

Control of bacterial virulence by cell-to-cell signalling molecules

Alina Maria Holban¹, Coralia Bleotu², Mariana Carmen Chifiriuc^{1,*} and Veronica Lazar¹

¹Department of Microbiology and Immunology, Faculty of Biology, University of Bucharest, Romania

²S. Nicolau Institute of Virology, Bucharest, Romania

*Corresponding author: Mariana Carmen Chifiriuc, e-mail: carmen_balotescu@yahoo.com

Due to increasing microbial resistance to classical antimicrobial drugs, novel alternative antimicrobial approaches are urgently needed. Virulence can be defined as a complex adapting mechanism providing pathogenic bacteria certain advantages in competing with commensal microbiota and host defence mechanisms. Bacteria communicate through small diffusible density dependent signal molecules termed autoinducers (AIs) or Quorum Sensing (QS) signalling molecules. QS signalling systems are used to control and coordinate the production of virulence factors required for colonizing and persistence in different environmental conditions, but also for interfering with host core signalling pathways. Even though QS AIs are recognized and active firstly on the producer strains, recent findings demonstrate that bacteria AIs can be recognized also by other prokaryote and eukaryote species, so they also mediate the interspecies and even inter-kingdoms communication. On the other hand bacteria are able to sense and respond to small signalling molecules produced by superior organisms, as plant and animal hosts. This inter-species cross-communication usually impacts on virulence of pathogenic bacteria and on the development of infectious process. By modulating bacteria virulence using their own regulatory molecules or inter-species compounds used in bacteria cell-to-cell communication we aim to emphasize an intelligent strategy to efficiently combat infections, without taking the risk of developing drug resistance and other harmful environmental and host related side effects.

Keywords virulence; Quorum sensing; cell-to-cell communication; antimicrobial impact

1. Introduction

Humans are facing a significant moment in modern health care where many antibiotics have lost their effectiveness in treating life-threatening and debilitating diseases. Since the world's population continues to increase quickly, agricultural markets are asked in meeting worldwide nutritional needs. In order to increase production for better support of life, plants and animals antibiotics or other chemical treatments are still given in order to enhance disease resistance and avoid infections that considerably reduce the production. Bacteria are the most well adapted organisms on Earth, therefore there is a matter of time for microorganisms to adjust their behaviour and acquire resistance. This aspect, along with the irrational usage of antibiotics by human individuals leads to alarming statistics regarding microbial resistance, bacterial pathogens outpacing our abilities to manage them. Therefore, there is a critical need to identify new antimicrobial compounds and to develop novel methods for disease prevention and treatment.

Unfortunately, the development of new drug leads has slowed dramatically over the past 10 years, and newer drugs that have been successfully developed are strictly reserved to treat only the most serious infections, so as not to repeat over-usage mistakes of the past [4]. Recent interest of researchers demonstrates that novel alternative strategies can be used in order to potentate the effect of regular drugs [8] by targeting or controlling their release [7], or by using natural [5, 6] and synthetic antimicrobial compounds [9]. Furthermore, completely ecological antimicrobial strategies aiming of using natural microbial derived compounds [10] used to hijack bacterial signalling pathways, as communication; in order to attenuate certain responses are under development.

2. Cell-to-cell signalling in bacteria

Bacteria were for a long time believed to exist as individual cells aiming to find nutrients and multiply. More than 30 years ago microbiologists found that bacteria can communicate by producing and responding to small diffusible molecules that act as signals. Since their first description in the marine luminescent bacterium *Vibrio fischeri*, where they function as the control mechanism of light production [1], multiple roles have been assumed to these molecules, termed autoinducers (AIs). It has been proven that cell-to-cell communication, or quorum sensing (QS), is a widespread phenomenon in bacteria that is used to coordinate gene expression among local populations and also to regulate genes that promote invasion, defence, spread and virulence in pathogenic bacteria. Because of their great impact on bacteria behaviour, there is an increasing interest on targeting QS communication systems in order to attenuate bacteria virulence and pathogenicity. Modulating communication of adversaries serves as an effective approach to disrupt cooperative actions among individuals or groups. Another key advantage proposed in targeting quorum sensing is based on the premise that a treatment that does not suppress growth of a cell will not exert a selective pressure to develop resistance to that treatment. Since QS is not an essential process, and QS mutants usually have not displayed growth defects this strategy may represent one of the best antimicrobial long-term methods [4].

Many classes of AIs have been described to date. The most intensely studied AIs are the N-acylhomoserine lactones (AHLs) of Gram negative bacteria also sometimes referred to as autoinducer-1 (AI-1), the peptides of Gram-positive bacteria and a class of universals AIs termed AI-2, used by both Gram negative and Gram positive bacteria, whose structures remain unknown in most species [2]. The presence of a novel AI has been recently postulated. AI-3 autoinducer has been described in *Escherichia coli* and it seems to act as an interspecies signalling molecule being involved in inter-kingdom cell-to-cell signalling [3]. There are also other quorum sensing signals that go beyond these classes, including *Pseudomonas* quinolone signal (PQS), and diffusible signal factor (DSF), present in many bacteria and also new molecules will undoubtedly be soon discovered as the study of quorum sensing expands quickly [4]. QS structures and circuits are essential for developing QS inhibitory or modulatory methods, also named Quorum Quenching (QQ).

