QS – systems communication of Gram-negative bacterial cells

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Abstract   *Quorum sensing* (QS) is a communication mechanism used by bacteria to recognize cell population density fluctuations and control gene expression, which is a critical role both in intra- and interspecies communication and controls microbe-host interactions. *QS* is the process in which the bacterial cells detect threshold concentration of signaling molecules in the external environment, and then after having exceeded this allowable threshold, they respond accordingly and modify their behavior by altering the expression of their genes. Regulation of gene expression in response to the density of bacterial cells in a population is a key phenomenon in the mechanism of *QS* and it is used by both Gram-negative and Gram-positive bacteria. In Gram-negative bacteria LuxR protein plays a key role in *QS* system as a type of transcription regulators and participates in a variety of biological behaviors with LuxI protein and signal molecules, including those encoding virulence factors and antibiotics biosynthesis, plasmid transfer, bioluminescence, and biofilm formation. New researches which highlight the unusual signaling molecules, novel regulatory components and heterogeneity in the *QS* system of Gram-negative bacteria are presented in this paper.

Systemy komunikacji QS w komórkach bakterii Gram-ujemnych

Słowa kluczowe   komunikacja międzygatunkowa, historia *quorum sensing*, *quorum sensing* u *Vibrio* i *Pseudomonas aeruginosa*, synteza autoinduktora AI-2

Streszczenie   *Quorum sensing* (QS) jest mechanizmem komunikacji używanym przez bakterie do rozpoznawania zmian w zagęszczeniu populacji i kontroli ekspresji genów, który jest ważny zarówno w komunikacji wewnątrz jak i pomiędzy gatunkowej oraz kontroluje interakcje bakteria-gospodarz. *QS* jest procesem, w którym komórki bakteryjne wykrywają progową koncentrację cząsteczek sygnałowych w środowisku zewnętrznym, a następnie, po przekroczeniu tego progu, odpowiadają swoiście i modyfikują swoje zachowanie przez zmiany w ekspresji genów. Regulacja ekspresji genów w odpowiedzi na gęstość komórek bakteryjnych w populacji jest kluczowym fenomenem w mechanizmie *QS* i jest stosowana zarówno przez bakterie Gram-ujemne, jak i Gram-dodatnie. U bakterii Gram-ujemnych białko *QS* odgrywa kluczową rolę w systemie *QS* jako rodzaj regulatora transkrypcji oraz wspólnie z białkiem LuxI i cząsteczkami sygnałowymi uczestniczy w różnych procesach biologicznych takich jak kodowanie czynników wirulencji i biosyntezie antybiotyków, w transferze plazmidów, bioluminescencji

i formowaniu biofilmu. W pracy zaprezentowano nowe badania, które przybliżają te niezwykłe cząsteczki sygnałowe, nowe komponenty regulatorowe i heterogenność QS-systemu u bakterii Gram-ujemnych.

Introduction

Conventionally, *quorum sensing* (QS) was defined as cell-cell communication among bacteria that effects in changes in transcription factor activity, and consequently, changes in gene expression. *QS*-directed behaviors were defined as those that need all of the bacteria in the population to act in harmony to make the behaviors successful (Fuqua et al., 1994; Bassler, 2002). Newer research extends these definitions by showing inter-kingdom communication (Pacheco, Sperandio, 2009), responses by intracellular small-molecule chemical signals (Srivastava, Waters, 2012), and heterogeneity in gene expression that is controlled by *QS* (Grote et al., 2015). Regulation of gene expression in response to the density of bacterial cells in a population is a key phenomenon in the mechanism of *QS* and it is used by both Gram-negative and Gram-positive bacteria (Schauder et al., 2001).

The general scheme of functioning of the *QS* in Gram-positive bacteria is similar to the systems of intercellular communication in Gram-negative bacteria and is based on four main elements which include: synthesis of biochemical signaling molecules (autoinducers) within the bacterial cell, active or passive release of signaling molecules into the environment, recognition of autoinducers by specific receptors after exceeding the threshold concentration and cell response reflected in changes in the regulation of gene expression (Sifri, 2008). Autoinducers accumulate in the environment as bacterial population density increases. Bacteria monitor changes in the concentration of autoinducers to introduce modifications in their cell numbers and to collectively change total forms of gene expression. Processes that are controlled by *QS*, such as bioluminescence, the production of biofilms or the secretion of virulence factors are unproductive when undertaken by a single bacterial cell, but become effective when undertaken by the group (Bassler, Losick, 2006).

