Pallaval Veera Bramhachari *Editor*

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Preface

In the last decades, progress on the knowledge of bacterial quorum sensing and biofilm formation has been advanced exponentially. Bacteria have intriguing and diverse social lives. A unique phenomenon where microbes communicate and synchronize their behavior by the accumulation of (AHL) signaling molecules. A reaction occurs when AHL accumulates to an adequate concentration. They exhibit coordinated group behaviors regulated by quorum sensing (QS) systems that detect the density of other bacteria around them. The regulation of social behavior in bacteria is key to several phenomena of medical relevance, including biofilm formation and the expression of virulence in pathogens. Explicitly, QS is the chemical communication process that bacteria coordinate changes in their collective behavior in response to population density. A contemporary challenge in the field is to comprehend how QS works in scenarios that mimic real host environments.

Initially, bacteriologists explored QS quite separately, not relating it to biofilm. Later on, it was discovered that the QS is a molecular system based on rRNA genes, whereas biofilm formation is the quantitative community analysis for microbes. The knowledge and information about biofilm have skyrocketed since then. Rapid advances in molecular biology have revolutionized the study of QS in microbes and improved the understanding of intra- and interspecies communications among microbial communities. The advent of molecular biology has offered a number of revolutionary new insights into the QS research in microbes.

We now have an in-depth knowledge apropos how bacteria employ QS signals to communicate with each other and to coordinate their activities. In recent years, there have been extraordinary advances in the recent understanding of the genetics, genomics, biochemistry, and signal diversity of QS. The world has started to understand the connections between QS and bacterial sociality. This foundation places us at the beginning of a new era in which researchers will be able to work toward new medicines to treat devastating infectious diseases and use bacteria to understand the biology of sociality. The application of QS as a target for the development of novel anti-infective agents is the major activity in providing "quality of life enhancement" from the public funding of research.

We strongly believe that this book would provide enough insights into the amazing world of microbial QS. The present book is an attempt to compile the novel information available on recent advancements on various functional aspects of QS systems in different gram-positive and gram-negative organisms. Finally, the book also elucidates a comprehensive yet a representative description of a large number of challenges associated with QS signal molecules, viz., virulence, pathogenesis, antibiotic synthesis, biosurfactants production, persister cells, cell signaling and biofilms, intra- and interspecies communications, host-pathogen and social interactions, and swarming migration in biofilms. It is essential reading for the novice and expert in the field of QS researchers, industrialists, as well as students. With these objectives in mind, the content of this textbook has been arranged in a logical progression from fundamental to more advanced concepts. We hope that this book stimulates your creativity and wish you success in your experiments.

This book is a stunning reflection of the seriousness with which the several scientific minds are dedicated to the welfare of the scientific community. I am extremely thankful to the contributors for paying continuous attention to my request and showing faith in my capabilities. I shall always remain highly obliged to all of them forever. These words cannot justify the worthiness of their efforts.

We successfully compiled our creative and thoughtful research work due to genuine concern and painstaking effort of many more well-wishers whose names are not mentioned, but they are still in our heart. So, the reward is surely worth for their efforts. I want to dedicate this book to my mother, S. Jayaprada (late).

Myself and contributing authors hope from the bottom of our hearts that this book will be a good guidebook and compass for research studies in bacterial quorum sensing. Bon voyage, all!

Machilipatnam, India Pallaval Veera Bramhachari

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About the Editors

Dr. Pallaval Veera Bramhachari is currently a faculty and head of the Department of Biotechnology at Krishna University, Machilipatnam, India. He has earlier served as postdoctoral research scientist at the Bacterial Pathogenesis Laboratory, Queensland Institute of Medical Research in Brisbane, Australia, and DBT postdoctoral fellow at the Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore. He completed his PhD in Microbiology from Goa University, India. His major research interests are the investigation and development of microbial exopolysaccharide (EPS), biosurfactants and biofilms from diverse environments. Furthermore, he expanded his research fields to the applied research in microbiology and cell biology including biochemical mechanism of bacterial EPS in sequestrating heavy metals, stress-induced proteins, novel biosurfactant molecules, bacterial-biofilm formations, and structure-function relationship of bacterial rhamnolipids. He has published more than 105 research articles including 2 international books in prestigious peer-reviewed international Scopus-indexed journals and presented 45 abstracts at various national and international conferences. He has served as editorial board and reviewer for a number of national and international journals. He is a member of many international scientific societies and organizations, most importantly, Indian Science Congress & Society of Biological Chemists, India. He successfully completed two major research projects. He has been conferred with various prestigious awards, notably, DST Young Scientist with a research project and nominated as Associate Fellow of Andhra Pradesh Academy of Sciences for the year 2016. He obtained two Indian patents in 2017. He has more than 12 years of teaching and research experience at the university level.