2.1. QS in Gram negative bacteria

Gram negative species as *Pseudomonas aeruginosa*, *E. coli* and *Salmonella sp.* are the most investigated for QS studies. The most well-known signalling molecules for Gram negative bacteria are N-acyl homoserine lactones (AHL), 2-alkyl-4-quinolone, γ -butyrolactones, furanones, long chain fatty acids derivatives, peptides, 4,5-dihydroxy-2,3-pentanediones derivatives (DPD), known as type 2 and/or 3 autoinducers [26]. The vast majority of gram negative QS systems that have been intensively studied utilize AHLs as signalling molecules. These molecules are comprised of an invariant homoserine lactone (HSL) ring attached to an acyl chain that can vary in length between 4 and 18 carbon atoms. AHLs are biosynthesized by members of the LuxI family of AHL synthases using the substrates S-adenosylmethionine (SAM) and an acylated acyl carrier protein (acyl-ACP) [11]. All AIs are produced at basal levels and their concentration increases with growth. Once a critical concentration threshold is achieved, interaction between AHLs and LuxR-type receptor proteins localized in the cytoplasm of the cell becomes favourable. LuxR family members are transcriptional regulators whose DNA-binding activities change upon ligand interaction, resulting in modulation of target gene regulation in response to AHL accumulation [4].

2.1.1. AHLs (AI-1) signalling

P. aeruginosa is the main model in investigating AHL AIs in Gram negative bacteria. In this versatile opportunistic pathogen two AHL QS systems were described, *las* and *rhl*. In the *las* system, N-3-oxododecanoyl-homoserine lactone (3OC12-HSL, OdDHL) AI is produced by the enzyme encoded by the *lasI* gene. When certain densities are achieved OdDHL binds to the transcriptional activator LasR, which in turn will dimerize and bind to target promoters to control gene expression. In the *rhl* system, the AI N-butyryl-homoserine lactone (C4-HSL) is produced by *rhlI* gene encoded enzyme. At certain concentrations C4-HSL binds to its cognate transcriptional regulator, RhIR, to control the activity of target promoters [12]. Even though they can function independently, *rhl* system is controlled by *las* system at both transcriptional and post-transcriptional levels. Both *las* and *rhl* systems regulate the timing and production of multiple virulence factors, including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, superoxidase dismutase and also biofilm formation [13, 14]. Moreover, high amounts of AHLs were identified in cystic fibrosis lungs, which mean they may impact on the progression of infectious process. Apart from regulating the expression of virulence factors, AHLs have been shown to directly interact with host cells and modulate key processes as host immune response and apoptosis [17-20].

In *P. aeruginosa las* and *rhl* systems are interconnected with a third signalling circuit that utilize 2-alkyl-4-quinolones (AQs) for signalling, 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS=Pseudomonas quinolone signal) and its precursor 2-heptyl-4 quinolone being the most relevant autoinducers of Pqs system [15]. Even though the molecular role and signalling are not fully understood, it has been demonstrate that Pqs system regulates numerous virulence genes including those involved in iron scavenging, PQS itself acting as an iron binding molecule [16]. After analysing transcriptome data, over 90 genes were found to be regulated by the Pqs system, but to date it has been demonstrate its impact on biofilm formation and several virulence factors, including elastase, pyocyanin and LecA lectin. PQS is also considered essential for full virulence of *P. aeruginosa* in multiple hosts [21].

2.1.2. AI-2 and AI-3 signalling

AI-2 is a key QS signalling molecule used by Gram negative species as *E. coli* and *Salmonella sp.* AI-2 was originally identified as one of the AIs controlling light production by the marine bacterium *Vibrio harveyi*, the gene responsible for AI-2 production being identified and named *luxS* [22]. Although the structure of AI-2 in *E. coli* is currently unknown, AI-2 molecule produced by *Salmonella enterica* serovar Typhimurium has been identified as (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran [23]. Studies revealed that LuxS controls the expression of the type-3 secretion system encoded by the locus of enterocyte effacement (LEE) Pathogenicity Island [24], virulence determinant that is required for the formation of the characteristic attaching and effacing (AE) lesions caused by these pathogens enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli*. Transcriptomic studies have revealed that LuxS is a global regulator in EHEC, controlling the expression of over 400 genes, many of these genes modulating bacterial

virulence such as flagellar motility, surface adhesion and Shiga toxin production [25]. Recent findings revealed that other signalling molecules are vital for inducing AE lesions in host enterocytes after EHEC infection, using a newly described two-component signalling system (TCS). QseBC is a bacterial TCS that is regulated by LuxS, with QseB being the response regulator and QseC the sensor kinase; and similar to LuxS it has been found that this TCS controls motility gene expression [27]. Studies revealed that QseBC is responsible for recognizing AI-3 signalling molecule in *E. coli*. Intriguingly it seems that QseBC also acts as recognising system for mammalian catecholamine stress hormones, QseC functioning as the receptor for the host hormones epinephrine and norepinephrine, indicating that small molecule signalling pathways in eukaryotes and bacteria can intertwine [28].

