Review paper

Quorum Sensing Inhibitors: The Novel Bacterial Infection Disrupting Agents

Abstract

With increasing misuse of antimicrobial substances in medical settings, it became imminent to search for alternative pathogen control means to aid in preventing disease spread. Among such alternatives is the action upon virulent gene expression of the pathogen itself. A possible approach in this direction is the search for quorum sensing inhibitors from different components of the environment. In the last decade, a great interest in the search for new and novel anti-quorum sensing substances from plant and microbial origin has been observed. In this review, we are trying to follow up what has been published in this direction showing areas of interest for researchers to follow.

1. Introduction

The consensus regarding prokaryotic cell discovery is that these simple organisms have a unicellular existence and only divide to produce more of their kind, and that they are solitary and respond to external stimuli independently of each other (Juhas, *et al*., 2005 ; Reading and Sperandi, 2006). However, detailed studies of prokaryotic life in different environments have lead to the conclusion that planktonic microbial growth rarely exists in nature (Vu, *et al*., 2009). Recently with increasing research, a radical change in this concept occurred. It was found that a large number of developmental processes and behavioral characters are not directly related to planktonic growth, but are more related to multicellular organization (Gera and Srivastava, 2006 ; Njoroge and Sperandio, 2009). The first incidence of such biological response came to light in the 1970s (Lyon and Muir, 2003 ; Bassler and Miller, 2006) with the discovery of luminescence production by a marine bacteria, *Vibrio fischeri* (Zhang and Dong, 2004 ; Gera and Srivastava, 2006 ; Vikram, *et al.*, 2010 ; Brackman, *et al*., 2011b). It was observed that when these bacteria are free-living in sea water they are non-luminescent and this was attributed to low density of bacterial cells (Uroz, *et al*., 2009). However, when

grown to high cell densities in the laboratory, they produce bioluminescence with blue-green light (Borg and Sams, 2014). Moreover, this bacterium commonly forms symbiotic relationships with some fish and squid species (Defoirdt, *et al*., 2008 ; Uroz, *et al*., 2009). These marine animals carry a specialized organ called the light organ, in which the bacteria are housed (Brelles-Marino and Bedmar, 2001 ; and Lyon and Muir, 2003 ; Gera and Srivastava, 2006 ; Uroz, *et al*., 2009). Turning from non-lumiscent to lumiscent in *V. fischeri* is the responsibility of autoinducing substances which were identified as Acyl homoserine lactone (AHL) (Defoirdt, *et al*., 2008). Bacteria use AHL signals to monitor their own population density and synchronize target gene expression, a cell-to-cell communication mechanism known as quorum sensing (QS) (Zhang and Dong, 2004 ; Uroz, *et al*., 2009 ; Vikram, *et al*., 2010).

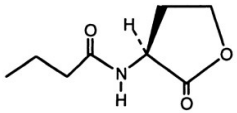
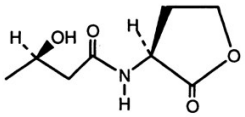
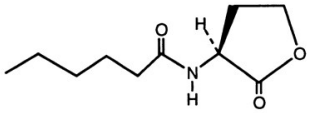
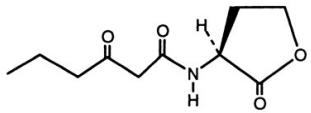
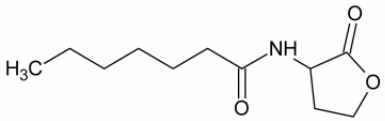
The term “quorum sensing” was first introduced in 1994 by Fuqua, *et al*. (Fuqua, *et al*., 1994 ; Dunn and Stabb, 2007 ; Williams, *et al*., 2007). QS is a process that enables bacteria to communicate by production, release and detection of secreted signaling molecules called autoinducers (Vikram, *et al*., 2010 ; Lintz, *et al*., 2011). This process enables a population of bacteria to regulate gene expression collectively (Ma, *et al*., 2013) which regulates behavior on a community-wide scale (Henke and Bassler, 2004 ; Zhou, *et al*., 2014) where gene expression is controlled in response to changes in cell-population density (Vikram, *et al*., 2010). As the density of bacteria increases, the extracellular concentration of the autoinducer increases until it reaches a threshold level (Borg and Sams, 2014). At this point, the bacteria respond with a population- wide alteration in gene expression (Henke and Bassler, 2004 ; Ng and Bassler, 2009 ; Lv, *et al*., 2014). Interestingly, bacteria can also control the local concentration of autoinducer by degrading signals that they have already been generated (Uroz, *et al*., 2009). Autoinducers may simply be degraded by the bacteria to

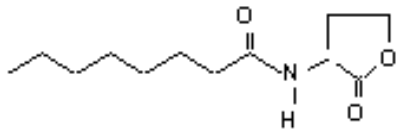
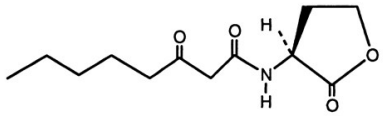
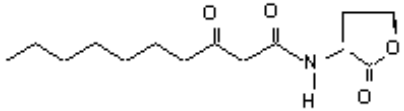
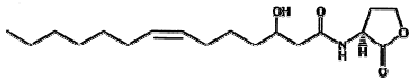
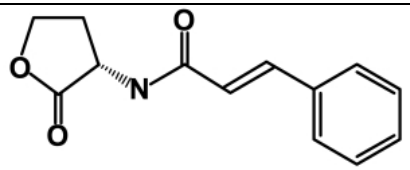
be used as nutrient source of carbon, nitrogen, or energy (Dunn and Stabb, 2007 ; Uroz, *et al*., 2009).

2.0 Quorum sensing autoinducers

QS is widespread in the bacterial world (Cotar, 2013 ; Lv, *et al*., 2014). Processes controlled by quorum sensing are unproductive when undertaken by an individual bacterium but become effective when undertaken by the group (Abu-Salah and Dhand, 2011). Cell-to-cell communication can occur within and between bacterial species, and between bacteria and their eukaryotic hosts (Henke and Bassler, 2004 ; Vikram, *et al*., 2010). QS signaling molecules have general criteria; their production occurs during specific stages of growth and under certain physiological conditions in response to certain changes in the environment (Vikram, *et al*., 2010). They accumulate extracellularly up to threshold concentration and are recognized by specific receptors. The end result is a change in gene expression thus eliciting an appropriate response (Winzer, *et al*., 2002 ; Zhou, *et al*., 2014).