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Part I
Introduction to Microbial Quorum Sensing

Introduction to Quorum Sensing Research in Diverse Microbial Systems

Pallaval Veera Bramhachari

Abstract Bacteria are able to produce and act in response to minute diffusible molecules called autoinducers (AI). These molecules amass as cell density enhancements and regulate the expression of set of genes to control diverse physiological functions by quorum sensing (QS). Several species of bacteria swap signal molecules to assist checking their own population densities. Until recently, it was contemplated that QS was an unusual phenomenon restricted to not many microbial species. Nevertheless, numerous novel exemplars of interbacterial and intrabacterial signaling mechanisms are documented. Amongst them, acyl-homoserine lactone (AHL) and QS signaling systems are perhaps the best implicated diverse chemical languages used by both Gram-positive and Gram negative bacteria respectively. QS systems have primarily smudged the difference involving unicellular and multicellular life forms. Several QS systems exceptionally essential to medicine and agriculture. These QS microbes could be undoubtedly expensive tools for biologists to inquire and comprehend the progress of cooperation and cell to cell communication, wherein the realistic applications of this acquaintance will befall well-known in conjunction with basic acquaintance.

Keywords Autoinducers (AI) · Quorum sensing (QS) · Acyl-homoserine lactone (AHL) · Cell to cell communication

Introduction

Microbes can coordinate population behavior and can adapt an array of behaviors that are essential for fitness with small molecules called acyl-homoserine lactone (AHL) which serves as a signal of cellular population density, triggering new patterns of gene expression for mounting virulence and pathogenesis [6]. Quorum sensing (QS) enables bacteria to communicate with members of their own species, with other species of bacteria, and with their eukaryotic host cells i.e., intra- and

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inter-species communication [5]. QS plays critical roles in regulating diverse cellular functions in microbes, including bioluminescence, pathogenesis, virulence, gene expression, biofilm formation and antibiotic resistance. Therefore these microbes have the competence to coordinate and regulate explicit sets of genes by sensing and communicating amongst themselves by utilizing variety of signals [2]. Interestingly the discovery that bacteria capable to communicate with each other tainted our discernment of many single organisms populating our milieu. However it is very imperative for pathogenic bacteria during infection of a host to either coordinate their virulence to escape the immune response or be capable to establish a triumphant infection.

Numerous QS systems have been investigated and established, together with many noteworthy systems that involve regulation of genes essential for triumphant establishment of symbiotic and pathogenic microbial interactions. Even though several QS systems are identified, perhaps the two most meticulously depicted systems are acyl-homoserine lactone (acyl-HSL) systems of many peptide-based signaling systems of several Gram positive species Gram-negative and species. QS systems in bacteria are usually classified into three categories: [1] LuxI/LuxR–type QS in Gram-negative bacteria that employ typical signal molecules viz. acyl-homoserine lactones (AHL); [2] oligopeptide-two-component-type QS in Gram-positive bacteria, that employ small peptides as signal molecules; and [3] luxS-encoded autoinducer 2 (AI-2) QS in both Gram-positive and Gram-negative bacteria. Remarkably every signal molecule is detected and countered by a specific sensing machinery and regulatory network [3].Given the fact that many bacteria employs QS mechanisms in conniving pathogenicity, virulence and biofilm formation yet the QS machinery comprises a new target for the development of antibacterial agents with prospective applications in scores of emerging fields. At present, as a minimum of four different strategies intending at meddling with QS have been proposed, including [1] inhibition of signal generation; [2] interference with signal dissemination; [3] blocking signal receptors; and [4] inhibition of signaling response system [1, 7]. QS research has many potential applications; most of these involve controlling bacteria by interfering with their signaling systems. For instance, many bacteria count on QS to control the expression of genes which produces biofilm and expression of virulence genes. Nevertheless, if the QS systems are blocked, we can avert these microbes from being extra precarious. Therefore the connection between biofilm formation and QS led to turmoil of studies to evaluate how microbial social behaviors influence this imperative mode of growth; however it was promptly discovered that this association clearly depends on complex environmental conditions [4].

