the development of an alternative strategy rather than the existing antibiotic therapy to control the pathogen. In the present chapter, we discuss the different quorum sensing pathways in *V. cholerae*, the common quorum quenching molecules that targets these pathways and a novel strategy of biofilm inhibition in *V. cholerae* using antibiofilm compounds in combination with antibiotics to control the disease. Co-dosing strategy reduce the dosage of antibiotics and such a combination therapy can in turn be used to control the spread of antibiotic resistance.

Keywords Antibiofilm compounds · Antibiotic resistance · Combinatorial therapy · Quorum sensing · Quorum quenching · *Vibrio cholerae*

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4.1 Introduction

Vibrio cholerae is the causative agent of the acute diarrheal illness cholera, once the most feared of all pandemics. Today, cholera is managed by oral rehydration therapy and broad spectrum antibiotics, yet the disease is insuperable during epidemics. In 2015, case fatality ratio (CFR) of the disease reported from 42 countries was 0.8%, which includes a total of 172,454 cases with 1304 deaths (World Health Organization 2016). Cholera outbreaks were reported from 16 countries in Africa, 13 in Asia, 1 in Oceania, 6 each in Europe and America. Globally it has been estimated that a total of 21,000–143,000 deaths occur every year from 1.3–4.0 million cholera cases (Ali et al. 2015). However, exact number of cases remains unknown because of the lack in a strong surveillance programs to monitor the disease in developing countries.

V. cholerae is commonly found in saline water bodies, but act as a facultative pathogen when sufficient infective dose reach the human host. There are about 206 serogroups of *V. cholerae* out of which O1 and O139 are known to cause outbreaks. *V. cholerae* O1 are further divided as classical and El Tor biotypes based on phenotypic and genotypic differences. The other serogroups of the bacteria are collectively known as non O1/O139 *V. cholerae* as they do not agglutinate either O1 or O139 polysera. These non O1/O139 *V. cholerae* were considered to be non-toxigenic until it caused a colossal outbreak in1992 (Dutta et al. 2013). However, though improper sanitation has been found to be an important factor for sudden cholera outbreaks, detailed understanding about the role of other factors that play role in sudden outbreaks are still unclear. There have been two hypotheses on the occurrence of sudden cholera outbreaks, the first being that the onset of infection in the population is by transmission of the pathogen from asymptomatic carriers to healthy individuals. This hypothesis explicates that the asymptomatic individuals infect local water bodies leading to explosive sporadic outbreaks of cholera (Frerichs et al. 2012; Eppinger et al. 2014). The second hypothesis assumes that the sporadic sudden cholera cases are initiated when a healthy individual acquires the pathogen from environmental autochthonous toxigenic vibrios (Huq et al. 1983; Alam et al. 2006). Temperature, salinity, nutrients and precipitation are important factors which influences the persistence of vibrios in environmental waters. Also, the pathogen comprises a major component of the commensal flora of phyto-zooplankton persisting as biofilms on them (Jutla et al. 2013).

Biofilms are surface attached, matrix enclosed, multicellular communities of bacteria found in association with both biotic and abiotic surfaces. *V. cholerae* exist in both planktonic and biofilm state during intestinal and aquatic phase of its life cycle. Role of biofilm in *V. cholerae* pathogenesis is well established as it provides the bacterium with enhanced transmission ability during epidemics and also enhanced tolerance to antimicrobial agents. *V. cholerae* biofilms, in its environmental phase provides protection from environmental stresses such as grazing protozoa, bacteriophages and nutrient limitation (Matz et al. 2005). Also, *V. cholerae* biofilms on chitin surfaces

induces its natural competence whereby the cells acquire new genetic materials such as resistance genes (Meibom et al. 2005). During inter epidemic periods, metabolically quiescent *V. cholerae* persist in the environment in biofilm state known as Viable but Non Culturable vibrios (VBNC) which cannot be cultured by normal traditional culturing methods. These cells become active only after a passage through the host or from signals produced by active cells present in the environment (Colwell et al. 1996; Bari et al. 2013). The pathogen biofilms in environment have important biological relevance as it provide protection to harsh environmental stresses and also increase the infective dose of cells entering a host capable of initiating the infection. Though development of *V. cholerae* biofilms within host is poorly understood, recent studies using rabbit ileal loop infection models have identified clusters of *V. cholerae* in microcolonies, supporting the fact that *V. cholerae* forms biofilms *in vivo* which will be subsequently excreted in stool thereby increasing the transmission of the disease cholera (Faruque et al. 2006). In the present chapter, we discuss the role of biofim in *V. cholerae* antibiotic resistance, the different quorum sensing systems in the bacteria and different quorum quenching mechanisms. The chapter highlights on the quorum quenching/antibiofilm therapy as an alternative imminent strategy to control the increasing antibiotic use and its probable advantages in decreasing the burden of antibiotic resistance in the pathogen.