QseC homologues have been identified in other pathogenic bacteria as: *Salmonella enterica* serovar Typhi (*S. typhi*), *S. typhimurium*, *Vibrio parahaemolyticus* and *Francisella tularensis* [30]. Although their role is not fully elucidated, it has been shown that they are involved in regulating the expression of virulence factors, in survival and colonization [29].

Besides QseBC, multiple regulators are involved in the control of virulence gene expression in *E. coli* and *Salmonella sp.* A second two-component regulatory system that controls the formation of AE lesions, QseEF has been recently described [31]. This system is composed of the histidine kinase QseE and the response regulator QseF. These two proteins are involved in the transcriptional control of the effector EspFu, which seems to be translocated into host cells by EHEC. Interestingly, a third gene in the same operon, *qseG*, separates *qseE* and *qseF*. QseG is an outer-membrane protein and it is also required for the translocation of type III secretion system effectors into host cells [31].

2.1.3. Other types of signalling molecules in Gram negative bacteria

First signalling molecules identified in prokaryotes were cyclic AMP (cAMP) derivatives, which are produced by adenylate cyclases and binds to CRP transcriptional factors family. cAMP directly or indirectly controls the production of multiple virulence factors as motility or toxins, interfering with other QS systems [38].

After a close genomic examination of LuxI and LuxR homologs among all sequenced Gram negative bacteria genomes, is clearly highlighted the increased amount of LuxR sequences comparing with LuxI. This unbalanced report suggests that response regulators are prevalent against effectors, which indicate the presence of some unidentified alternatives that could serve as LuxR binding molecules. This idea has been proved after identification of a novel family of cyclic dipeptides, named diketopiperazines (DKP). DKP have been identified in many Gram negative culture supernatants and are able to bind to the LuxR type proteins. It has been postulated that DKP can interfere with many QS systems in bacteria by acting as agonists or antagonists of AHLs [36].

Phenazines, pigmented nitrogen containing proteins can also be involved in cell-cell signalling. Phenazines exhibit a toxic effect against many prokaryotes and eucariotes, offering a clear advantage of the producing species. Pyocyanin (5-N-methyl-1-hydroxyphenazine), present exclusively to *P. aeruginosa* is the most well investigated phenazine. This blue pigmented molecule is also one of the most important virulence factors in this pathogen, being involved in both acute and chronic infections by suppressing lymphocyte proliferation and damaging epithelial tissue in order to target multiple cellular functions [37].

Other types of molecules involved in the QS mechanisms of Gram negative bacteria are represented by small diffusible signal molecules (DSF = diffusible signal factor), which can be chemically characterized as derivatives of cis-11-methyl-2-dodecenoic acid. These molecules have initially been identified for *Xanthomonas campestris*, *Stenotrophomonas maltophilia* [32], *Xylella fastidiosa* [33] and *Burkholderia cenocepacia* [34]. DSF molecules have multiple roles, as they are involved in regulating the expression of extra cellular enzymes, dispersion of biofilms, toxins resistance and survival [26, 35].

2.2. QS in Gram positive bacteria

Signal molecules used for Gram positive bacteria QS signalling varies from those of gram negative bacteria, and thus far no Gram positive bacteria have been shown to produce AHLs. Gram positive QS signalling systems typically use small post-translationally processed peptides as signal molecules. These peptide signals interact with the sensor element of a histidine kinase TCS. In Gram positives QS is used to regulate the development of bacterial competence in *Bacillus subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis* and virulence in *Staphylococcus aureus* [39]. *S. aureus* has served as a model to study bacterial peptide signalling, since this organism is a member of the human microbiota, being found in approximately 30% of the adult population [40]. Despite its widespread prevalence in healthy subjects, *S. aureus* can be also a harmful opportunistic pathogen, revealing multiple virulence factors involved in its very rapid transmission. Furthermore, this Gram positive pathogen has been increasingly associated with antibiotic resistance and incriminated to form biofilms on many surfaces, including indwelling devices such as urethral stents [42].

S. aureus virulence is dependent on temporal expression of a diverse array of virulence factors, including both cell-associated products, such as protein A, collagen, and fibronectin-binding protein and secreted products including lipases, proteases, alpha-toxin, toxin-I, beta-hemolysin, and enterotoxin [39]. One of the factors involved in *S. aureus* virulence control is its peptide-based QS system, encoded by the accessory gene regulator (*agr*) locus [40]. The

autoinducer in this *agr* QS system is an oligopeptide termed the autoinducing peptide (AIP), encoded by *agrD* gene. AIP is trimmed and secreted by a membrane-bound protein AgrB [2], the active AIP being a 7–9 aminoacids long peptide, with a 5-membered thiolactone ring [42]. Extracellular AIP binds AgrC, a membrane-bound sensor kinase, which leads to activation of AgrA right after AgrC autophosphorylation. The *agr* system is intricately involved in the regulation of virulence genes, predominantly from two promoters, P2 and P3, producing RNAII and RNAIII [40]. P2 promotes the transcription of the *agr* operon from the RNAII transcript, which includes *agrA*, *agrB*, *agrC* and *agrD*. Active AgrA may be a phosphorylated homodimer inducing transcription of P2 and P3 promoters. Transcription of P3 leads to the production of RNAIII, the effector molecule of the *agr* system [42]. RNAIII is a 514 nucleotide regulatory RNA, which also functions as the mRNA for the δ -toxin, the 5' end being thought to upregulate α -haemolysin, while the 3' end seems to be required for the repression of protein A synthesis. RNAIII reduces the expression of surface adhesins, and increases the production of capsule, toxins and proteases [2, 42].