This book illustrates the importance and significance of Quorum sensing, its vital roles in regulating varied cellular functions in microbes, including virulence, bioluminescence, pathogenesis, biofilm formation, gene expression as well as antibiotic resistance. Microbes can coordinate population behavior with small molecules called auto inducers (AI) which serves as a signal of cellular population density, triggering new patterns of gene expression for mounting virulence and pathogenesis. Therefore these diverse microbes have the competencies to coordinate and regulate explicit sets of genes by sensing and communicating amongst themselves

utilizing variety of signals. Nonetheless these intricate quorum communications raises numerous fundamental questions which are increasingly attracting the attention of scientists.

In this book we focus on how bacteria can coordinate an activity and synchronize their response to external signals and gene regulation in selected bacteria and fungi. The theme of the proposed book revolves around the basic understanding of QS systems as well as the importance of QS systems controlling the several physiological behaviors in bacteria and fungi. Nonetheless these intricate quorum communications raises numerous fundamental questions which are increasingly attracting the attention of scientists in medicine, agriculture and industry. Natural anti-quorum sensing strategies already exist. There are numerous opportunities for novel biotechnological applications to delay/augment QS controlled functions in bacteria. More molecules are yet to be discovered. Thus, understanding the microbial QS machinery and outcome has essential implications to appreciate the multifarious host-pathogen interactions and may perhaps endow with innovative targets for antimicrobial therapies that block or interfere with their crisscross microbial communication networks.

Significance

The detection of the extensive use of QS systems in diverse microbes is fundamental in steering several researchers to explore the secret behind multicellular behaviors rather than on individual physiological processes. There is a blaze of research progress on bacterial QS, and the field persists to inflate quickly. Conversely, investigations on how bacterial QS factors regulate at transcriptional and translational level in biofilms remains in their early stages. An apparent challenge in the field ascertains, what factors of a biofilm influence the onset of QS and consequent gene expression. An additional key challenge is to resolve functional complications of multi species biofilm quorum sensing. Future investigations will clearly address several questions in the promising field of intricate bacterial social behaviors. The answers to these questions will certainly endow with innovative insights and revelations. We understand that there are several diverse small molecule-dependent interactions amid microbes and their hosts. There is definitely more to be discovered and sort out fundamental differences among these multiple QS signaling systems. Understanding these issues will be significant as we move towards translating basic studies of QS to congregate future prospects, including functional studies of the microbiome.

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Conflict of Interest The author declares that there is no conflict of interest.

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Intra and Inter-Species Communication in Microbes: Living with Complex and Sociable Neighbors

G. Mohana Sheela, A. M. V. N. Prathyusha, Nageswara Rao Reddy Neelapu, and Pallaval Veera Bramhachari

Abstract Quorum sensing is a signaling mechanism wherein the microbes interact with each other through diverse chemical signals, known as auto inducers. Microbes not only synthesize, secrete, detect and respond to the chemical signals but also sense the signals that they do not synthesize in their immediate environment to discriminate their neighbors from others. Intra and inter-species communications between microbes surrounded by biofilm could be antagonistic, such as competition over nutrients and growth inhibition, or synergistic. These comprise the mixed biofilm development by co-aggregation; metabolic cooperation where one species utilizes a metabolite produced by its nearest species, along with augmented resistance to antibiotics or immune responses host. Interestingly bioluminescence, virulence factor expression, antimicrobial resistance, sporing and maturation of microbes also depend on mixed communications. These favourable interactions in mixed biofilms have important environmental, industrial and clinical connotations. The present review emphasizes the current knowledge relating to intra and species auto inducers and their role in activation of genes along with the receptors and signal molecules released by host cells.