Table 1: Some AHLs, their producing microorganisms and some of their observed density-dependent regulatory effects (Guan, *et* al., 2000; Fray, 2002; Pierson, 2005; Ahlgren *et al*., 2011).

AHL Molecule	Structure	Bacteria	Behavior/Function
N-butanoyl-homoserine lactone (C_4-HSL)		*Serratia liquefaciens*	Cell motility/swarming
N-3-hydroxy-butanoyl-homoserine lactone ($3OHC_4$-HSL)		*Vibrio harveyi*	Bioluminescence
N-hexanoyl-homoserine lactone (C_6-HSL)		*Chromobacterium violaceum*	Pigments/antibiotics/chitinase
N-3-oxohexanoyl-homoserine lactone ($3OC_6$-HSL)		*Erwinia carotovora* *Pseudomonas aureofaciens*	Pathogenicity/antibiotics Biocontrol activity/antibiotics
N-heptanoyl-homoserine lactone (C_7-HSL)		*Rhizobium leguminosarum*	?

N-octanoyl-homoserine lactone (C_8-HSL)		*Ralstonia solanacearum*	Pathogenicity in plants
N-3-oxooctanoyl-homoserine lactone ($3OC_8$-HSL)		*Agrobacterium tumefaciens*	Conjugation
N-3-oxododecanoyl-homoserine lactone ($3OC_{10}$-HSL)		*Pseudomonas aeruginosa*	Pathogenicity
N-3-hydroxy-7-cis-tetradecenoyl-homoserine lactone ($3OHC_{14}$-HSL)		*Rhizobium leguminosarum*	Growth inhibition/rhizosphere genes
Cinnamoyl-homoserine lactone		*Bradyrhizobium* spp.	Stem nodulation

QS signals produced by microorganisms interfere effectively with QS signaling in other organisms (Vikram, *et al*., 2010 ; Cotar, 2013). The bacterial QS system shows a high degree of signal specificity because of the structural differences in QS signals and the binding domain of the receptor proteins (Table 1) (Rajesh and Rai, 2014 ; Sieper, *et al*., 2014). Many QS signals, especially those from the same family of bacteria, share a certain degree of structural similarity, which may suggest the possibility of cross species signal interference (Zhang and Dong, 2004 ; Sieper, *et al*., 2014). Bacteria can resist signal interference by producing enzymes that destroy the induction signals from other species (Rajesh and Rai, 2014). AHL- lactonases produced by *Bacillus* spp. (Liu, *et al*., 2005), *Agrobacterium tumefaciens* and *Arthobacter* spp. are good example (Uroz, *et al*., 2009). AHL-acylase which breaks the amide linkage between fatty acid chain and homoserine lactone moiety has been identified in *Variovorax paradoxus*, *Ralstonia* sp., and *P. aeruginosa* (Zhang and Dong, 2004 ; Liu, *et al*., 2005).

Dong, *et al*. (2002) screened 800 bacterial isolates from soil and plants for AHL inactivation substances. Twenty isolates out of 800 exhibited AHL inactivation ability. The best 8 isolates that gave the highest activity were characterized by their 16S rRNA. The bacterial isolates were very closely related to *Bacillus thuringiensis* with different subspecies. *B. thuringiensis* has both plasmid and DNA genes but it was found that the AHL inactivation ability was due to genes located in the chromosome not the plasmid since some of the isolates that lacked a plasmid still displayed AHL inactivation activity. Dong, *et al*. (2002) suggested that the insecticidal-active *B. thuringiensis* can be used as biocontrol agent against bacterial diseases that are mediated by AHL QS mechanism.

Bacteria are highly adaptive organisms capable of rapidly responding to changes in their environment (Lv, *et al*., 2014). The key to adaptation is the large reservoir of genes and the pathways of gene control that turn on and off in response to environmental changes or changes in growth (Roy, *et al*., 2011 ; Boncompte, *et al*., 2011). QS signals are highly accumulated at the mid-exponential phase (Borg and Sams, 2014) but the accumulated signals disappear rapidly after the bacterial cells enter the stationary phase, which is attributed to the expression of lactonases (Rajesh and Rai, 2014). Switching off the QS-controlled gene expression could ensure sufficient energy and resources for synthesis of essential stress-responsive proteins and enzymes (Zhang and Dong, 2004 ; Rajesh and Rai, 2014). Additionally, the AHL signal concentration may allow the population of bacteria to distinguish between growth in planktonic culture and growth in a biofilm, and so it allows the population to modulate gene expression.

2.1 QS in Gram negative bacteria

In gram negative bacteria, the most used system is LuxI/R quorum sensing system (Borg and Sams, 2014). LuxI-type enzymes synthesize the AHL autoinducers (Lintz, *et al*., 2011), while LuxR-type proteins (cytoplasmic receptor protein) bind their cognate autoinducers and control transcription of target genes (Figure 1). At low cell density, bacteria produce a basal level of AHL and release it outside the cells (Lv, *et al*., 2014; Brackman and Coenye, 2015). As the bacteria multiply, they produce more AHL that accumulates in the surrounding, then diffuse back to the cell and activates the R protein by binding to it forming R-AHL complex (Lintz, *et al*., 2011). This complex interacts with target promoter and induces expression of target genes (Henke and Bassler, 2004; Zhang and Dong, 2004 ; Lv, *et al*., 2014). In general AHL molecules that have been identified contain 4-14-carbon acyl side chains and either an oxidized, hydroxyl, or no substitution at the third carbon (Kievit and Iglewski, 2000 ; Sieper, *et al*., 2014).

2.2 QS in Gram positive bacteria

Gram positive bacteria use modified oligopeptides as autoinducers, which are exported from the cell via an ATP-binding cassette transporter (Kleerebezem, *et* al., 1997) and can be detected by a two-component signal transduction protein called "sensor histidine kinase" which is a membrane bound protein (Kalia, 2013 ; Brackman and Coenye, 2015). The information is channeled to target genes through a phospho-relay to a response regulator protein (Zhang and Dong, 2004 ; Estrada, *et al.*, 2010) (Figure 1).

2.3 Autoinducer-2 (AI-2)

A third, less known class of the QS autoinducers that are found in both Gram negative and Gram positive bacteria is the AI-2 (Henke and Bassler, 2004 ; Juhas, *et al*., 2005 ; Swift, *et al*., 2008; Brackman and Coenye, 2015). AI-2 is considered a universal signal which functions in interspecies cell-to-cell communication. In addition, AI-2 control the virulence factors production of *Neisseria meningitides*, *Vibrio cholera*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Salmonella typhimurium* and *Actinobacillus* spp. (Xavier and Bassler, 2003 ; Swift, *et al*., 2008 ; Zhou *et al*., 2014). In general bacteria have evolved mechanisms to compete with each other at the level of QS (Lyon and Muir, 2003 ; Cotar, 2013) (Figure 1).