Short history of *QS* discovery

The mechanism of recognizing the quantity of bacteria was first observed in Gram-negative bacteria *Vibrio fischeri* and later in *V. harveyi*. Because these two species are present in the marine environment for a long time, it was believed that the mechanism is unique and concerns only the two species of the *Vibrio* genus. However, in the 90s it was found that the phenomenon of *QS* also relates to other Gram-negative bacteria (Suárez-Moreno et al., 2012).

The first information about the ability to communicate bacterial cells in populations appeared in literature in 1970, thanks to research conducted by two scientists, Nealson and Hastings (O'Toole, 2016). In their publication in the "Journal of Bacteriology" for the first time they described the phenomenon of bioluminescence in marine bacterium *V. fischeri*.

V. fischeri lives in symbiosis with Hawaiian squid *Euprymna scolopes*, which uses the ability of bioluminescence of bacteria as a camouflage for defense against predators. Squid has the ability to control the intensity of the emitted light thanks to the special organ situated at its bottom side, called a light organ, which is the "home" for the *V. fischeri* cells (Verma, Miyashiro, 2013). *E. scolopes* is nocturnal and lives in shallow marine waters off the coast of Hawaii. During starry nights, when the intensity of light coming from the atmosphere through the water column is

high, thanks to the light receptors located on the back, the squid can receive and record the light, then using special shutter, according to the needs, it can close or open the light organ filled with bacteria. *E. scolopes* opens or closes the shutter so that the amount of light produced by bacteria is perfectly the same as intensity and wavelength of light reaching the back of the squid, which causes that its shade and contours are unseen and as a result it is invisible. Using the light of bacteria, the squid lights up itself and defends against attacks of potential predators, which without seeing the squid's shadow cannot trace it. At dawn, it pumps out of its body to the environment about 95% of the bacterial cells, which being dispersed in water are incapable to generate light.

Interesting for scientists was not the fact of production of light by *V. fischeri*, but when the light was produced. It was noted that the bacteria show the ability to emit light only inside the squid's organ, but they lose it in a dilute suspension as free-living cells in marine waters. One question was bothering, how bacteria, such primitive organisms are able to detect a situation in which they are alone (no luminescence) from those when they are in the community (the appearance of luminescence) (Bassler, Losick, 2006).

The presence of small, chemical signaling molecules, called anutoinducers was recorded very quickly. After exceeding the threshold concentration of these molecules in the bacterial growth medium, which also indicates that the bacterial cells reach a suitable number, there is a synchronized reaction of whole population - in the case of bacteria *V. fischeri* – the production of light (Taga, Bassler, 2003). It was concluded that the entire population by using chemical signals inform all organisms that constitute it about the need for proper change of certain behaviors.

Thus, there are two conditions at the basis of the phenomenon of bioluminescence of *V. fischeri*. The first one is the availability of carbon and energy sources in the specialized light organs of the host, presenting favorable conditions for rapid growth and development of bacteria, so that the population can achieve a high density (Jaworski et al., 2005). The second condition is the ability to achieve high levels of the released signaling molecules only in the colonized squid's organ. It has been shown that the number of cells of *V. fischeri* in 1 ml of sea water does not exceed 100, while in the light organs of *E. scolopes*, it can reach a value of 10^{10}–10^{11}/1 ml (Fuqua et al., 1996). Low availability of nutrients in the sea water hampers the development of both the population and the accumulation of secreted autoinducers in the environment of growth, which results in their leaving and no light emission by bacteria. Once it has been discovered how the bacterium *V. fischeri* produces light, the next step was to use the tools of molecular biology to know better the mechanism (Bassler, Losick, 2006). It has been found that the diffusive signal molecules are molecules of N-acyl-L-homoserine lactone. These autoinducers demonstrate species specificity, due to the presence of various substituents attached to the side of the N-acyl chain. *V. fischeri* bacteria secrete synthesized by themselves molecules of N-(2-Oxohexanoyl) homoserine lactone, which accumulate in the medium of cells' growth. After acquiring the appropriate number of cells in a population, and thus the proper concentration of autoinducers in the environment and threshold value will become exceeded, the production of light by bacteria will start (Kołodyński, Jankowski, 2005).