Keywords Auto inducers · Intra and inter communication · Quorum sensing · Multi-species biofilm formation

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Authors G. Mohana Sheela, A. M. V. N. Prathyusha, Nageswara Rao Reddy Neelapu and Pallaval Veera Bramhachari have equally contributed to this chapter.

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Introduction

The biofilms identified on medical devices or tissues of a host or on agricultural produces are bacterial cells living together as a community. Effective colonization of any pathogen as a biofilm requires recruiting cells of the same species or diverse species. If the cells of same species are recruited then biofilm is known as monospecies microbial biofilm, whereas if the cells of the other species are recruited then biofilm is known as polyspecies microbial biofilm. A monospecies microbial biofilm can recruit cells of the same strain or other strains of the same species. Communication within a monospecies microbial biofilm (same strain or other strains) or with other species of polyspecies microbial biofilm requires an effective communication scheme called quorum sensing (QS). QS controlled processes are in general contemplated ineffective when an individual bacterium acts alone, however extremely productive in groups of cells with a unified response [11]. Thus, quorum sensing materializes to unclear the difference amid unicellularity and multicellularity and therefore allows bacteria to function as a multicellular organism. QS facilitates cells to execute a broad range of functions, for instance defense against toxins ([22, 32, 39]), starvation responses to nutrients [21], competition with other microbes for nutrients and survival [20] and institute symbiotic association with more species.

Bacteria achieve intra-species and inter-species communication in course of the production, secretion and detection of small molecules [25]. Many of these compounds, termed "auto inducers", elicit beyond a definite threshold concentration via transmembrane signaling via phosphorylation and eventually gene regulation. The bacterial signaling compounds belong to a array of chemical classes, together with the furanosyl borate ester autoinducer-2 (AI-2), alkylhydroxyquinolines, N-acylhomoserinelactones (AHLs), cis-2-dodecenoic acid, (PQS), or α-hydroxyketones (AHKs), CAI-1 and LAI-1 [25, 43]. These diverse communications helps in (i) detecting the density of the cells of the biofilm; (ii) differentiating species and detecting them in a polyspecies microbial biofilm; (iii) differentiating strains and detecting them in a monospecies microbial biofilm; (iv) sometimes the communication in some species can help in detecting the signal and misguiding the other species in the biofilm; and (v) interkingdom communication to communicate between bacteria-fungi, bacteria-plants and bacteria-mammalian cells. If, density of the cells in the population is detected, then information can be used for effective utilization of the nutrients. Detecting low density of the same species or strain population in monospecies microbial biofilm can help in releasing the virulence factors to increase the density of the population. When different strains or species are sensed, then information is used to inhibit other strains or species by releasing inhibitors or by regulating the gene expression as per the requirement. Sometimes, when bacteria are competing in the mixed population or multiple species especially in niches like gastrointestinal tract, the signal or communication is removed by the bacteria to trick the other bacteria as if its density is low and is a monospecies microbial biofilm.

Literature reports three communication systems (i) intraspecies communication system; (ii) interspecies communication systems and (iii) interkingdom communication system which are well studied, characterized and established. These communication systems help in performing the above mentioned roles. Therefore, this chapter reviews the use of intraspecies and interspecies communication system to (i) detect density of the cells; (ii) differentiate same species or strain; and (iii) interfering or being tricky with communication of the other bacteria. At the same time this chapter also elaborates on interkingdom communication system between bacteria-fungi, bacteria-plants and bacteria-mammalian cells.

Intraspecies Communication in Bacteria

Bacteria discharge chemical signals into neighboring environment and sense them when approaches their contiguity. A bacterium evaluates the neighbor's number in its adjacency using the unique sensing strategy. Consequent to sensing a critical cell density, bacteria synchronize a number of cellular processes and physiology in density-dependent manner is called quorum sensing [4]. QS mechanism controls a several functions in bacteria viz. biofilm formation, motility, bioluminescence, virulence etc. (Fig. 1). It is noteworthy that bacterial species utilizes a multisignal network QS configuration to accomplish specific activities, which depend on specific environmental niches. A bacterium integrates multiple QS systems are to decipher discrete information transmitted through specific signals. Intraspecies communication is so far significantly investigated, easy due to the ease of working with pure cultures of bacteria [23, 25].