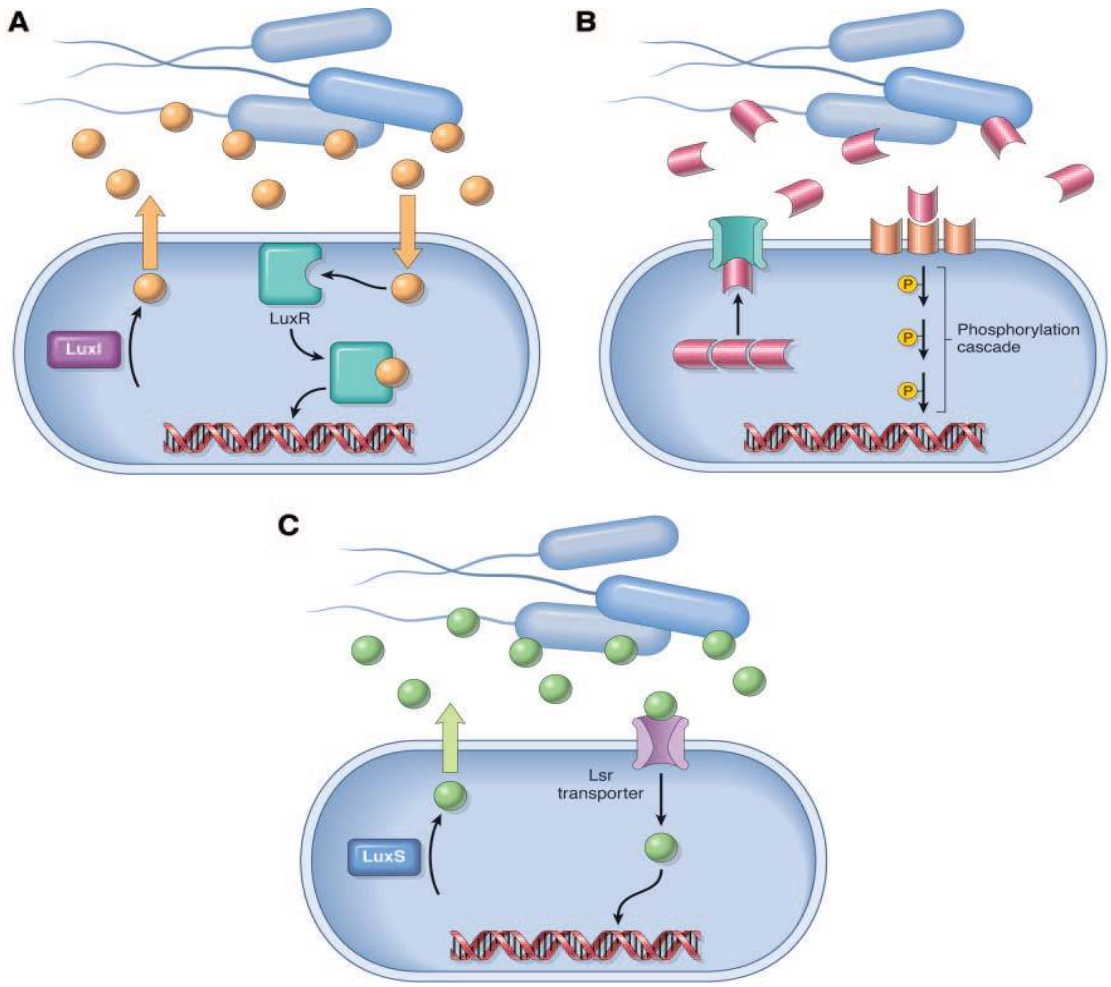


Figure 1: Schematic representation of quorum sensing signaling pathways. The bacteria synthesize and released the AIs (regardless of their chemical nature) which will diffuse to the surrounding environment until they reach a certain threshold concentration; then they will diffuse back to the cell, bind to a specific receptor and activate the transcription of target genes. A; LuxI/R in Gram negative bacteria.B; AIP in Gram positive bacteria. In this model, the AIs will not diffuse back into the cells; instead, they will bind to a surface receptor which will induce a signal transduction cascade that will ultimately result in altered gene expression. C; LuxS/Lsr transporter in Gram negative and Gram positive.

3. Role of QS

QS plays a role in regulating bacterial functions such as the synthesis of antibiotics, the production of virulence factors, exopolysaccharide biosynthesis, motility, bacterial swarming, plasmid conjugal transfer and transition into the stationary phase (Givskov, *et al.*,

1996 ; Brelles-Marino and Bedmar, 2001 ; Abu-Salah and Dhand, 2011). QS also allows the bacteria to control certain molecular functions such as DNA replication, RNA transcription, cell division, and amino acid metabolism (Estrada, *et al.*, 2010). Such control over these functions may provide competitive advantages to bacteria to maintain themselves in ecological niches (Vikram, *et al*., 2010). Moreover, the ability of bacteria to counteract the QS signaling of their competitors could also significantly increase or multiply their competitive strength in ecosystems (Zhang and Dong, 2004 ; Roy, *et al.*, 2011 ; Sieper, *et al*., 2014).

Among the numerous biological processes mediated by QS is the formation of biofilm (Lazer, 2011 ; Rajesh and Rai, 2014 ; Brackman and Coenye, 2015). Bacteria can form biofilm in any place where they can form colonies i.e. on a wide array of biological and non-biological surfaces including water piping, natural aquatic systems, plant surfaces, tooth enamel, and implanted medical devices (Stephens, 2002 ; Kociolek, 2009 ; Lv, *et al*., 2014). Over 99% of microorganisms on Earth live in biofilm forms (Vu, *et al*., 2009). Biofilms are physical structures made up of microcolonies which serve as the basic unit of the greater biofilm structure (Zhou, *et al*., 2014). These microcolonies are considered as bacterial cells embedded in a matrix of EPS (Abu-Salah and Dhand, 2011; Brackman and Coenye, 2015). Bacteria in biofilm proliferate on the attachment surfaces and continue to expand until the community growth is limited by substrate availability due to increase diffusion distance (Heilmann, *et al*., 1997 ; Hong, *et al*., 2012). At this time, the biofilm reaches a steady state and is considered as a mature biofilm, which consist of towers and mushrooms of cells embedded in exopolysaccharide (EPS) (Hentzer and Givskov, 2003 ; Lemon, *et al*., 2008).