The genetic studies found that the system responsible for regulating the process of bioluminescence in *V. fischeri* includes two genes: *luxI* and *luxR* (Kołodyński, Jankowski, 2005). These two genes encode regulatory proteins, LuxI and LuxR, the first one acts as the synthase of autoinducer and the second one is a receptor of this autoinducer. LuxR cytoplasmic protein recognizes and binds to a signaling molecule, specific to itself, and forms a complex that induce the transcription of genes responsible for the synthesis of the light emitting luciferase enzyme complex (Waters, Bassler, 2005). Luciferase enzyme complex is encoded by five structural genes *luxCDABE*, being

a part of the operon *luxICDABE*. Connection of LuxR protein with the autoinducer changes the spatial conformation of the LuxR protein, and as a result it comes to the exposition of its binding domain. The LuxR protein, amended in this way recognizes and binds to a promoter of the *luxICDABE* operon, allowing for the transcription of genes of this operon (Figure 1). What is important, the complex AHL/LuxR not only initiates the synthesis of proteins of luciferase complex (LuxCDABE), but also the synthesis of LuxI protein, responsible for the production of signaling molecules. The same complex has also the ability to connect to the promoter of the *luxR* gene and on the basis of the negative control it may inhibit synthesis of LuxR regulatory protein (Engebrecht, Silverman, 1984; Shadel, Baldwin, 1991). This repression is required, for example in the case of strong induction when proteins of *luxICDABE* operon are produced in excess. It shows that the production of light by *V. fischeri* is actually a precisely controlled process, regulated by the concentration of signaling molecules, synthesized and released into the environment by the bacterial cells living in the population (Shadel, Baldwin, 1991).
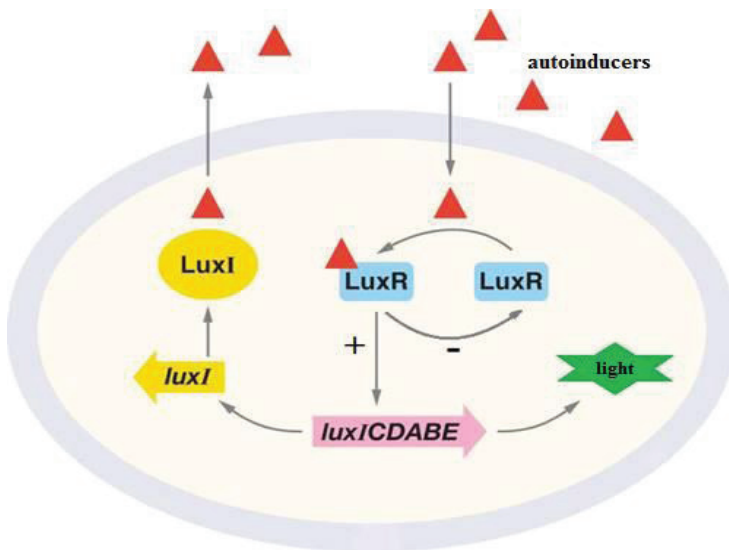
LuxR/autoinducer complex activates (+) transcription of *luxICDABE* operon encoding expression of luciferase enabling luminescence and initiating synthesis of LuxI protein responsible for the production of autoinducers. LuxR/autoinducer complex simultaneously inhibits (–) the production of LuxR protein

Figure 1. *Quorum sensing* regulation system in bacterium *Vibrio fischeri*

Source: Waters, Bassler (2005).

QS communication system

As it has been already mentioned above, the sensing mechanism of bacteria was first observed in bacteria *Vibrio fischeri* and later in *V. harveyi*. Because these two species are present in the marine environment, it was believed for a long time that the mechanism is unique and is restricted

only to the two species of the *Vibrio* genus. However, in the 90s it was found that the phenomenon of *QS* is much more widespread, and also relates to other Gram-negative bacteria (Súarez-Moreno et al., 2012). Gram-negative bacteria usually use a system LuxI/LuxR *QS* homologous to the system responsible for the bioluminescence of *V. fischeri* (Rutherford, Bassler, 2012).