Gram-positive bacteria utilize peptides (Auto inducing peptides) as signaling pheromones generally, while N-acyl homoserine lactones are employed by gram-negative bacteria as small molecules. However, some aromatic alcohols discharged by fungi known to function as AIs for intraspecies signaling [7]. Gram-positive bacteria communicates using peptides and senses through receptor-histidine kinases (RHKs) entrenched in membrane however in gram-negative bacteria small molecules can disseminate through the cytoplasmic membrane which bind to regulatory proteins within the cell to trigger transcriptional changes. Peptides and small molecules subsist and respond through membrane-bound or cytoplasmic receptors in all classes of bacteria [19].

Many gram-negative bacteria employ acylhomoserine lactones as intraspecific signals in density reliant gene regulation. First acyl-HSL, was documented in marine bacterium *V.fischeri.* Numerous bacteria including *A.tumefaciens, P.aeruginosa* and *R.leguminosarum* produce a broad range of acyl-HSLs, differs in length of acyl moiety in addition to degree of oxidation at C3 position. Acyl-HSLs are known to signal through a protein known as LuxR and are produced by an enzyme known as LuxI [15]. For instance, *V.harveyi*, a marine bacterium closely related to *V. cholerae*, utilizes three AIs HAI-1 (AHL), CAI-1 and AI-2 to regulate intra-species, intra-genera and inter-species communications respectively (Fig. 2). However,

Fig. 1 Quorum sensing or communication among bacteria of a biofilm regulating several activities

N-(3-hydroxybutanoyl)-homoserine lactone, is synthesized by LuxLM protein [2, 6]. Despite the fact, LuxLM is not a part of LuxI homologues, it employs analogous biosynthetic pathway to LuxI-family protein. However, the synthesized AI-1 is identified by the response regulator protein LuxN that is related to two-component sensor kinase in Gram-positive bacteria [5] and transmit signal through LuxR. (CAI-1) is synthesized by CAI-1 auto inducer synthase (CqsA) acts on SAM and decanoyl-CoA. CqsA enzymes exist in all *Vibrio sp.*, moreover they can generate various CAI-1 moieties that have different acyl chain lengths and modifications. *Vibrio* spp. counter to each other's CAI-1s with diverse affinities than to their own CAI-1 s, which perhaps implies that CAI-1 is employed for intra-*Vibrio* communication [33, 34].

Notably, gram-positive bacteria communicate using modified oligopeptides (AIPs), which are reasonably bigger than AHL. Therefore the oligopeptides do not diffuse freely through the cell membrane and hence, the cells need two-component phosphorelay cascades consisting of a membrane-bound receptor/sensor histidine

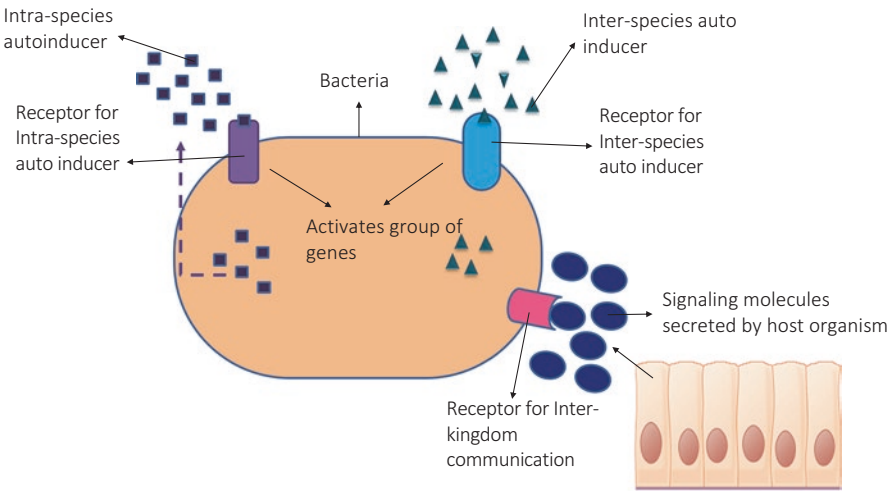

Fig. 2 Intra and Inter species auto inducers and receptors enable bacteria to communicate with others of their own species and different species or genus