The formation of bacterial biofilms *in vitro* involves certain steps for all kinds of bacteria that are capable of constructing biofilm structure (Figure 2) (Hong, *et al*., 2012) starting with the reversible attachment of bacterial cells to the surface and followed by

irreversible attachment that is mediated by the exopolysaccharide (EPS) materials. The formation of microcolonies which are considered as the first stage of biofilm maturation then follows (Zhou, *et al*., 2014). Formation of mature biofilm which has a 3D structure that contains cells packed to each other and connected with channels to transport nutrient and removal of waste must then occur (Hong, *et al*., 2012). The last stage is the detachment and the dispersion of cells from the biofilm and initiation of new biofilm (Bjarnsholt and Givskov, 2007 ; Annous, *et al*., 2009).

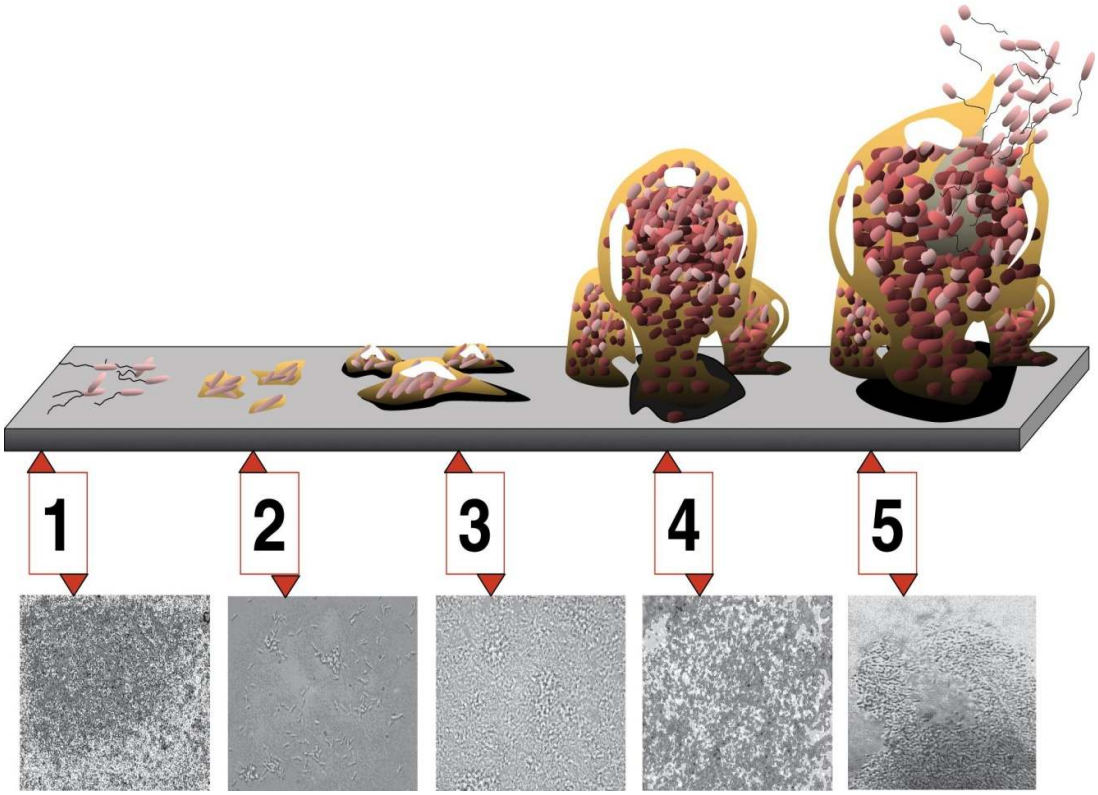


Figure 2. Diagram of the *P. aeruginosa* biofilm-maturation pathway. Most free-living bacterial cells have the ability to attach to a wide variety of biotic and/or abiotic surfaces via numerous adhesion apparatuses and secreted substances. Attached cells will proliferate on a surface and start producing mainly exopolysaccharides which will enable them to actively move into microcolonies. The high-density microcolonies differentiate into mature biofilms by a 3OC12-HSL-dependent mechanism (Parsek and Greenberg, 2000). A mature biofilm exhibits three-dimensional structures such as mushroom or tower shapes.

It must be kept in mind that eradication of pathogenic biofilms would be facilitated by better understanding of their development and metabolism (Stephens, 2002 ; Abu-Salah and Dhand, 2011 ; Hong, *et al*., 2012). Prevention of biofilm formation in/on biomedical devices is usually via prophylaxis with antimicrobial agents that prevent biofilm formation by eradication of the first microbial attachment to the device (Danese, 2002).

Biofilm-forming bacteria are responsible for most human infections (Abu-Salah and Dhand, 2011; Brackman and Coenye, 2015), including infections in the urinary tract, middle ear, mouth, heart, and lungs, as well as those associated with medical devices such as catheters, prostheses, artificial heart valves and replacement joints (Kociolek, 2009 ; Njoroge and Sperandio, 2009 ; Valentine, *et al*., 2014; Alasil, et al., 2015)

The major problem with biofilm formation is the increase of antibiotic resistance from 10 - 1000 times more than free living bacteria (Vikram, *et al*., 2010 ; Packiavathy, *et al*., 2012). By forming biofilm, bacteria may resist antibiotics by reducing the penetration of the antibiotic through the polysaccharide protected area (Abu-Salah and Dhand, 2011 ; Hong, *et al*., 2012 ; Brackman and Coenye, 2015), or a suitable anaerobic environment within the biofilm may be provided (Childs, 2006 ; Kociolek, 2009 ; Zhou, *et al*., 2014). The organization of the biofilm can also provide more specialization of the bacteria which increases the protection (Costerton, *et al.*, 1999). In addition, biofilm environment allows a large number of bacteria to be in close proximity (Bjarnsholt and Givskov, 2008 ; Lemon, *et al*., 2008 ; Vikram, *et al*., 2010). This dense and diffusion-limited biofilm matrix provides ideal conditions for the accumulation of signal molecules and a protected environment for bacteria to induce quorum sensing regulated virulence factors (Hentzer and Givskov, 2003 ; Valentine, *et al*., 2014). Biofilms can be made by a single or multiple bacteria species (Mah and O'Toole, 2001 ; Lv, *et al*., 2014)

4. Plant-associated bacteria and quorum sensing

Plant associated epiphytic bacteria form a film or colonies on the surfaces of a wide variety of plants (Annous, *et al*., 2009). It is reported that approximately 30-80% of epiphytic bacteria are capable of forming biofilms. This ability is considered as a survival strategy for the bacteria to withstand harsh environments on the plant surfaces (Kanagasabhapathy, *et al*., 2006 ; Annous, *et al*., 2009). In addition, exopolysaccharides (EPS) that are released in biofilm aid in protecting bacterial cells from desiccation (Annous, *et al*., 2009).