4.2 Association of Antibiotic Resistance and Biofilm

Lately, *V. cholerae* strains resistant to these commonly used antibiotics have appeared with increasing frequency in India, as well in as other countries (Bilecen et al. 2015; Gupta et al. 2016). The pathogen develops resistance to antibiotics through several mechanisms. Biofilm formation in *V. cholerae* has been associated with its resistance gene acquisition. Horizontal gene transfer promotes evolution and genetic diversity of the pathogen. Gene transfer among the environmental and clinical strains in the natural environments has led to the emergence of multidrug-resistant bacteria (Martínez 2012). Also, previous studies have demonstrated that EPS matrix prevents diffusion of antimicrobial agents thus providing a protective niche to the bacterial pathogen which is best known to increase transmission via biofilm microcolonies (Teschler et al. 2015). The common antibiotics taken against cholera such as the quinolone antibiotics, tetracyclines and erythromycin does not reach the bacteria that resides within the biofilm. However these cells get exposed to sub-inhibitory concentrations of the antibiotics leading to adaptive evolution of the bacteria to these antibiotics (Bengtsson-Palme and Larsson 2016). Draft genome sequence analysis of an evolved Haitian variant *V. cholerae* strain isolated from a recent outbreak in south India revealed the presence of aminoglycoside gene, *strB* and *strA*, sulphonamide resistance gene, *sul2* and phenicol resistance genes, *floR* and *catB9* suggesting the real time evolution of *V. cholerae* to the commonly used antibiotics (Narendrakumar et al. 2017).

4.3 Genetic Determinants of *V. cholerae* Biofilm Formation

V. cholerae biofilm formation is initiated by the attachment of cells to biotic or abiotic surfaces by Type IV pili (TFP). The bacteria have three types of TFP namely, toxin co-regulated pili (TCP), mannose –sensitive hemagglutinin (MSHA) pilin and chitin- regulated pili (chiRP) of which MSHA pili play a major role in biofilm development. After surface attachment, the pathogen produces an extrapolymeric matrix composed of proteins, nucleic acids and Vibrio exopolysaccharide (VPS). Matrix proteins such as RbmA, RbmC, Bap1 along with VPS maintain the integrity of these biofilms. The VPS genes are clustered as *vpsI* and *vpsII* and located at two different positions in the large chromosome of *V. cholerae* separated by the rbm gene cluster. *vpsI* cluster comprises of *vpsA-K* genes (VC0917-27) and *vpsII* cluster comprises of *vpsL-Q* (VC0934-9) (Fong et al. 2010). Several studies have reported that the VPS genes are important for the colonization and biofilm development both *in-vitro* and *in-vivo*. Expression of genes such as *vpsA* (VC0917), *vpsB* (VC0918), *vpsC* (VC0919) and *vpsN* (VC0936) are found to be up-regulated during the initial colonization stages and final stages of infection in animal model experiments. VpsH (VC0924) was identified to be at detectable levels in cholera patients on *In vivo*-induced antigen technology (IVIAT) (Hang et al. 2003). In frame deletion mutation of vps gene clusters significantly reduced the ability of *V. cholerae* to produce biofilm. However, not all genes in the *vpsI* and *vpsII* gene clusters were important for its biofilm formation. *V. cholerae vps* clusters are positively and negatively regulated by VpsR, VpsT and HapR, CytR respectively (Teschler et al. 2015). VpsR and VpsT were identified to be important for the maximal expression of *vps* genes and mutation studies in either of the genes significantly reduced the biofilm formation in *V. cholerae* (Yildiz et al. 2001). The positive regulators VpsR and VpsT have been identified to be homologous to the two-component regulatory systems that are involved in sensing and responding to environmental stimuli with a sensor histidine kinase that regulates *vps* genes expression.

4.4 Quorum Sensing and Biofilm Formation

Cell to cell communication in bacteria for the regulation and expression of specific genes is known as bacterial quorum sensing (QS). Quorum sensing is dependent on the bacterial cell density and concentration of chemical signal molecules known as autoinducers (AIs) produced by the bacteria. These AIs are released into the environment which accumulate and signals other bacteria of the same kind for collective expression of specific genes for definite functions such as virulence, biofilm formation, bioluminescence etc (Williams and Camara 2009). Many of the molecular mechanisms for intracellular signaling in bacteria are now well studied. Different quorum sensing small molecules produced by microbes include Acyl Homoserine Lactones (AHL), Peptide auto-inducers and Auto-inducer 2.

4.4.1 Acyl Homoserine Lactones (AHL)

AHLs are produced within the bacterial cells and released into the environment. AHLs produced by different bacteria differ in the R-group side chain length which can vary from 4 to 18 carbon atoms and the carbonyl group at the third carbon. AHL signals contains a homoserine lactone linked by a amide bond to the acyl side chain. The first identified AHL molecule in QS was in the marine bacterium *Vibrio fischeri* which were responsible for the bioluminescence in the light organs of Hawaiian bobtail squid, *Euprymna scolopes* (Ruby 1996). After this discovery, AHL signal molecules were identified from different Gram- negative bacteria including *V. cholerae.* AHL signal molecules are chiefly catalyzed by LuxI enzyme family and perceived by LuxR cytoplasmic DNA binding proteins. Based on the length of the side chain, AHL molecules can be distinguished as short side-chain AHL moleculaes and long side- chain AHL molecules. Short chain AHLs diffuse freely across the cell membrane whereas the long chain AHLs requires active efflux pumps to export them from within the cell. Different bacteria are known to produce different AHL molecules and few of them produce diverse AHL signals (Huma et al. 2011). There are also reports of bacteria like *Pseudomonas simiae* and *Pseudomonas brenneri* isolated from Ny-Alesund, Arctic at 79°N producing varied AHL molecules at different temperatures (Kalia et al. 2011; Dharmaprakash et al. 2016).