kinase protein with an intracellular response regulator to sense extracellular oligopeptides. AIP is synthesized from processing of propeptide, AgrD, modified and exported by AgrB thereby AIP binds to and triggers activation of the receptor-histidine kinase, AgrC. Nonetheless the activation resulted in improved transcription of the exceptional regulator, RNAIII, eventually leading to activate the transcription of the genes in the quorum sensing regulons [26, 27].

QS is usually noticed where microbes of a single species or different species team up and compete with each other. However, existence of more than one QS system in the individuals of a same species permits the emergence of numerous types of connections among these QS systems. Communication occurs not only between the species but also among the individuals of same species. For instance the communication among the individuals of different species observed in *V. harveyi, E. coli, S. typhimurium, V. cholerae*, and *E. faecalis* [30, 31]. The analogous arrangement of QS systems was observed in *V. harveyi, V. cholerae* and *V. vulnificus* while helps control biofilm production and virulence factors [24, 40]. The series arrangement of QS systems was evidenced in *P. aeruginosa* that causes a hierarchical activation of every system and controls multiple lung adhesion factors and virulence factors [1, 18]. *B.subtilis* and *P. aeruginosa* also possess more than one QS system that aleinate each other and only one amid the systems is favored, permitting the bacterium to choose one of two proxy lifestyles. It is presumed that the subsistence of multiple arrangements of QS systems might play a central role in processing environment specifically and as a consequence, dictating preferred and robust combined cellular response [30].

Inter-Species Communications in Microbes

Mechanism of intercellular communication as a function of population density exists in several bacteria. These signaling circuits are based on the release of diffusible molecules to the extracellular medium and their detection and subsequent modification of global gene expression above definite threshold concentration. Many bacteria utilize cell-cell communication systems to regulate horizontal gene transfer. Interspecies signaling between *P.aeruginosa* and *B.cepacia* generally occur collectively in the lungs of people with cystic fibrosis, where they are identified with high mortality and morbidity [14]. For example QS transcription factor ComA in *Bacillus sp*. typically utilize small extracellular peptides as cell-cell communication signals to regulate sporulation, competence, and exoenzyme production, polysaccharides, and other secondary metabolites [8]. The production of AI-2 a furanosyl borate diester molecules is widespread amongst diverse species of Gram-negative and Gram positive bacteria and these signal molecules are treated as ubiquitous signal for interspecies communication in bacterial community [4, 29] (Fig. 2).

Interspecies interactions among *S.maltophilia* and *P. aeruginosa* in mixed biofilms do not encode *LuxS* or any known type of N-AHL synthase [38, 45]. On the contrary, the genome of *P. aeruginosa* does not carry an *rpf* gene cluster. Thus both strains do not emerge to produce signaling molecules of same structural class no more can produce AI-2. However interspecies signalling between the two species was shown to be mediated by diffusible signal factor (DSF), persuades both *P.aeruginosa* biofilm architecture and the synthesis of proteins that donate to resistance of this strain to cationic antimicrobial peptides (CAMPs). Noteworthy that *P. aeruginosa* does not have a luxS gene and hence does not produce AI-2. Nevertheless, this pathogen can detect AI-2 produced by bacteria within the oropharyngeal flora with subsequent effects on virulence gene expression [13]. In a comparable approach, *Escherichia, Salmonella* and *Klebsiella* sp. doesn't possess the ability to produce N-AHL, nevertheless it can hold the Lux R type protein SidA which can detect exogenous N-AHLs produced by other bacteria [41]. It is interesting to note that, plant pathogenic bacteria employ complex signalling systems to regulate the expression of virulence genes at the cellular level and within populations. In plant pathogenic *Xanthomonas* spp. and *X. fastidiosa*, the key QS signal molecules are unsaturated fatty acids, called diffusible signal factors (DSFs) [12]. Additionally, a new type of QS signal, the small protein Ax21, has been explored in recent times [16, 17].