It has been revealed that the survival of *S. aureus* is also dependent on genes regulated by a second QS system, RAP/TRAP. In this system, the proposed AI, RNAIII activating protein (RAP) is believed to be secreted by an as yet unknown mechanism. This AI enters the cell and activates the target of RAP molecule (TRAP). The activated TRAP upregulates *agr* expression and promotes cellular adherence, which is essential for biofilm formation [43].

The *agr* system is estimated to regulate over 70 genes and only about 23 codify for known virulence factors [44]. Virulence factors regulated by *agr* encompass two classes: the first class contains virulence factors involved in attachment to the host and immune evasion, while the second class contains genes involved in the production of exoproteins associated with invasion and toxin production. It has been postulated that the activation of the *agr* system essentially switches the bacterium from an adhesive, colonizing commensal to an invasive and aggressive pathogen [2].

Recently, four distinct groups of *agr* polymorphism have been identified and these were categorized as I–IV. Even though each group has a distinct AIP and any API is able to bind to the receptors from all groups, each AIP is only able to activate the receptor from the same group. Apart from groups I and IV, which are able to cross-activate, it has been proved that all other groups are cross-inhibiting [44]. There remains no doubt that the *agr* system is involved in *S. aureus* virulence; however, exactly how it contributes remains controversial and currently there is a great interest for deciphering this mechanism.

Cell-to-cell communication is therefore a widespread phenomenon in bacteria that is used to coordinate gene expression among local populations and to modulate competition and virulence [45]. With the ongoing emergence of antibiotic-resistant pathogens, therapeutic strategies aiming to modulate QS communication, and therefore virulence, seem to be one of the most suitable ecological alternatives.

3. Categories of QS modulators

Even though each QS system utilized by bacteria is unique and specific, all QS circuits share a background mechanism comprised of signal production, signal accumulation, and signal detection. All QS modulators that have been characterized so far have been found to target at least one of these three steps. Therefore there have been described quorum-sensing inhibitors for signal degradation and inactivation, for inhibition of QS signal biosynthesis, and inhibition of signal detection [4]. Another clustering item refers to the provenience of QS modulator, which can be natural, or chemically synthesized QS agonist or antagonist. Here we review the most relevant strategies of attenuating bacteria virulence by using QS modulators and QQ.

3.1. Natural QS modulators

Naturally originated compounds are always preferred in biomedical field, since they are biodegradable and usually very efficient, being perfect candidates for ecological anti-infectious strategies. Recent studies propose the usage of natural eukaryotic- derived vegetal [6, 46–49] and animal compounds [53], or microbial derived compounds [50–52] for attenuating bacteria virulence and modulating QS. With the widespread availability of bacterial reporter strains that provide robust autoinducer responses with easily measurable products, such as luciferase, pigments, and enzymes, the ability to test samples of naturally occurring compounds seems to be limited only by the ability to gather and acquire sizeable amounts of starting material [4].

3.1.1. Microbial QS modulators

The human microbiota is composed by more than 1000 species and it is estimated that there are 10 times as many bacterial cells within the gastrointestinal tract as there are human cells within our bodies. The normal microbiota plays essential roles in mammals nutrition, physiology, development, immunity, and behaviour, such that disrupting the structure and balance of this community leads to dysbiosis and disease [45]. It has been proposed that competition between resident microbes and pathogens is influenced by the expression of virulence factors by pathogens and by the nutritional requirements of both populations [73]. One of the most important aspects in a quorum-sensing bacterial population is density-dependent fitness benefits [74], bacteria density being crucial in infections. These dynamics can steer the survival, colonization, and clearance of pathogens in the gut. After entering in the host gut pathogens have to

face hostile condition in order to survive and multiply. Excepting host defence mechanisms, commensal bacteria are another challenge pathogens have to face. During early stage infection pathogens produce many virulence factors in order to be able to adhere, colonize and multiply within the host. Virulence factors produced by pathogens also serve as a competitive advantage against highly number of commensals [45]. On the other hand, commensals fight back limiting nutritional resources and interfering with pathogens communication and virulence, finally leading to pathogen clearance during late infection [45]. Bacteria composing normal microbiota are vital for pathogen clearance, since it have been shown that axenic animals are unable to clear infections, even if low dose of pathogen are used [73].

Our recent studies have shown that different sub inhibitory concentrations of phenyl lactic acid, produced by probiotic strains of *Lactobacillus sp.* may attenuate the virulence and pathogenicity of certain clinical strains of *P. aeruginosa* and *S. aureus* [51], this molecule being proposed to act as a QS modulator. It have been shown that *L. acidophilus* secretes a molecule(s) that either acts as a QS signal inhibitor or directly interacts with bacterial transcriptional regulators, controlling the transcription of *E. coli* EHEC O157 genes involved in colonization. Authors demonstrate that this probiotic strain significantly modulate the production of AI-2 signalling molecule in *E. coli*, the expression of important virulence-related genes and Shiga toxin production [75].