4.4.2 Peptide Auto-inducers

Many Gram-positive bacteria utilize peptides as signal molecules for QS. They are usually secreted oligopeptides which results from post-translational modifications. Peptide signals can differ in size from 5 to 87 amino acids and also can contain modifications like lactone or thiolactone linkages. These peptide auto-inducers require special export mechanisms like ATP binding cassette transporters. After the accumulation of sufficient peptide auto-inducer in the environment, the signal molecules are perceived by histidine sensor kinase protein of a two-component regulatory system in the bacteria leading to expression of specific genes. Competence signal peptides (CSP) produced by streptococcal species is an example for peptide auto-inducer (De Spiegeleer et al. 2015). Bacteriocins are also categorized to be a peptide AI (Zhao and Kuipers 2016). ComX bacterial peptide AI induces sporulation in *Bacillus subtilis* by activating the ComP/ComA two-component phosphorylation cascade. Activation of the ComP/ComA component in turn leads to increased gene expression of the transcriptional activator ComK which in turn activates the expression of genes required for sporulation (van Sinderen et al. 1995). In *Staphylococcus aureus,* peptide AI system *agr* play an important role in virulence and pathogenicity of the organism (Baldry et al. 2016). The *S. aureus* peptide AI is detected by AgrC/AgrA sensor kinase/response regulator which gets phosphorylated. The phosphorylated ArgA upregulates RNAIII which positively regulates the expression of virulence genes of *S. aureus*.

4.4.3 Auto-inducer 2

Auto-inducer 2 is a furanosyl borate diester signal molecule. AI-2 is produced by many Gram positive and Gram negative bacteria and is believed to be an evolutionary link between the two QS systems. AI-2 facilitates cross species communication in bacteria. The AI-2 system was also first reported from the marine bacterium *V. fischeri*. For bacteria like *E. coli* and *Salmonella typhimurium*, a group of genes called *lsr* gene cassette are induced that encode AI-2 components of the machinery (Pereira et al. 2013). Periplasmic protein LsrB act as the receptor for AI-2 signal molecules which will be delivered inside to the cell cytoplasm by an ABC transporter. This transportation of the signal molecule into the cytoplasm is coupled to its phosphorylation by LsrK kinase (Xavier and Bassler 2005). The prime function of the phosphorylated AI-2 is to deactivate the transcriptional repressor LsrR which in turn activates the positive feedback of AI-2. The synthesis of AI-2 is facilitated by the enzyme LuxS which converts S- ribosyl homocysteine to homocystein and 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor compound of AI-2 (Marques et al. 2011).

4.4.4 Quorum Sensing Signals in Vibrionaceae Family and Biofilm Formation

Vibrionaceae comes under the Gammaproteobacteriae comprising facultative anaerobic Gram negative bacteria such as *V. cholerae*, *V. parahaemolitycus, V. harveyii, V. anguillarum, V. vulnificus, V. fischeri* etc. The family includes *Vibrio* and *Photobacterium* genera. Of these genera are many top priority human diarrheal pathogens such as *V. cholerae* and *V. parahaemolyticus* and fish pathogens such as *V. anguillarum* and *V. vulnificus.* There are also many bacterial symbionts such as *V. pomeroyi*, *V. aestuarianus*, and *V. fischeri* in this family (Yang et al. 2011). QS systems have been identified in many bacteria of the Vibrionaceae family such as *V. harveyi* (Bassler et al. 1997), *V. cholerae* (Zhu et al. 2002), *V. anguillarum* (Milton et al. 1997; Buchholtz et al. 2006), *A. salmonicida* (Bruhn et al. 2005), *V. vulnificus* (Valiente et al. 2009) and *Photobacterium phosphoreum* (Flodgaard et al. 2005). In a study to determine global phylogenetic distribution of AHL molecules in bacteria belonging to Vibrionaceae family using biological monitors and LC-MS identification, it was identified that acyl homoserine lactones, including AHLs with odd numbers of carbon, was the most abundant signaling molecule. The study also revealed an AHL fingerprint correlated to specific phylogenetic subclades in Vibrionaceae family (Rasmussen et al. 2014). However, quorum sensing systems rapidly diverge in nature and signal orthogonality and mutual inhibition frequently occur among closely related diverging systems of Vibrionaceae. Different homologues of LuxI/LuxR proteins have been identified that respond to each of the different AHL

molecules. Also, the degree of sensitivity of LuxI/LuxR to different AHL molecules differ (Tashiro et al. 2016).