In a study by Veselova, *et al*. (2003), 300 strains representing 6 genera and 18 species of soil-borne and plant-associated gram negative bacteria were successfully isolated. AHL production was observed in 17.5% of the screened bacterial strains. All strains of *Erwinia herbicola* produced AHL, as well as 41 out of 239 (17%) strains of *Pseudomonas* species, and all isolated strains of *Xanthomonas ampelina*, *X. campestrist*, *X. malvacearum*, *X. translucens*, *X.* v*esicatoria*, and *Pantoea stewarii*. AHL production was detected using two reporter strains: *Agrobacterium tumefaciens* and *Chromobacterium violaceum* CV026. It was found that plant- associated bacteria synthesized AHL more frequently than did free soil-borne strains. It is suggested that the closer the relationship of the bacteria with the plant host, the higher the probability that it produces AHL (Veselova, *et al*., 2003).

Schreiber, *et al*. (2005) found that the bacteria associated with plant surfaces increased water permeability of the covering cuticle. By this, the bacteria had the ability to change basic physiochemical properties of leaf surfaces. The bacterial ability to change leaf surface properties such as wet ability and cuticular permeability was found to increase epiphytic fitness, since these changes caused increased rate of organic and inorganic compound leaching from the leaf interior through the cuticle to the leaf surface. These

changes improved the living conditions for these bacteria in their natural habitat (Schreiber, *et al*., 2005).

Several bacterial species that interact with plants including pathogenic, non-pathogenic and symbiotic bacteria have been shown to produce AHL- like compounds (Rajesh and Rai, 2014). Generally, in plant-associated bacteria, QS is used to control a broad range of traits in both plant and bacteria such as growth inhibition and nodulation in plant host and the production of antibiotics in both (Brelles-Marino and Bedmar, 2001 ; Braeken, *et al*., 2008 ; Estrada, *et al*., 2010).

Higher plants might also synthesize and secret compounds that mimic the activity of the bacterial AHL (Sieper, *et al*., 2014). The exudates of the pea seedlings contain several activities that mimic AHL signal and stimulate or inhibit AHL- regulated actions (Gonzalez and Keshavav, 2006 ; Karamanoli and Lindow, 2006). Pea and crown vetch-secrete compounds inhibited AHL mediated violacein production in *C. violaceum* CV 026 reporter strain, while rice, soybean, and tomato activated AHL- dependent swarming in *Serracia liquefaciens*. Plant-produced AHL could be used to modify the behavior of symbiotic or pathogenic bacteria (Brelles-Marino and Bedmar, 2001). Actually, little is known about the exact role of these compounds in plants but they may be important for the co-evolution between plants and their associated bacteria (Brelles-Marino and Bedmar, 2001 ; Sieper, *et al*., 2014).

Barnard, *et al*. (2007) found that QS played an important role in the regulation of secondary metabolite production, virulence factors, and biofilm formation of *Erwinia* and other soft-rotting bacteria.

5. Quorum sensing inhibitors (QSI)

The discovery of antibiotics in the 20th century helped in controlling a large number of life-threatening diseases (Hentzer, *et al.*, 2003 ; Saga and Yamaguchi, 2009). Along the 20th century, the excessive and misuse of antibiotics has lead to the development of multidrug-resistant strains (Chopra, *et al*., 1997 ; Hentzer, *et al.*, 2003 ; Kalia, 2013; Al asil, et al., 2015). Accordingly, the development of alternative treatment methods is a must need (Maeda, *et al*., 2012 ; Mahasneh, 2013). Considering the biological functions that are under the control of QS mechanism and the rapid development of antibiotic-resistant bacterial species, researchers are looking for practical approaches to interfere with microbial QS (Uroz, *et al*., 2009). Such approaches may be titled as 'anti-pathogenic', 'quorum quenching', or 'signal interference' (Zhang and Dong, 2004 ; Vikram, *et al*., 2010).

There are certain criteria for selecting quorum sensing inhibitors (QSI). To be useful QSI should be small sized molecules with the ability to efficiently reduce QS regulated gene expression (Yang, *et al*., 2009), have high specificity for a given QS regulator (Rajesh and Rai, 2014) and be chemically stable. QSIs with such characteristics pose a challenge to bacteria which are unable to resist them with time, and by this it will have no effect on the beneficial normal flora (Kalia, 2013).

Researchers developed several ways to inhibit quorum sensing which include inhibition of autoinducer synthesis, autoinducer receptor antagonism, sequestration of autoinducers, degradation of autoinducers, inhibition of autoinducer transport/ secretion, and blocking autoinducer receptors via antibodies (Nanting, *et al*., 2009 ; Uroz, *et al*., 2009).

The algae *Delisea pulchra* which is endemic to the South-eastern coast of Australia (Manefield, *et al*., 1999) produces halogenated furanones that inhibit the swarming of *Serratia liquefaciens* and inhibit biofilm development by *Pseudomonas aeruginosa* (Bjarnsholt and Givskov, 2007). Halogenated furanones are structural analogs of AHL, and function by reducing the half-life of LuxR type proteins (Brelles-Marino and Bedmar, 2001). At the same time, these halogenated furanones also inhibit quorum sensing without interference with the LuxI/R system, which strongly suggests that it may interfere with several classes of autoinducer signals (Henke and Bassler, 2004 ; and Khmel and Metlitskaya, 2006) figure 3.

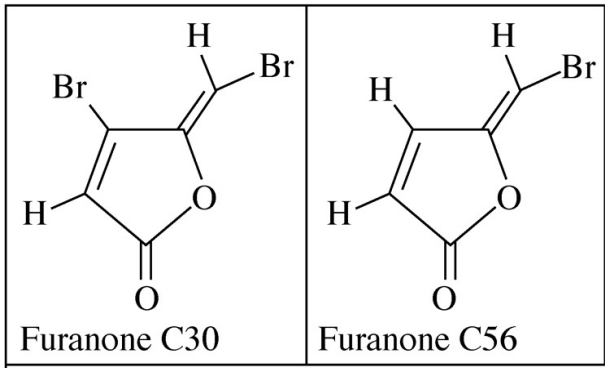


Figure 3: structure of furanones C30 and C56

Martinelli, *et al*. (2004) studied the effect of 30 naturally and chemically synthesized furanones on violacein production in *C. violaceum*. They found that some of these furanones had the ability to interfere with bacterial growth and lead to reduction of violacein as a result of growth interference. Most of the other furanones have a direct effect on the violacein production through a competitive interaction with LuxR protein and have no interference with bacterial growth.