It have been revealed that a soil-dwelling species of *Bacillus* produces a lactonase (AiiA), which is able to hydrolyze the homoserine lactone ring of all known AHLs molecules. *In vivo* experiments showed that when transgenically expressed in the QS plant pathogen, *Erwinia carotovora*, the bacteria had greatly attenuated virulence and caused only minor soft rot symptoms, comparing with wilde-type *Erwinia*. Various AHL lactonases not belonging to the AiiA clade have also been identified. Metagenomic analyses facilitated the discovery of QlcA, BipB01, BipB04, BipB05, and BipB07, and screens for AHL-degrading bacteria led to the identification of QsdA of *Rhodococcus erythropolis* strain W2, AiiM of *Microbacterium testaceum*, AidH of *Ochrobactrium sp.* strain T63, and QsdH of *Pseudoalteromonas byunsanensis* strain 1A01261 [4]. Certain *in vitro* and *in vivo* studies have confirmed the overall value of these lactonases in quorum quenching and disease prevention.

Not only bacteria derived signalling molecules can be effective in modulating QS-dependent virulence, but also certain fungal signalling compounds. Rasmussen and his collaborators have shown that certain compounds produced by *Penicillium* species, as patulin and penicillic acid have an inhibitory role on QS molecules, affecting the expression of 45-60% of the genes regulated by QS in certain microorganisms [76]. It has been shown that mycorrhized and non-mycorrhized fungal species, belonging to the both *Ascomycota* and *Basidiomycota* phylum, have the capacity to hydrolyze OdDHL through a lactonase activity, but the responsible molecules remain unknown. Farnesol, a signalling molecule involved in inducing the transition from hyphal to the yeast state in *C. albicans* can alter the production of toxic phenazines, like pyocyanin, in *P. aeruginosa*. Moreover, farnesol may generate oxygen reactive species in a great number of microbial species [77]. This process seems to play an important role in the competition between bacteria and fungi and may significantly impact on host response and infectious process.

3.1.2. Algae-derived QS modulators

Many photosynthetic taxons ranging from algae to superior plant species have been proved to interfere with bacteria cell-to-cell communication, virulence and pathogenesis.

The unicellular soil-freshwater algae *Chlamydomonas reinhardtii* and *Chlorella spp.* were found to secrete substances that mimic the activity of the N-acyl-L-homoserine lactone (AHL) signal molecules used by many bacteria for quorum sensing regulation of gene expression [54]. This effect seems to involve some of algae proteins that are affected by bacteria infections, as chaperonins, nitrogen regulatory protein PII, and GTP-binding proteins. The algal mimic compounds were able to cancel the stimulatory effects of *E. coli*, *P. aeruginosa* and *V. fischeri* AHLs on the accumulation of seven of these proteins, providing evidence that the secretion of AHL mimics by the alga could be effective in disruption of quorum sensing in naturally encountered bacteria [54].

The most well investigated algal QS modulators seem to be furanones and their derivatives. Brominated furanones were among the first recognized small-molecule inhibitors of quorum sensing. Though concerns regarding their toxicity remain a blockade for their commercial or therapeutic use, furanones have served as helpful molecular probes in understanding signalling and the consequences of its inhibition [4]. Several algal species, as *Delisea pulchra*, were found to have broad-spectrum antimicrobial activities targeting both Gram negative and Gram positive bacteria. Results revealed that *Delisea* – derived furanones interfere with AHLs recognition systems and may inhibit QS dependent phenotypes as swarming motility in *Serratia liquefaciens* [55]. The same research group indicate that this effect is mainly due to a competitive interaction between AHL and furanone for the LuxR-type receptor protein [55]. Furanones have proved to significantly inhibit bioluminescence in *V. harveyi*, disrupting QS regulated gene expression when used in micromolar concentrations [56]. Furanone mediated inhibition of QS signalling was investigated also in versatile pathogen *P. aeruginosa*, the results revealing a great virulence gene expression disruption. Hentzer et al. demonstrated reduced activities of exoprotease, pyoverdine, and chitinase, and also biofilm disruption after addition of low concentrations of furanones. *In vivo* studies revealed that furanones remain active in biological systems, decreasing mortality in *P. aeruginosa* lethal dose infected mice [57].

Furanones signalling in bacteria is far of being understood at molecular level, but recent studies suggest that the AI-2 pathway may be involved in this cross-signalling in both Gram negative and Gram-positive bacteria. It has been

demonstrated that chemotaxis and flagellar biosynthesis genes of *E. coli* that were reportedly induced by exogenous AI-2 were inhibited when the furanones were added to cultures [58]. The mechanism by which this occurs remains yet unclear. AI-2 signalling in Gram positive bacteria is also unknown, since no signalling pathways have been described, even if staphylococcal biofilms were inhibited from growing on materials used in medical devices when furanone compounds were integrated into them [59].