Most of the members in the Vibrionaceae family are also good biofilm formers. QS controls biofilm formation in Vibrionaceae by different mechanisms such as regulating coordinated behavior and synchronized environmental response (Mireille Aye et al. 2015; Okutsu et al. 2015), regulating the synthesis of biofilm matrix (Tseng et al. 2016), indirectly upregulating biofilm formation by increasing bacterial motility (Yang et al. 2014) and also regulate dispersal of matured biofilm to initiate a new developmental cycle of biofilm formation (Emerenini et al. 2015). A recent study has proved the ability of *Vibrio parahaemolyticus* to regulate biofilm formation and enhancement of colonization by QS (Vinoj et al. 2014). Also studies on genetic basis of QS mediated regulation of virulence related gene Hfq, motility/extracellular protein Pep and colony phenotype intermediated protein valR was studied in biofilm forming *V. alginolyticus* (Chang et al. 2010; Cao et al. 2011; Liu et al. 2011). VanT, a homologue of *Vibrio harveyi* LuxR, is known to regulate biofilm formation in *V. anguillarum* (Croxatto et al. 2002). AphA, DNA binding regulators in vibrios belonging to padR family proteins which is important for the QS in vibrios at low cell density is known to regulate biofilm formation, motility and virulence in pandemic *V. parahaemolyticus* (Wang et al. 2013). It is also interesting to note that there is about 85% homology between the AphA protein of *V. parahaemolyticus* and *V. cholerae* suggesting a possibility of similar regulation in *V. cholerae* also.

4.4.5 V. cholerae *Quorum Sensing Systems*

V. cholerae posseses two well characterized and one predicted quorum sensing mechanism as compared to *V. fischeri*. The well-established systems of *V. cholerae* includes the CqsA/CAI-1/CqsS system and LuxS/AI2/LuxPQ system (Ke et al. 2014). In *V. cholerae,* there are two AIs such as the Cholera Auto-inducer (CAI), S-3-hydroxy-tridecan-4-one and Auto-inducer-2 (2S, 4S- 2- methyl-2,3,3,4-tetrahydroxy tetrahydrofuran-borate) and two cognate receptors. The CAI-1 signals are synthesized by CqsA and sensed by CqsS system sensor (Ng and Bassler 2009). AI-2 is synthesized by LuxS and the cognate sensor is LuxP/Q. The components of the third predicted QS system in *V. cholerae* is unknown.

At low bacterial cell density when the AIs are at minimum concentration, the QS system sensors CqsS and LuxP/Q transfers a phosphate group to the cytoplasmic integral protein LuxU which in turn phosphorylates LuxO (Milton 2006). The phosphorylated LuxO in association with the σ^{54} initiate the repression of *hapR* via the activation of a putative repressor. HapR is a transcriptional repressor of the genes which have specific functions in *V. cholerae* biofilm formation and virulence (Amy et al. 2009). Previous studies have reported that a deletion mutant of *hapR* produces strong biofilm compared to its wild type counterpart. At a higher cell density, the AIs exported out reaches the maximum threshold level converting the LuxQ and CqsS kinases to phosphatases. This backflow of phosphate group from the LuxO

destabilizes the repression of putative protein on *hapR* activating its expression. HapR activates Haemagglutinin Protease A (HapA) which disperses the *V. cholerae* in biofilm to planktonic cells.

Another major component that determines the biofilm formation of *V. cholerae* is the concentration of a secondary messenger, c-di-GMP (Cotter and Stibitz 2007). c-di- GMP messengers are synthesized by proteins containing specific GGDEF motifs and its degradation is carried out by proteins containing domains with EAL or HD-GYP motifs. Previous studies reports the presence of 62 genes that encode proteins governing c-di-GMP levels in *V. cholerae* (Galperin 2004). Increase in the concentration of the secondary messenger within the bacterial cell induces the activation of the transcriptional activator *vpsT*. VpsT in turn activates the genes in the vps clusters leading to the increase in extracellular matrix production and increased biofilm. However, the master transcriptional repressor gene HapR is identified to regulate the c-di-GMP mediated biofilm formation at two levels. HapR has been identified to repress 14 genes that encodes for proteins that synthesis c-di-GMP. Also, HapR directly represses the *vpsT* gene which prevents the downstream activation of *vps* genes.

4.5 Quorum Quenching

Quorum quenching (QQ) or QS interference is a strategy wherein the cell to cell communication of bacteria is interrupted by specific compouds. QQ strategy came up with the understanding that bacterial cells have the ability to communicate with each other to collectively regulate various important traits such as virulence, biofilm formation, antimicrobial resistance (Sharma and Jangid 2015; Shiva Krishna et al. 2015). This communication occurs via various signaling molecules which are recognized by specific receptors. The signal molecule binding to the receptor further activates a cascade of molecular signaling which ultimately results in the specific gene regulation. Theoretically, mechanisms that can effectually interfere this signaling/communication between the bacteria can be used as a QQ molecule. A QQ molecule can act on either the signaling molecule, receptor to the signal, regulatory proteins in the signaling cascade.

4.5.1 Quorum Quenching by Signal Molecule Degradation

There are QQ enzymes like AHL-lactonase, AHL-acylase and paraoxonases (PONs), which degrade AHL signals.