He, *et al*. (2012) studied the effect of furanone C-30 on the ability of *Streptococcus mutans* to form biofilm. Different concentrations of furanone and different time points of

biofilm formation (4, 14, and 24 h) were investigated. They also examined the structure of the biofilm and its thickness by the confocal laser scanning microscope which revealed reduction in biofilm volume and thickness with increasing furanone concentration. He, *et al*. (2012) found that the furanone C-30 used in this study can inhibit biofilm formation by *S. mutans* and it does not affect the bacterial growth rate itself.

Another interesting and applicable use of furanones was examined by Shoharani and Agrawal, (2010) where they studied the enhancement of fermented milk shelf life by using furanone compounds. They isolated 22 bacterial isolates from fermented milk; 50% of the isolates were *Leuconostoc mesenteroides* (probiotic bacteria), and the other 50% of isolates were *Pseudomonas* spp. Some *Pseudomonas* are responsible for milk spoilage; its autoinducer (hexanoylhomoserine lactone) was detected in the milk. The same authors tested 2 types of furanones: 2(5H)-furanones and bromofuranones which showed ability to reduce some of the virulence factors produced by *Pseudomonas spp* and, as a result, the use of such furanones to increases the shelf life of milk up to 9 days instead of 4.

Baveja, *et al.* (2004) tested the ability of furanones to physically adhere to biomaterials to inhibit *Staphylococcus epidermidis* and its slime production. It was found that there was no significant change in the material characteristics after furanone coating, while the bacterial load and slime production on all furanone-coated materials were significantly reduced.

Alasil, et al. (2015) tested the activity of novel species of *Panibacillus* strain 139SI which was isolated from soil samples, to produce QSI substances against QS- controlled virulence factors and biofilm formation of *Psedumonas aeruginosa* both *in vitro* and *in vivo*. They found a significant decrease in the QS- controlled LasA protease, LasB elastase

pyoverdin, and biofilm formation. In addition, the extract significantly prolonged the survival times of rats and facilitated the clearance of biofilm infection from rats infected lungs.

Nithya, *et al*. (2010 a) tested the activity of 46 marine bacterial extracts against *P. aeruginosa* biofilm formation *in vitro*. Eleven extracts that have activity as antibiofilm formation were successfully isolated from *Bacillus pumilus, B. indicus, B. arsenicus, Halonacillus trueperi, Ferrimonas balearica,* and *Marinobacter hydrocarbonoclasticus*. Three of these extracts had the ability to inhibit biofilm formation up to 95 % at their lowest inhibitory concentration, and they also had the ability to destroy fully developed mature biofilm.

Nithya, *et al*. (2010 b), showed that *B. pumilus* isolated from the Palk Bay, had the ability to reduce the accumulation of AHL in *C. violaceum* which interferes with the QS system. In addition, a significant reduction in the virulence factors of *P. aeruginosa*, and 61 % inhibition of biofilm formation of *Serratia marcescens* were observed. During the characterization of the active secreted materials it was found that the activity was due to acylase enzyme.

Bakkiyaraj and Pandian, (2010) studied the activity of coral-associated *Actinomycetes* against *S. aureus* and MRSA biofilm formation both *in vitro* and *in vivo*. The methanolic extract showed no inhibitory activity against the growth of the bacteria; the activity was only observed against the biofilm formation. The *in vitro* result was confirmed by testing the activity of the extract *in vivo* using the nematode *Caenorhabditis elegans*. Thenmozhi, *et al*. (2009) tested the extract of coral-associated bacteria and was able to inhibit biofilm formation of *Streptococcus pyogenes* with no antimicrobial activity, suggesting QSI only substantiated by the ability to reduce violacein production by *C. violaceum* up to 80 %.

Medicinal plants have also been studied for their activity against biofilm formation of bacteria and the interference with QS as inhibitors for the film formation (Vikram, *et al.*, 2010). Quave, *et al*. (2008) have tested the activity of the extract from Italian medicinal plants against the adhesion, biofilm formation and planktonic growth of MRSA. Five tested plant extracts showed no activity against planktonic cells which indicated limited bacteriostatic activity. At the same time, they exhibited dose dependent inhibition of biofilm formation and bacterial adhesion. These plants were: *Arundodonax, Ballotanigra, Juglansregia, Leopoldiacomosa,* and *Marrubiumvulgare.*

Packiavathy, *et al.* (2012) examined the antibiofilm and QSI activity of *Cuminum cyminum* extract and found that it strongly interferes with AHL-regulated physiological functions that are coupled with biofilm formation. Adonizio, *et al*. (2008) studied the activity of six South Florida plant extracts against the virulence factors produced by *P. aeruginosa*. It was found that the extract of *Conocarpus erectus, Chamaesyce hypericifolia, Callistemon viminalis, Bucida buceras, Tetrazygia bicolor* and *Quercus virginian,* exhibited QSI activity against *C. violaceum* and *A. tumefaciens.* They observed that plant materials that contain epiphytic bacteria have higher QSI activity in comparison with their washed counterpart. It was concluded that these associated bacteria increased the QSI activity of the plant extract. In this same study the activity of these plant extracts against LasA protease, LasB elastase, pyoverdin production, biofilm formation and QS genes was tested. All the tested plant extracts showed effect on the QS gene profile, and only *C. erectus, B. buceras, C. viminalis* extract showed inhibitory activity over the tested virulence factors and biofilm formation, with minor effect over growth.

5.1 Microbial quorum sensing inhibitors

Discovering the microbial world as a source of therapeutic agents began at around the middle of the 20th century (Chopra, *et al*., 1997 ; Hentzer, *et al*., 2003 ; Rasmussen, *et al*., 2005 ; Saga and Yamaguchi, 2009). Even with this short discovery period, it is estimated that nearly 10 % of all known biologically active products are of microbial origin, and now microbially derived biologically active substances became the foundation of modern pharmaceuticals (Saga and Yamaguchi, 2009 ; Penesyan, *et al*., 2010). An emerging source of new bioactive materials in most recent studies is an epiphytic bacterium that is associated with unusual marine environments, animals and plants (Mearns-Spragg, *et al*., 1998 ; Sintim, *et al*., 2010). The exploration of relatively untouched, unusual and extreme environments can also assist in finding novel microorganisms that produce new chemical molecules of new biological characteristics.