3.1.3. Plant-derived QS modulators

Plants have been used since antiquity for their healing and antimicrobial properties. Although their action on bacteria development at molecular level is mostly unknown, they are used for attenuation of bacteria virulence, adherence and biofilm formation and also for modulating QS communication [46, 49].

Essential oils also known as volatile oils are concentrated hydrophobic liquids containing volatile aroma compounds extracted from plants. Many medical applications have been proposed for essential oils in history, ranging from skin treatments to remedies for cancer and infection treatment [60]. Studies have revealed that rose, geranium, lavender, peppermint, cinnamon, clove and rosemary oils are very potent QS inhibitors mediating QS dependent phenotypes in Gram negative species. Clove, cinnamon, lavender and peppermint oils revealed a promising anti-QS activity in *Chromobacterium violaceum*, significantly inhibiting pigment production. Clove essential oil have proved to interfere also with *P. aeruginosa* QS, reducing social phenotypes as swarming motility [61].

Even if they have proved their medical efficiency during time, essential oils use is restricted by certain physico-chemical, as high volatility. Therefore, recent studies aiming to reduce their volatility and enhance essential oils stability during time developed nanostructured vectoring systems [5-8, 46] and proposed improved adequate assessing methods [62]. Apart of stabilizing active volatile compounds nanohybrid phytoactive systems can be also used for controlled release and specific targeting [46]. Our recent work demonstrate that *Citrus maxima* essential oil exhibit improved antimicrobial and anti-adherence properties against *S. aureus* when used as a Core/shell/extra-shell layer coating a magnetite core [5]. Furthermore Anghel et al. demonstrate that major volatile compounds Eugenol and Limonene significantly interfere with QS controlled phenotypes in *S. aureus* and *P. aeruginosa*, following magnetite coating *in vitro*. The results revealed that both Eugenol and Limonene based nanophytoactive systems significantly inhibiting adherence and biofilm formation [46]. Even though the molecular mechanisms by which essential oils and their major volatile compounds interfere with bacteria QS are mostly unknown, our recent studies demonstrate that *Rosmarinus officinalis*, *Mentha piperita*, *Salvia officinalis*, *Eugenia caryophyllata* and *Citrus maxima* essential oils directly modulate the expression of genes involved in QS circuits in both *S. aureus* and *P. aeruginosa*. The data demonstrate that *E. caryophyllata* essential oil down-regulate expression of *rhlI*, *rhlR*, *lasI* and *lasR* genes, the key points of AHL QS signalling in *P. aeruginosa*. Also, *S. officinalis*, *R. officinalis* and *E. caryophyllata*, essential oils, and limonene and eugenol volatile compounds have proved to significantly repress *agrI* gene in *S. aureus* demonstrating molecular interference of essential oils with bacteria QS circuits *in vitro* [63]. Furthermore, the results revealed that *S. aureus* and *P. aeruginosa* virulence is significantly repressed when bacteria is grown in medium containing essential oils, since production of soluble virulence factors, as exoenzymes and toxins are inhibited [63].

In vivo studies demonstrate that plants may produce many bacteria QS inhibitors during infection. It have been shown that in growing onion bulbs infected with *P. aeruginosa*, several compounds as pantolactone, 4,5-dihydro-4,5-dimethylfuran-2(3H)-one, myristic acid, and linoleic acid are produced [64]. Therefore, it was postulated that this compounds may be produced by plants and act as defence system. Pantolactone and myristic acid have proved to act as potent QS modulators in *P. aeruginosa*, significantly inhibiting pyocyanin production, protease, lipase and polygalacturonase activity, without impacting on bacterial growth [64].

Various species of the plant genus *Dalbergia* are traditionally used as medicine for sundry ailments and some of them have been recently shown to quench the virulence of Gram positive and Gram negative bacteria. Screening n-hexane extracts of leaves, roots and barks of endemic Malagasy *Dalbergia* species for their capacity to antagonize QS mechanisms in *P. aeruginosa* showed that many reduced the expression of the QS-regulated genes *lasB* and *rhlA* [65]. However, only the extract of *D. trichocarpa* bark (DTB) showed a significant reduction of QS-gene expression without affecting *aceA* gene encoding a QS-independent isocitrate lyase. Preliminary structural characterization of these potent biofilm disrupters suggests that they belong to the phytosterols, a class of vegetal hormones involved in cell-to-cell signalling. Further characterization of DTB impact on QS revealed that the QS systems *las* and *rhl* are inhibited and associated phenotypes affected. Swarming and twitching motilities, biofilm formation and the production of pyocyanin, elastase and proteases were hampered in the presence of the DTB extract. The strong inhibition of motility and biofilm formation suggests that the DTB extract contains agents disrupting biofilm architecture, which is an important observation in the context of the design of new drugs targeting biofilm-encapsulated pathogens [65].

3.1.4. Mammalian-derived QS modulators

Animal hosts have also adapted to bacterial pathogens for a better survival. Since QS inhibitors have proved to exist in plants in nature and have been shown to attenuate bacteria virulence and infectivity, researchers have explored

mammalian signalling compounds in finding molecules to protect human and animal hosts against infections by modulating cell-to-cell communication.