4.5.1.1 Quorum Quenching by AHL-Lactonase

AHL- lactonase act on the QS signal, AHL by hydrolyzing the homoserine lactone ring (Dong and Zhang 2005). The first AHL-lactonase, encoded by the *aiiA* gene was identified from a *Bacillus* sp. isolate 240B1. The enzyme has two zinc ions at their active site which initiates a nucleophilic attack at the substrate's carbonyl carbon group. The lactone ring interact with the enzymes Zn^1 and the substrate's carbonyl oxygen interact with Zn^2 thereby weakening the carbonyl bond breaking the lactone ring to yield an open-ring product. Due to its specificity in action, AHL-lactonase is the most precise AHL-degradation enzyme which can hydrolyse both short chain and long chain AHLs efficiently. The ability of AiiA to disrupt QS in *V. harveyii* and *V. cholerae* and thereby inhibit their biofilm formation has been well documented (Bai et al. 2008; Augustine et al. 2010). AiiA enzyme have been successfully cloned into plants such as potato, tobacco, eggplant, cabbage, carrot and celery plant to produce *Erwenia carotovora* infection resistant crops (Dong et al. 2001). Also, *Escherichia coli* containing cloned *Bacillus thuringiensis aiiA* was identified to express greater amount of the enzyme compared to the parent strain increasing its industrial applications (Lee et al. 2002). Another AHL lactonase AttM identified from Agrobacterium tumefaciens with only 24.8% identity to AiiA was characterized to have bacterial fitness properties within the plant tumor (Haudecoeur et al. 2009).

4.5.1.2 Acylases

AHL-Acylases, like lactonases interfere with bacterial QS to attenuate major functions such as virulence, motility and biofilm production. Acylases have been identified to decrease 3OC12HSL and C4HSL accumulation within the bacterial cells which drives the virulence factor production machinery. Acylases have been identified both in Gram-positive and Gram- negative bacteria. AHL-acylase AhlM identified from Streptomyces strain M664 could cleave both medium and long chain AHL signal molecules. AHL acylases AiiC and Aac identified from Anabena strain PCC7120 and Shewanella respectively were proved effective in disrupting biofilm formation of fish pathogen *V. anguillarum in-vitro* (Morohoshi et al. 2008).

4.5.1.3 Oxidoreductases

A third class of AHL- degrading enzymes are the oxidoreductases that inactivates AHL molecules via oxidation or reduction of the acyl chain of the signal molecule. P450 monooxygenase and the NADH-dependent enzyme BpiB09 are the two well-studied AHL signal molecule degrading oxidoreductases. Heterologous expression of BpiB09 have been identified to decrease AHL accumulation in *Pseudomonas aeruginosa* subsequently inhibiting swarming motility, biofilm formation and pyocyanin production (Bijtenhoorn et al. 2011; Kumar et al. 2015).

4.5.2 *Quorum Quenching by Inhibition of Signal Molecule Synthesis*

Apart from degrading signal molecules that play important role in bacterial cell to cell communication, there have been many compounds that inhibit or decrease the signal molecule synthesis thereby interfering QS. Most of the signal molecule synthesis inhibitors work by indirectly inhibiting precursor molecules which are important for signal molecule synthesis. For example, small molecule triclosan act on enoyl-ACP reductase, an important precursor of AHL synthesis and immucillin A (ImmA) inhibits 5-MAT/*S*-adenosyl-homocysteine nucleosidase (MTAN) which is crucial for both AHL and AI-2 synthesis (Hoang and Schweizer 1999; Singh et al. 2005). Very low concentration of nucleoside analogues have been found effective to inhibit MTAN activity in virulent *V. cholerae* strains (Gutierrez et al. 2009).

Recent studies on *V. cholerae* QS precursor molecule (S)-4,5-dihydroxy-2,3-pentanedione (DPD) showed that nucleoside analoges of DPD delineate QS in *V. cholerae, V. harveyi, V. anguillarum, V. vulnificus and S. typhimurium* (Meijler et al. 2004; Lowery et al. 2008; Smith et al. 2009; Brackman et al. 2009). Adenosine analogues have been found to have potent antibiofilm activity by blocking AI-2 based QS.

4.5.3 *Quorum Quenching by Inhibition of AHL Receptor*

The CqsS receptor of *V. cholerae* and *V. harveyi* share extensive homology. However, when it comes to inhibition of CqsS receptors in both the bacteria, possess different overall stringencies for ligands. Many small molecule inhibitors that block the binding of signal molecule to the receptor thereby cutting off the QS signaling cascade has been identified recently. High throughput screening studies have identified many small molecule inhibitors of QS receptors in *V. cholerae* (Peach et al. 2011).

4.6 Antibiofilm Activity of Natural Compounds Against *V. cholerae*

Natural products are a good source of compounds that have various biological activities including antimicrobial and antibiofilm properties. These products are considered safe to administer and are believed to cause lower degree of antimicrobial resistance unlike antibiotics. Many of these compounds are identified to inhibit bacterial virulence or biofilm formation by interfering with the QS of bacterial pathogens. List of important QQ molecules have been listed in Table 4.1.