Mai, et al. (2015) isolated QSI crude extract from *Leucetta chagosensis* Dendy 1863. They successfully isolated isonaamine A, isonaamidine A and three new alkaloids, these are isonaamine D, di- isonaamidine A and leucettamine D. Isonaamidine A and isonaamine D were identified as inhibitors of three quorum sensing pathways of V. harveyi (CAI-1, AI-2 and harveyi autoinducer), with strongest activity observed for isonaamidine A against AI-2 QS mechanism.

Skinderose, *et al*. (2008) studied 284 extracts of marine organisms from the Great Barrier Reef, Australia, and tested for their ability to produce QSI active substances. Of these extracts, 23% were active in inhibiting LuxR mediated QS, and 56% were active in specific *P. aeruginosa* QS system. Some of these extracts, such as the extract of the sponge *Luffariella variabilis*, was active in both systems.

Kanagasabhapathy, *et al*. (2009) were successful in isolating 96 epibiotic bacteria associated with the marine brown alga *Colpomenia sinusa.* They used *Serratia rubidaea* as indicator strain for quorum sensing inhibitory model. They found that 12 % of the 96 isolates were able to produce QSI, whose activity was observed by inhibition of colour without affecting growth. The active isolates belonged to *Bacillaceae, Pseudomonadaceae, Pseudoalteromonadaceae*, and *Vibrionaceae*.

Weng, *et al*. (2012) studied the QSI activity isolated from different bacteria. Twelve putative isolates out of 500 in the initial screening were isolated. Three of these isolates belonged to the genus *Pseudomonas*. Two strains were able to produce QSI that inhibited *N*-butyl- homoserine lactone. The third strain produced QSI against *A. tumefaciens* autoinducer.

Ma, *et al*. (2013) studied the quorum quenching activity of bacteria from tobacco. A total of 1177 leaf-associated isolates were screened for their ability to disrupt AHL-mediated QS using the biosensor *C. violaceum* CV026. One hundred and sixty-eight strains (14 % of isolates) interfered with AHL activity. One hundred and six of these isolates enzymatically degraded the AHL. While the remaining isolates inhibit QS by other ways of chemical interruptions. Seventy-nine percent of the enzymatically active isolates have lactonase activity. Phylogenetic analysis using 16S rRNA revealed that the leaf associated bacteria belonged to *Bacillus spp*., *Acinetobacter spp*., *Lysinibacillus spp*., *Serratia spp*., *Pseudomonas spp*., and *Myroides spp.*

Abed, *et al*. (2013) isolated QSI compounds from extremophilic microorganisms from hypersaline mat. They successfully isolated five bacterial strains belonging to *Marinobacter*, *Halomonas*, and one archaeal strain belonged to the genus *Haloterrigena*. Extract of all

isolates exhibited QSI activity against *C. violaceum* CV017. Dichloromethane extract of *Marinobacter* resulted in isolation of four related diketopiperazines (DKPs).

5.2 Quorum sensing inhibitors produced by plants

Medicinal plant has long been used in folk medicine and alternative medicine as a source of new antimicrobial agents against different bacterial species (Talib and Mahasneh, 2010 ; Dandpat, *et al*., 2013). Several medicinal plants have been tested for their ability to produce QSI, in order to find suitable safe alternatives for natural furanones (Al- Hussaini and Mahasneh, 2009 a and b). In the studies of Al-Hussaini and Mahasneh the ability of several plant extracts to have QS inhibition activity using the *C. violaceum* (ATCC 12472) testing system was reported. The activities of eight Jordanian plants, using different plant parts were tested. The best antiquorum activity was reported for *Sonchus oleraceus* and *Laurus nobilis*, and moderate activity for the other tested plants was recorded.

Vattem, *et al*. (2007) tested the activity of 13 plant extracts at sublethal concentrations against violacein production by *C. violaceum* and the effect on the swarming motility of *E. coli* 0157:H7 and *P. aeruginosa* PA01. The results indicated that all the tested extracts have activity as QSI, and they explained the mechanism of inhibition as a combination of interference with AHL activity and modulation of the synthesis of AHL. In addition, it was indicated that any phytochemical extracts that inhibited QS also inhibited swarming of pathogenic bacteria, known to be modulated by QS. A very similar study by Koh and Tham, (2011) tested the ability of Chinese medicinal plants to produce QSI. Eight of the selected plants exhibited QSI activity and these were: *Prunusarmenaca, Prunella vulgaris, Nelumbonucifera, Panaxnotoginseng, Punicagranatum, Areca catechu,* and *Imperata cylindrical*. They considered such plants as a rich source of compounds to combat pathogenic bacteria and reduce development of antibiotic resistance.

Choo, *et al*. (2006) studied the activity of vanilla extract over the QS of *C. violaceium* CV026. It was found that at certain concentrations of the extract, inhibitory activity over the violacein production, which occurs in a density dependant manner, was exhibited. By this finding, it was concluded that the extract of vanilla beans exhibited inhibitory activity over the QS system, and they suggest that the intake of vanilla can promote health and reduce pathogenicity.

Zaki, *et al*. (2013) examined the antiquorum sensing activity of some ornamental and medicinal plants of Egypt. The activity of 23 plants was tested by using agar well diffusion method against *C. violaceium* ATCC 12472, six plant extracts exhibited anti-QS. These were the leaves of *Adhatoda vasica Nees, Bauhinia purpurea L., Lantana camara L., Myoporum latetum G.,* the fruits of *Piper longum L.,* and the aerial parts *of Taraxacum officinale.*

Khan, *et al*. (2009) evaluated the QSI activity of plant essential oil against *C. violaceium and P. aeruginosa.* Out of 21 essential oils tested, 4 oils showed varying levels of QSI. The best QSI activity was observed in *Syzygium aromaticum* (clove) oil, followed by the activity of cinnamon, lavender, and peppermint oils. Clove oil showed 78.4 % reduction in violacein and 78 % reduction in swarming motility of *P. aeruginosa*. On the other hand, Olivero, *et al*. (2011), studied the QSI activity of another set of *piper* essential oils. The studied piper species (*Piper bredemeyeri, Piper brachypodom,* and *Piper bogotence*) exhibited more than 50 % inhibition of violacein pigment without affecting bacterial growth and these results highly suggest that plant essential oils can be used as possible antipathogenic drugs.