This mechanism is compounded with four common features that are found in nearly all known Gram-negative *QS* systems (Bassler, 2010) and these are: signal molecules acyl-homoserine lactones (AHLs), proteins from the family of autoinducer synthase (LuxI), proteins from the family of transcriptional regulators (LuxR) and target genes. The function of LuxI protein as the synthase of autoinducer comes down to catalyze the reaction of joining S-adenosylmethionine (SAM) with an acylated, carrier protein, ACP which results in the formation of AHL molecule (Rutherford, Bassler, 2012). The autoinducers AHLs or other molecules that are synthesized from S-adenosylmethionine (SAM) are able to diffuse passively through the bacterial membrane and to accumulate in bacterial environment until they reach the critical concentration. Autoinducers are bound by specific receptors that reside either in the inner membrane or in the cytoplasm. Then, the sensor of LuxR signal detects the autoinducer and connects to it, interacting with the promoter sequence of an operon which leads to the induction of transcription of selected genes. The specific structure of LuxR allows for the dual role of this protein – N-terminal fragment of the polypeptide chain is responsible for the recognition and binding with the AHL, and the C-terminal fragment of the chain interacts directly with DNA. In the presence of autoinducer molecule, LuxR protein is activated and changes its conformation, and so induces the transcription of target genes (Figure 2) (Rutherford, Bassler, 2012). The newly-discovered enzyme system has been described to degrade efficiently different AHLs of various Gram-negative pathogens (Zhang et al., 2017). The enzymatic degradation of *QS* molecules is called *quorum quenching* (QQ) and it has been considered as a promising anti-virulence therapy to treat biofilm-related infections and battle antibiotic resistance.
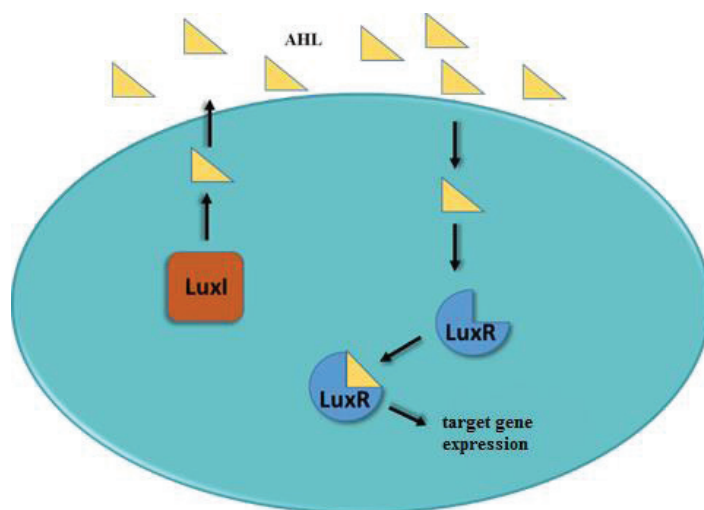
AHL – acyl-homoserine lactone, LuxI – protein responsible for AHL production, LuxR – signal sensore

Figure 2. General scheme of *quorum sensing* system in Gram-negative bacteria

Source: Siepka, Gładkowski (2012).

Homologs of LuxI/LuxR system have been identified so far in over 100 species of Gram-negative bacteria (Rutherford, Bassler, 2012). Many bacterial pathogens using *QS* mechanism controls the production of virulence factors – for example such systems as: LasI/LasR and RhlI/RhlR in *Pseudomonas aeruginosa*, SmaI/SmaR in *Serratia marcescens* or CviI/CviR in *Chromobacterium violaceum* (Rutherford, Bassler, 2012). However, it should be noted that genes controlled by *QS* encode not only the classical virulence factors, but also proteins involved in basic metabolic processes of cells (Sifri, 2008). It is estimated that a significant part of the bacterial genome (4–10%) and proteome (20% or more) may be regulated by mechanisms of mutual cell communication.

Then, it is known that Gram-negative bacteria often use several autoinducers, however now new studies are highlighting the molecular factors that bring the receptors strange specificity in individuating between closely related molecules. *QS* information is often joined by small RNAs that control target gene expression and that also function in reaction loops. (Papenfort, Vogel, 2010).

QS system in *Pseudomonas aeruginosa*

P. aeruginosa, the Gram-negative bacteria is a bacterium that causes chronic lung infections in patients suffering from cystic fibrosis created on biofilm formation (Høiby et al., 2010). This pathogenic phenotype is particularly serious in patients with HIV co-infection. Selective pressure applied by anti-infective treatments positively selects multidrug-resistant *P. aeruginosa* strains. Moreover, this effect challenges the antibiotics treatment of this pathogen (Pesci et al., 1999). Resistance is acquired either by joining plasmid-encoded resistance genes or by spontaneous resistance mutations of *P. aeruginosa* (Lister et al., 2009).