Table 4.1 List of important quorum quenching molecules in Vibrionaceae family

Quorum quencher	Active against	References
Hexyl-4,5-dihydroxy-2,3-pentaedione	*V. harveyi*	Schaefer et al. (1996)
		Lowery et al. (2009)
4-hydroxy cis or trans analogs	*V. fischeri*	Olsen et al. (2002)
N-sulfonyl-HSL	*V. fischeri*	Castang et al. (2004)
Furanone C-30	*V. anguillarum*	Rasch et al. (2004)
N-(heptyl-sulfanyl acetyl)-L-HSL (HepS-AHL)	*V. fischeri*	Persson et al. (2005)
AiiA enzyme	*V. cholerae*	Augustine et al. (2010)
Oceanobacillu/ Halomonas extract	*V. cholerae* and *V. cholerae. parahaemolyticus*	Nithya et al. (2010)
Artctic actinomycetes extract	*V. cholerae*	Augustine et al. (2012)
Resveratrol	*V. cholerae*	Augustine et al. (2014)

4.6.1 Antibiofilm Activity of Phytochemicals

Plant extracts and plant compounds have been used since time immemorial to treat diarrheal diseases and other stomach ailments. Traditional medicines of India and China use plant extracts for treating bronchitis, asthma, gastric ailments, phlegm, dysentery, leukorrhea, kidney trouble, urethritis, and dropsy (Mitra et al. 2007).

Resveratrol, a phytochemical present mostly in the skin of grapes, blueberries, raspberries, mulberries etc have been identified to target upstream targets of QS system, reducing *V. cholerae* biofilm production upto 80% (Fig. 4.1). Molecular docking studies, AphB, a LysR-type regulator important in the QS of *V. cholerae* was identified to be the putative target for resveratrol (Augustine et al. 2014).

Vikram et al. (2011) demonstrated that flavonoid compounds such as naringenin and quercetin isolated from citrus fruits have been identified to reduce biofilm formation in *V. harveyii* and *V. cholerae* by acting as antagonists of AHL and AI-2 (Vikram et al. 2011). Also, several studies are available showing the anti-virulence activity of plants like 'neem', apple, hop, green tea and elephant garlic via inhibition of bacterial growth or the secreted cholera toxin (CT). Mangrove plant extracts have been known for their mosquito larvicidal, antifungal, antiviral, anti-cancer and anti-diabetic activity. Leaves and bark extracts of mangrove plants *Ceriops tagal* and *Pemphis acidula* revealed to have potent antibiofilm ability against a wide set of microorganisms such as *P. aeruginosa, Klebsiella pneumonia, V. parahaemolyticus*, *S. aureus* and *V. cholerae* (Arivuselvan et al. 2011). Cinnamaldehyde and its derivatives have been found effective as an antibiofilm compound against all Vibrio sp., by interfering AI-2 mediated QS pathway by decreasing the DNA-binding ability of

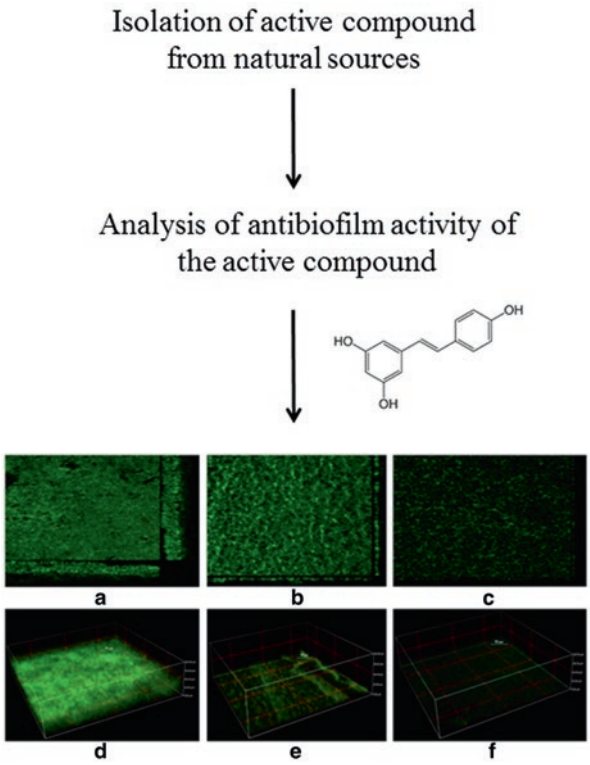

Fig. 4.1 Isolation of active compounds from natural sources and analysis of antibiofilm activity of the compound against *V. cholerae*. CLSM images of *V. cholerae* biofilm inhibition using resveratrol. (*a*) untreated culture. (*b*, *c*) Biofilm treated with 15 & 20 μg/ml of resveratrol. (*d*) 3D view of biofilm thickness of untreated. (*e, f*) 3D view of biofilm thickness of cultures treated with 15 and 20 μg/ml resveratrol

LuxR (Brackman et al. 2008). Also, in *V. cholerae*, cinnamaldehyde analogues have been proved to have antibiofilm (Niu et al. 2006; Brackman et al. 2011). Water soluble extract of Cranberry has found to have potent antibiofilm property against *V. cholerae* acting via down-regulating the *vps* operon by modulating the level of c-di-GMP in the QS pathway. Also it has been shown that Cranberry extracts can block the initial attachment of *V. cholerae* into the host enterocytes during an infection and also inhibit cholera toxin production by regulating LuxO-HapR pathway (Dinh et al. 2014).