It has been proved that after entering the host cells bacteria QS molecules are inactivated or cleaved by mammalian enzymes [67], most of which are currently unknown. QS inactivation seem to be molecule specific, a study performed in human epithelial airway tissues revealing that after treatment with *P. aeruginosa* AHLs, OdDHL is cleaved shortly after addition, but not C4HSL [67]. Human paraoxonases are highly conserved antioxidant enzymes, for which have been also proved the ability to hydrolyze lactones. Despite their antioxidative properties, however, the presence of three highly conserved lactonases in animals in the absence of any significant endogenous lactones was considered intriguing and researchers aimed to identify natural substrates for these enzymes. It has been proved that paraoxonases can interfere with bacteria QS, degrading *P. aeruginosa* produces AHLs and through blocking this bacterium communication, also reduce the extent of infection [66].

In recent years, some mammalian cell-to-cell signalling molecules have proved to interfere also with bacteria communication. It has been demonstrated that the pathogens are able to recognize and respond to various host signalling molecules, such as the opioid dynorphin [68], brain natriuretic peptide (BNP) hormones [69], IFN γ [70] and also catecholamines [27-31, 71, 72]. These eukaryotic signalling molecules are able to interfere also with bacteria cell-to-cell communication and these cross-signalling impacts on bacteria virulence and progression of infectious process [26]. The most investigated eukaryotic molecules which impacts on host-bacteria communication are stress related hormones. Recent data demonstrate that *P. aeruginosa* can intercept opioid compounds released during host stress and integrate them into core elements of QS circuitry leading to enhanced virulence. The results revealed that synthetic (U-50,488) and endogenous (dynorphin) kappa-agonists opioids stimulate *P. aeruginosa* virulence *in vitro* by significantly increasing pyocyanin production [68]. This phenotype seems to be influenced by the ability of kappa-opioids to interfere with bacteria key elements of the quorum sensing circuitry such as the global transcriptional regulator MvfR and the quorum sensing-related quinolone signalling molecules PQS and HHQ. Furthermore, *in vivo* significance of kappa-opioid signalling of *P. aeruginosa* was demonstrated in mice by showing that dynorphin can be released from the intestinal mucosa following ischemia/reperfusion injury, activates quinolone signalling in *P. aeruginosa*, and enhances its virulence against probiotic species of *Lactobacillus* and nematode *Caenorhabditis elegans* [68].

Studies investigating the role on neuroendocrine stress hormones on bacteria species, suggested that catecholamine noradrenaline (norepinephrine, NA) may act as a QS surrogate molecule, developing similar effects with peptide bacteria autoinducers (AI), generically called hormone-like molecules [3, 24-28]. Furthermore, the same research group indicate QseC type receptors as common receptors for catecholamines and a novel autoinducer – AI-3, and propose QseEF two component system being responsible for adrenergic and stress sensing in *E. coli* [24-28]. However, some question marks still remain regarding the ligand specificity of this receptor, since the complete structure and role of AI-3 is still not known.

Karavolos and collaborators observed that exposure of *S. typhi* to neuroendocrine hormones resulted in increased haemolytic activity. A proteomics-based dissection of the haemolytic phenotype identified a significant reduction in levels of outer membrane protein A (OmpA) after exposure to physiological concentrations of adrenaline or noradrenaline. This seems to be attributed to increased levels of the small RNA (sRNA) chaperone protein Hfq and the sRNA *micA* repressing *ompA* expression. The haemolytic response was specific to membrane vesicles, and was not observed in an *S. typhi* strain lacking the sRNA, *micA* [72]. The authors also revealed that these effects could be reversed by the addition of the β -adrenergic blocker propranolol. Another remarkable finding is that the neuroendocrine hormone-mediated haemolysis required the CpxAR two-component signal-transduction system [72] and was independent of the *E. coli* O157:H7 bacterial adrenergic receptor orthologue QseBC, the only bacteria adrenergic signalling pathway reported so far [3, 28].

Another bacteria pathogen which seems to sense and respond to adrenergic hormones by QS – dependent modulation of virulence is *P. aeruginosa*. It has been reported that the hormone norepinephrine increases *P. aeruginosa* PA14 growth, virulence factor production, invasion of HCT-8 epithelial cells, and swimming motility in a concentration-dependent manner. Transcriptome analysis of *P. aeruginosa* exposed to 500 microM, but not 50 microM, norepinephrine for 7 h showed that genes involved in the regulation of the virulence determinants pyocyanin, elastase, and the signalling QS molecule PQS were upregulated [71]. Also, the production of rhamnolipids, which are important in *P. aeruginosa* infections, was not significantly altered in suspension cultures upon exposure to 500 microM norepinephrine, but decreased on semisolid surfaces. Swarming motility, a phenotype that is directly influenced by rhamnolipids, was also decreased upon 500 microM norepinephrine exposure. Hedge and co-workers suggested that the increase in the transcriptional activation of *lasR* but not that of *rhIR* and the increase in the levels of PQS suggest that the effects of norepinephrine are mediated primarily through the *las* quorum-sensing pathway [71]. Opposite with these results, another research group demonstrate that catecholamines are able to inhibit *P. aeruginosa* virulence by repressing represses the expression of *toxA* and the siderophore genes [37]. It has been revealed that norepinephrine enhances the growth of bacteria by supplying iron from serum iron binding proteins, as transferrin. This provision of iron seems to repress the expression of exotoxin A gene, *toxA*, the pyoverdine genes *pvdD* and *pvdE*, and their regulators, *pvdS*, *regA*, and *pchR*, suggesting that norepinephrine accomplishes this repression through *PvdS* and *PchR* regulatory proteins [37].