P. aeruginosa uses two recognized AHL autoinducers as well as non-AHL autoinducers for *QS*. Precisely, cyclic dipeptides (2,5-diketopiperazines, DKPs) are produced by tRNA-dependent on cyclodipeptide synthases (Campbell et al., 2009) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) is produced by proteins that are encoded by gene cluster *ambBCD,* the non-ribosomal peptide synthase (Lee et al., 2013). In addition, a quinolone (2-heptyl-3-hydroxy-4-quinolone, known as PQS) is used as an autoinducer, which is synthetised by proteins that are encoded by the *pqsABCDH* genes, and with the two AHLs it controls the construction of biofilms and the production of virulence factors (Lee, Zhang, 2015). Quinolones are generally recognized for their antibiotic and anticancer activities (Heeb, 2011), which reveals the multi-functionality of particular autoinducers (Papenfort, Bassler, 2016).

P. aeruginosa utilises *QS* for cell-to-cell communication to regulate the expression of virulence factors and for biofilm formation what allows disrupting the host immune systems and causes chronic infections. Examples of virulence factors, as wrote above, are LasA, LasB, and also Exotoxin A (ToxA) which is a transferase that is allied with cellular death (Ballok, O'Toole, 2013). The elastases LasA and LasB were shown to have an effect on cell wall elasticity and in consequence delay the therapeutic process (de Bentzmann et al., 2000). *P. aeruginosa* also produces, for example, hydrogen cyanide, which is a powerful inhibitor of cellular respiration and is associated with compromised lung function in patients (Ryall et al., 2008). *P. aeruginosa* in contrast to *V. fischeri* that uses only one *QS* circuit, displays the three *QS* routes named *Las*, *Rhl*, and *Pqs* that are interconnected with each other. These signalling routes are hierarchically regulated: the *Las* system activates both the *Rhl* and *Pqs* systems (Jimenez et al., 2012), while *Rhl* can destroy *Pqs* and *Pqs* activates *Rhl* (Welsh et al., 2015). Gallagher et al. (2002) suggested the

involvement of protein PqsE in *Pqs* signaling, rather than *Pseudomonas* quinolone signal (PQS) biosynthesis.

Interspecies communication

Bacteria usually co-occur with other bacterial species in multi-species communities in external environment or inside the host (e.g. in the gastrointestinal system or in the oral cavity). Both Gram-negative and Gram-positive bacteria are capable to recognize and consider autoinducing signaling molecules of other species (Jayaraman, Wood, 2008). It was also revealed that pathogenic bacteria can interact with eukaryotic host cells with operating each other's autoinducing signals (Jayaraman, Wood, 2008).

The concept of bacterial interspecies communication was first introduced in 1997 by research conducted by the American team led by Bassler et al. (1997). In their work, the authors have demonstrated the existence of a signaling molecule (called AI-2 autoinducer) responsible for inducing bioluminescence in free-living marine bacteria, *Vibrio harveyi*. This molecule, often referred as "universal" (Xavier et al., 2007) is used to communicate bacterial cells in mixed populations living together in the same environment (Federle, 2009). Since the discovery of the AI-2 autoinducer, numerous studies have been conducted in which the mechanisms for controlling the emission of light by *V. harveyi* were deeply analysed. Currently, the model of regulation of luminescence in cells of this bacterium is well known and described in many publications.

The ability of *V. harveyi* to recognize AI-2 autoinducer produced by its own cells as well as by other bacterial species allows it to monitor not only the density of its own population, but also populations of other species occupying the same niche. This results in a coordinated change in the expression of selected genes and consequently, the appropriate response of the entire population, allowing for effective adaptation to the prevailing environmental conditions (Pereira et al., 2013). The production of the AI-2 autoinducer is not only limited to the *V. harveyi* species. The activity of this molecule has been demonstrated in over 100 different Gram-positive and Gram-negative bacteria (Hirakawa, Tomita, 2013). Detailed studies are ongoing for better understanding the role that AI-2 autoinducer plays in the *QS* mechanism, and to know all the biological activities of bacteria controlled by this molecule. The ability to produce a universal signaling molecule such as the AI-2 autoinducer by various species of bacteria allows us to claim that this is the oldest evolutionary autoinducer in the world of microorganism, which was developed before the bacteria split into two living Gram-positive and Gram-negative (Schauder, Bassler, 2001).