4.6.2 Antibiofilm Activity of Marine Compounds

Compounds from marine sources have been a rich source of bioactive compounds. Marine organisms, plants and even sediment compounds have shown antibacterial and antibiofilm activity against a wide array of bacterial pathogens. Quorum quenching from marine sources gained its importance from early discoveries of

antibiofilm activity of these compounds against biofouling bacteria (Kalia et al. 2014). Marine bacterial exopolysaccharaides have been able to disrupt Vibrio sp., QS and thereby inhibit virulence gene expression and biofilm formation. Many marine bacteria such as *Oceanobacillus and Halomonas* have been identified to produce small molecule QQ enzymes that are able to disrupt *V. cholerae* and *V. parahaemolyticus* biofilm. Bacteria such as *Bacillus indicus*, *B. pumilus* and *Bacillus* sp. SS4 isolated from Palk Bay (Bay of Bengal) were shown to cause substantial inhibition of QS based biofilm formation in Gram-negative bacteria such as *Vibrio* species, *Serratia marcescens* and *P. aeruginosa* PAO1 (Nithya et al. 2010, 2011; Musthafa et al. 2011). Other major sources of antibiofilm compounds are halogenated furanones produced by *Delisea pulchra,* secretions from *Chlamydomonas reinhardtii* which mimic bacterial QS signals, bromoperoxidase produced by algae *Laminaria digitata* which deactives AHL molecules, *Ahnfeltiopsis flabelliformis,* red algae that produces α-D-galactopyranosyl-(1 → 2)-glycerol (Floridoside), betonicine and isoethionic acid which produces QS analogues (Gram et al. 1996; Manefield et al. 2000; Borchardt et al. 2001; Teplitski et al. 2004; Kim et al. 2007; Musthafa et al. 2011).

4.6.3 Activity of Synthetic QS Analogues

Many synthetic analogues have been identified to target QS molecules of Gram negative bacteria including *V. cholerae*. Though most of the QQ studies of synthetic quorum sensing inhibitors have been carried out in *V. fischeri*, the compound being translated to target QS systems of *V. cholerae* is not very difficult. Probable QQ compounds that could be used against *V. cholerae* are 4-hydroxy cis or trans analogs of HSL ring of signal molecule 3OC8HSL which have been found effective in inhibiting *V. fischeri* LuxR based QS reporter system, N-(heptyl-sulfanyl acetyl)-L-HSL (HepS-AHL) identified as potent LuxR inhibitor in *V. fischeri,* N-sulfonyl-HSL (with a pentyl chain), identified as an potent LuxR analogue in *V. fischeri*, Hexyl-4,5-dihydroxy-2,3-pentaedione identified to interfere QS in *V. harveyi* and thereby reduce its bioluminescence and Furanone C-30 which reduce virulence gene expression of *V. anguillarum* (Schaefer et al. 1996; Olsen et al. 2002; Castang et al. 2004; Rasch et al. 2004; Persson et al. 2005; Lowery et al. 2009). Nanoparticle therapy has also been proved to be effective against bacterial biofilms (Dobrucka and Długaszewska 2015; Szweda et al. 2015; Ahiwale et al. 2017). Quest for a single quorum quenching molecule that can limit quorum sensing in multiple pathogens is underway (Koul and Kalia 2017).

4.7 Advantage of Antibiofilm Drugs Over Antibiotics

Biofilms are generally insensitive to antibiotics. However, use of antibiotics or antimicrobial agents is effective against planktonic bacteria. Planktonic bacterial cells are known to be 1000- times sensitive to antibiotics compared to their biofilm counterparts (Rasmussen and Givskov 2006). Antibiotics and other antimicrobial agents generally act by inhibiting the growth of bacterial cells by interfering with their major metabolic or biosynthesis pathway killing them. Biofilm mode of life helps bacteria to survive the harsh antibiotic treatment. Within the biofilm, bacteria have the ability to exist as heterogeneous population and have a reduced metabolic activity (Mah et al. 2003). Bacterial cells with reduced metabolic activity have been identified to be inherently more resistant to antimicrobial therapies (Coates and Hu 2008).

Prolonged persistence of such antimicrobial compounds can induce toxicity to not just the target microorganism but also to non-target beneficial microbes existing in the particular environment. Another major problem in using antimicrobial agents over long period of time is the emerging antibiotic resistance. Multidrug resistant bacteria have now days become a global problem aggravating mortality due to infections (Neu 1992). This escalation of antimicrobial resistance among bacterial pathogens worldwide has necessitated an urgent need to look for alternative strategies to combat bacterial infections by reducing virulence rather than by killing the bacteria.

4.8 Combinatorial Therapy of Antibiofilm Agents and Antibiotics

Since the discovery of persistent antibiotic resistant cells in bacterial biofilms, alternative approaches to control bacterial diseases by using a combination of quorum sensing inhibitors that inhibit or disperse the biofilm and antibiotics that kill the dispersed bacterial cells have gained importance. Co-dosing of QSI/biofilm inhibitors with sub-inhibitory concentration of an antibiotic that can kill the bacteria that become sensitive to antibiotics upon release from biofilms in turn reduces the chance of antimicrobial resistance as these compounds target biofilm/QS pathways and does not affect bacterial growth. A pictorial representation of the combinatorial therapy that could be used to treat cholera has been depicted in Fig. 4.2.