Even though there is a great interest, controlling bacteria virulence by natural QS modulators is still a new and poorly explored field, with great perspectives for biomedical applications.

3.2. Synthetic QS modulators

Since the technological progress brings novel insights into discovering the intimate molecular support of bacterial QS signalling molecules, researchers aim to develop targeted synthetic QS modulators. In *S. aureus* QS signalling is controlled by *agr* system, which controls the production of Agr peptides that have been found to contain an unusual thiol ester-linked cyclic structure. Mayville et al. demonstrated that synthetic Agr thiolactone-containing autoinducing peptides interfere with *S. aureus* virulence both *in vitro* and *in vivo* [78].

Recent work on modulating AHL signalling in Gram negative bacteria lead to identification of novel small molecule inhibitors binding to N-acyl-homoserine lactone synthase TofI in *Burkholderia glumae*. In this bacterium the main AI synthesized by TofI is C8-HSL, which seems to control virulence, motility, and protein secretion. Chung et al. characterized two previously unknown QS inhibitors identified in a focused library of acyl-HSL analogues. Their functional and X-ray crystal structure analyses showed that the first inhibitor, J8-C8, binds to TofI, occupying the binding site for the acyl chain of the TofI cognate substrate, acylated acyl-carrier protein. Closer inspection of the mode of J8-C8 binding to TofI provides a likely molecular basis for the various substrate specificities of acyl-HSL synthases. The second inhibitor, E9C-3oxoC6, competitively inhibits C8-HSL binding to TofR, the cognate receptor of C8-HSL [79]. Singh et al. proposed several femtomolar transition state analogue inhibitors of 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) from *E. coli*. 5'-Methylthio-Immucillin-A (MT-ImmA) derivatives has proved to be efficient inhibitors for MTAN. Substitution of the methylthio group with a p-Cl-phenylthio group gives a more powerful inhibitor since provide a better dissociation constant K(i). Among tested synthetic inhibitors, the most powerful inhibitor was 5'-p-Cl-phenylthio-DADMe-Immucillin-A (pClPhT-DADMe-ImmA) with a K(i) value of 47 fm (47 x 10⁻¹⁵ m). These are among the most powerful non-covalent inhibitors reported for any enzyme, binding 9-91 million times tighter than the MTA and SAH substrates in *E. coli*. The inhibitory potential of DADMe-Immucillins has proved to support a fully dissociated transition state structure for *S. pneumoniae* MTAN also. Therefore, powerful inhibitors of MTAN are candidates to disrupt key bacterial pathways including methylation, polyamine synthesis, methionine salvage, and quorum sensing [80].

In *P. aeruginosa* it has been shown that a structurally unrelated stable mimic seems to interact specifically with AHL QS circuit. The triphenyl mimic seems to interact specifically with LasR but not with QscR. *In silico* analysis suggests that the mimic fits into the OdDHL-binding site of LasR and makes key contacts with LasR. The authors suggest that the triphenyl mimic can be used as an excellent scaffold for developing quorum-sensing inhibitors, and its stability and potency make it ideal for biotechnology uses such as heterologous gene expression [81].

Even though is a recent described molecule, AI-3 control of virulence in *E. coli* has prompted investigators to develop small molecules that could inhibit this signalling system. To this end, Rasko et al. have recently described the identification of one such molecule, through a high-throughput screening of a large library of compounds. The authors identified N-phenyl-4- {[phenylamino]thioxomethyl}amino}-benzenesulfonamide (LED209) as an inhibitor of QseC and bacterial virulence, both *in vitro* and *in vivo*. LED209 inhibits QseC autophosphorylation, virulence factor production and AE lesion formation by EHEC. Additionally, LED209 can inhibit virulence factor production and host colonization by *S. typhimurium* [82, 2].

4. Concluding remarks

Since the initial discovery of quorum sensing the mechanistic understanding of various QS circuits and appreciation for their importance in the pathogenesis of many bacterial species have ascended. The opportunity of targeting quorum sensing as a means to improve anti- infectious strategies or prevent infection has been met with strong confidence in the wake of the antibiotic resistance problem that currently obstructs treatment of many bacterial pathogens. It is clear now that the boundary between QS and bacterial virulence represents a promising field from which novel, effective anti-virulence strategies can emerge. Presumably, therapies that affect bacterial behaviour will not be as prone to resistance as are the targets of traditional antibiotics that result in outright killing of bacteria or inhibition of their growth. Therefore, therapeutic approaches that interfere with small signalling molecule-controlled pathways could have longer functional projection than novel developed antibiotic drugs. Apart of their efficiency and low resistance acquiring perspective, QS modulation brings an ecological alternative to classical therapies. The real challenge in the future relies in efficient transposition of this knowledge into real therapeutic approaches that could boost our nearly exhausted supply of effective antibiotics.

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