Synthesis of the molecule of AI-2 autoinducer

As it was described above, the chemical signaling molecules biosynthesized by Gram-positive and Gram-negative bacteria differ in the type and number of amino acids and the length of the acyl chain. Different structure of each molecule makes them species-specific, which means that they can be recognized and transmitted only by cells that belong to a particular species but are not recognized by other species of bacteria, even those closely related (Schauder et al., 2001). Unlike acylated homoserine lactones and oligopeptides, AI-2 autoinducer is non-species-specific and chemical structure of its molecules and its synthesis pathway are identical, regardless of the species of bacteria.

Analysis of the AI-2 biosynthesis pathway indicates that the autoinducer is generated as a result of transformation of basic for the metabolism compound, S-adenosylmethionine (SAM) (Matejczyk, Suchowierska, 2011). SAM is the main donor of methyl groups for methylation of both DNA, RNA as well as many proteins in the cell (Schauder et al., 2001). It is also the donor of other function groups necessary for the proper course of many cellular processes such as the synthesis of phospholipids or vitamins (Pereira et al., 2013). It should be noted that SAM is a substrate for both biosynthesis of AI-2 autoinducer and for the synthesis of species-specific acyl-HSl molecules. Transferring the methyl group from SAM to the various terminal acceptors catalysed by methyltransferases leads to the formation of intermediate product in cells, S-adenosylhomocysteine (SAH). Since SAH is cytotoxic to the cell, it is rapidly transformed by the Pfs nucleosidase, which acts to remove adenine to produce S-ribosyl homocysteine (SHR) (Schauder et al., 2001). Next, the LuxS enzyme, the expression product of the *luxS* gene, catalyses the fission of SHR to homocysteine and 4,5-dihydroxy-2,3-pentandione (DPD). Organisms with the *sahH* enzyme can decompose SAH directly into adenosine and homocysteine (Pereira et al., 2013).

DPD is a highly reactive molecule that is convertible and able to form complexes with other compounds, what suggests that different molecules derived from DPD conversion may be signals recognized by different bacterial species as AI-2 autoinducer (Waters, Bassler,2005). Two DPD-derived signaling molecules have been identified in *Vibrio harveyi* and *Salmonella typhimurium* on the basis of analysis of crystal structure of the complexes that form these molecules with corresponding AI-2 receptor proteins (in *V. harveyi* the function of the AI-2 receptor performs LuxP protein, in *S. typhimurium* – LsrB protein). It has been found that in *V. harveyi* the AI-2 autoinductor is (2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate produced by cyclization and complexation of boron by DPD. In turn, in *S. typhimurium*, the function of AI-2 performs (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran, which does not contain boron (Waters, Bassler, 2005).

The discovery of boron in the AI-2 signaling molecule synthesized in *V. harveyi* cells is surprising because only few known functions of this element in nature have been known so far (Waters, Bassler, 2005). The presence of boron in AI-2 autoinducer in *V. harveyi* is probably due to the high concentration of this element in the aquatic environment in which the bacteria live. In the terrestrial environment, the concentration of boron is much lower, which makes this element an unlikely component of AI-2 molecules in bacteria found in those ecosystems (for example in *S. typhimurium*). On the basis of these early observations concerning the AI-2 molecule, we can put forward a thesis that bacteria use a conservative pathway for biosynthesis of intermediate product DPD, whereas the final form of AI-2 is determined by the chemical composition of the specific environment (Waters, Bassler, 2005).

The existence of other biologically active derivatives of DPD is not excluded. Moreover, there is a possibility of presence in the bacterial cells, two or more receptor proteins recognizing various DPD derivatives. This would allow the bacteria to alter the expression level of particular genes depending on the information transferred by each signal (Waters, Bassler, 2005).

Conclusions

QS is a signaling machinery that is common in bacteria and includes the exchange of small molecules between bacteria and they are able to adapt the activity of gene expression to the population density in the close environment. This allows bacteria to recognize who their neighbours are and create phenotypes that are helpful for the group and that are useful for their survival.

New discovery shows how *QS* systems work using similar rules of action, which are rooted in the physical and chemical properties of the AIs, the matching receptors and their downstream regulators. The enzymatic degradation of *QS* molecules called *quorum quenching*, *QQ* has been considered as a promising anti-virulence therapy to treat biofilm-related infections and battle antibiotic resistance. Better understanding of *QS* details gives chance to combat bacterial infections by the attenuation of their *QS* communication systems and virulence.

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