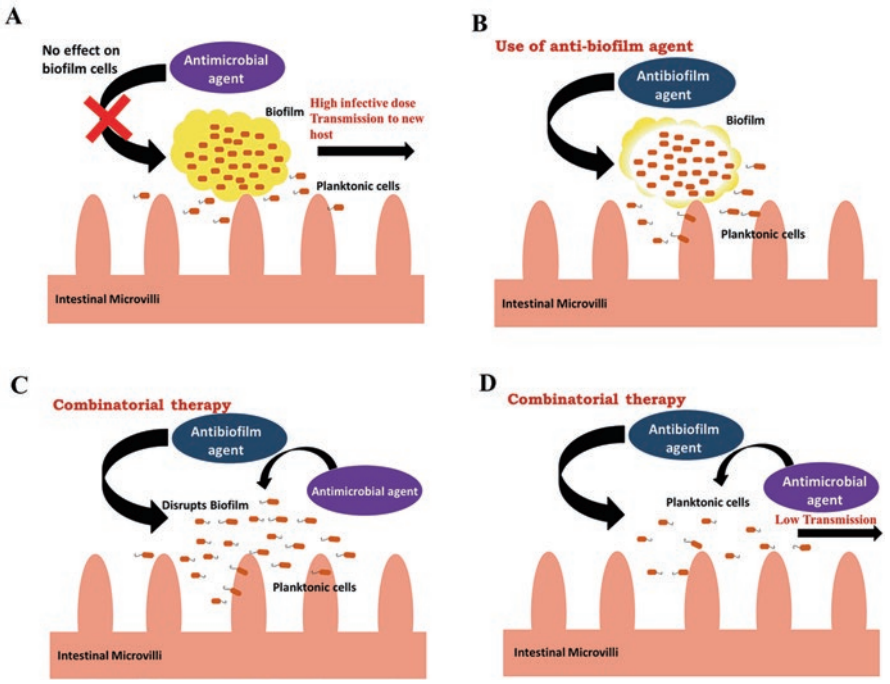

Fig. 4.2 (**a**) Conventional therapy of antibacterial agent alone (**b**) Use of anti-biofilm agent (**c** & **d**) Combinatorial therapy using anti-biofilm agent and antibacterial agents

4.9 Conclusion

The escalation of antimicrobial resistance among bacterial pathogens worldwide is becoming a critical concern. This necessitates an urgent need to look for alternative strategies to combat bacterial infections by reducing virulence rather than by killing the bacteria. In this context, scientists from all over the world are trying to develop novel therapeutic strategies that give importance to bacteriostatic compounds rather than bacteriocidal drugs. Similar to many opportunistic pathogens *V.cholerae* also rely on Quorum sensing, a bacterial cell to cell communication system for biofilm formation and virulence character expression. Quorum quenching or antipathogenic approach and biofilm inhibition will be the promising alternative strategies to contain the disease in future. Vibrios that reside within mature biofilms are highly resistant to antibiotics and host immune response due to the complex architecture and composition of the extracellular matrix. Recent studies have shown the key role

played by the biofilm mode of life adapted by vibrios in the emergence of resistant strains, pathogenicity, host colonization and survival in the natural as well as human niches of vibrio species. Small molecules that can inhibit biofilm formation of *V.cholerae* and/or disrupt biofilms will greatly reduce transmission potential of the bacteria especially in epidemic situations. Inhibiting quorum sensing mechanism appears to be an ideal alternative to conventional therapy as *V.cholerae* suppresses both virulence and biofilm formation at high cell densities.

4.10 Opinion

Previous studies have strongly associated biofilm formation to the virulence of *V.cholerae*. Thus targeting biofilm formation is considered as a potential anti-virulent strategy to treat infections caused by bacterial pathogens. To the best of our knowledge, there is no antibiofilm compound that is clinically approved thus far. In this context, discovering potential Quorum Quenching/anti-biofilm compounds against vibrios is highly warranted. This can be achieved by employing two strategies: (1) Screening natural sources especially from anti-diarrheal plants (formulation of herbal extracts), actinomycetes (small molecules) and well characterised phytochemicals and peptides. (2) Identification of conserved biofilm inhibiting/quorum quenching targets by employing multi-omic approaches such as transcriptomics and proteomics. The identified targets could be used in modern computational based drug discovery approaches for accelerating the development of broad spectrum anti-biofilm compounds against the major pathogens in the Vibrionaceae family. It has also been suggested that targeting the quorum sensing system and biofilm forming ability, which could disarm the bacteria may offer a affirming avenue to fray both pathogenesis as well as antibiotic resistance. Hence, by taking it as a top priority research agenda will definitely help to move forward to control the disease. Studies in some bacterial species have successfully demonstrated that these quorum sensing inhibitors can specifically bind to target proteins and inhibit virulence gene expression. However, the efficacy of these anti-quorum sensing drug targets for the inhibition of *V.cholerae* quorum sensing has not been extensively evaluated. A strengthened international networking and improved coordination among researchers active in cholera research program will accelerate the program at large.

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