6. *Pseudomonas aeruginosa* biofilm and QS

P. aeruginosa is a Gram-negative bacterium that can tolerate a wide range of environments (Antunes, *et al*. 2010). This bacterium is an opportunistic pathogen and is commonly associated with nosocomial infections and infections of severely burned individuals. It is a leading cause of death in severe respiratory infections, such as chronic lung infections in cystic fibrosis patients (Boncompte, *et al*., 2011 ; Valentine, *et al*., 2014; Alasil. Et al., 2015). Infections with *P. aeruginosa* are difficult to eradicate due to their high levels of antibiotic resistance and growth in biofilm (Cotar, 2013 ; Singh et al., 2015).

P. aeruginosa has a large bacterial genome (Hoiby, *et al*., 2001 ; Roy, *et al*., 2011). It was estimated that the expression of large numbers of genes of *P. aeruginosa* is controlled by QS systems (Chungani, *et al*., 2001 ; Annous, *et al*., 2009 ; Roy, *et al*., 2011). *P. aeruginosa* produces two AHL molecules, 3OC12-HSL and C4-HSL, which bind to the transcriptional regulators, LasR and RhlR, respectively (Lintz, *et al*., 2011 ; Hong, *et al*., 2012 ; Valentine, *et al*., 2014). Activated LasR complex activates the RhlR for expressing its virulence (Abu-Salah and Dhand, 2011 ; Hong, *et al*., 2012). In addition, the two QS systems are connected to each other by quinolone (Qsc), which binds to QscR receptor protein (Lintz, *et al*., 2011). The three receptors (LasR, RhlR, and QscR) have independent and overlapping regulons, such that LasR regulates RhlR and LasI regulates QscR (Lintz, *et al*., 2011 ; Kalia, 2013).

The size and complexity of the genome may reflect evolutionary adaptation allowing the bacteria to survive in diverse environments and resist the effects of a variety of antimicrobial substances (Schaber, *et al*., 2007 ; Roy, *et al*., 2011 ; Boncompte, *et al*., 2011). These are considered as important factors for their ability to form biofilm (Hoiby, *et al*., 2001; Roy, *et al*., 2011). *P. aeruginosa* produces a large number of virulence factors, which

makes it highly pathogenic for susceptible patients (Shaber, *et al*., 2007 ; Roy, *et al*., 2011). These factors include, but are not limited to, lipopolysaccharide, exoenzymes, endotoxins, and alginate (Hentzer, *et al*., 2002 ; Bjarnsholt and Givskov, 2007 ; Lintz, *et al*., 2011 ; Hong, *et al*., 2012 ; Valentine, *et al*., 2014).

Animal models for biofilm formation can be established in both rats and mice by intratracheal inoculation of bacteria in alginate beads and assayed using bacterial counts, histopathological changes, immune responses, and the efficiency of the antibiotic treatment (Hoiby, *et al*., 2001 ; Moser, *et al*., 2009 ; Bjarnsholt, *et al*., 2010 ; Brackman, *et al*., 2011a; Alasil, et al., 2015). Modulating QS will prevent biofilm formation or may weaken the established film (Annous, *et al*., 2009). In the study by Wu, *et al*. (2004) the activity of synthetic furanones against *Pseudomonas* lung infection model of mice was examined. *Pseudomonas* was first immobilized in alginate beads and then inserted intratracheally in the lungs of the mice. Different time treatment and different concentrations of furanones were given to the mice. They found that the administration of furanones to the mice as a treatment lead to the reduction of *Pseudomonas* load and accelerated lung clearance, as well as reducing the severity of lung pathology. Moreover, in the lethal lung load of *Pseudomonas*, furanone significantly increased the survival time of the mice.

In a study by Rasmussen, *et al*. (2005) 100 extracts of 50 *Penicillium* species were screened, 33 of these extracts exhibited QSI activity. Two compounds that were identified previously and showed to have QSI activity were isolated; these are patulin and penicillic acid (figure 4). In this study, patulin was found to enhance the biofilm susceptibility to tobramycin treatment. Lung infected mice in this study showed the ability to rapidly clear their bacterial loads when treated with patulin and other fungal extracts, because they had the ability to activate neutrophils.

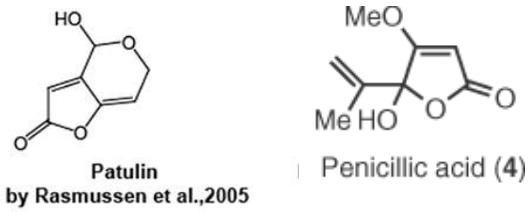


Figure 4: Structure of patulin and penicillic acid

7.0 **Bacterial resistance to quorum sensing**

Recently, large numbers of articles that describe additional natural sources of QSI have been published (Vikram, *et al*., 2010 ; Cotar, 2013). These inhibitors have been found in nutraceuticals food, herbal (Adonizio, *et al*., 2006 ; Choo, *et al*., 2006 ; Weng, *et al*., 2012), and fungal sources (Rasmussen, *et al*., 2005). The presence of QSI from natural foods is extremely interesting because, in most cases, vegetables and herbs are nontoxic to humans and readily available (Bjarnsholt and Givskov, 2007 ; Dandapat, *et al*., 2013). In bacteria that have more than one type of autoinducer or QS system, it may be necessary to disarm each system, starting with the QS system that lies most upstream (Balaban, *et al*., 2008).

Conventional antibiotics favor the evolution of resistance because they pose a strong selective pressure on bacteria (Defoirdt, *et al*., 2010). QS disruption has been shown to be an effective anti-pathogenic strategy, and by this it is considered to be a promising alternative to antibiotics (Vikram, *et al*., 2010). It is highly believed that the bacteria are unlikely to develop resistance because it poses no or little selective pressure (Defoirdt, *et al*., 2010 ; Zhu, *et al*., 2011 ; Kalia, 2013). Another point of view is that the bacteria may develop resistance to QSI, but it is suggested that the chance of resistance to QSI is smaller than conventional antibiotics. There is a need to be constantly watchful and always looking for more promising candidate safe drugs (Kalia, 2013).

Conclusion

Alternative strategies for antibiotic use are a must strategy since our world enters a strong antibiotic resistant era by microorganisms. The QSI substances are just one aspect to be further investigated and developed in the everlasting quest for new chemotherapeutic agents that can solve such types of world problems. Several evidences and researches ensure the importance of such substances to be used as alternative pharmaceutical products.

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