Technological advances in next-gen sequencing allowed to apply RNA sequencing of two species at the same time (dual RNA-seq technique) and so as to openly learn the gene expression of two interacting species devoid of the need to actually separate cells or RNA [44]. This method is considered functional to various types of interspecies communications viz. host-pathogen, commensal and mutualistic interactions. Plethora of latest studies investigated the potent host-bacterial interactions: for example, *S.pneumoniae* [3] a murine *Y.pseudotuberculosis* [28]; *P.aeruginosa*, [10]; *S.aureus* [37]. In a recent report, the enzyme sortase A has been evidenced for

its interspecies adherence property of firmicutes to dental surfaces. Recent studies have provided evidence that deleting of srtA gene inhibits saliva mediated adherence and aggregation of *S.mutans* and *S.gordonii* thereby reduces the caries formation [36]. Interestingly *P.aeruginosa* was shown to partake in inter species communication through signaling by cis-2-unsaturated fatty acids of diffusible signal factor (DSF) family. Sensing these signals involve the histidine kinase PA1396 and leads to modulation of biofilm formation and improved resilience to diverse antibiotics.

Interkingdom Communication

Plethoras of recent papers have proven that QS molecules can influence gene expression in eukaryotes as many eukaryotic hormones structurally resemble AHLs. In general this has been described as interkingdom signaling [35]. In mammals there are three extensive groups of steroids, hormones, proteins/peptides and amino-acid derivatives. Amine and peptide hormones cannot diffuse through the cell membrane and bind to cell-surface receptors, while steroid hormones will diffuse through plasma membranes and bind to intracellular receptors. Peptide hormones include the insulin, glucagons and epidermal growth factor (EGF). Steroid hormones are resulted from cholesterol, and amines are produced from tyrosine. Interestingly amine hormones comprise the noradrenaline (NA), catecholamine adrenaline and dopamine. These hormones are employed in inter-kingdom signaling with microbes. Cugini and group depicted that farnesol produced by *C. albicans* interferes with the signal-specific quinolone QS system in *P. aeruginosa*. The *Pseudomonas* quinolone signal (PQS) binds to the LysR-type PqsR (MvfR) regulator to activate the expression of diverse virulence factors [9]. The structural determinant of microbial CAI-1 and derivatives was also tested for interkingdom signaling responses of *C.elegans* [42]. Nevertheless, little is known on a molecular level about the response of mammalian and protozoan cells to prokaryotic QS signals.

Conclusions and Future Perspectives

The cellular signaling systems of microbial pathogens are potential targets for novel disease control approach for the reason that are inclined to be essentially unlike from those of eukaryotic organisms and they eventually regulate biofilm formation and multiple virulence factors. The deliberation of the evolution of social behaviors of microbes is an enthralling endeavor that can enthuse every researcher in the area to appraise the benefits and continuance of inter and intercellular communications in environment. Nonetheless the homolog of LuxI-LuxR QS system was earlier reported in several bacteria, including LasI-LasR, RhlIRhlR, QscR, TraI-TraR and CviR. However the specific biological function for this profligate signal specificity

is still unknown, it is contemplated that the later class of receptors may be used only for inter-species signaling. Although there are fascinating indications for a LuxS dependent universal signal molecule in bacteria, staunch proof concerning the chemical nature of the compound and its signaling mechanism in all the organisms is currently wanting. Yet, there are many more questions that remain unanswered: Few important questions regarding quorum sensing are: whether diverse environmental niches decide on QS network architecture or QS ligand specificity? How do bacterial cells prioritize one type of auto inducers? And what are the necessities that facilitate QS systems to tune their signal responses to changing stimuli?

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Quorum Sensing Systems and Persistence

Laura Fernandez-García, Lucia Blasco, Rocío Trastoy, Rodolfo García-Contreras, Thomas K. Wood, and Maria Tomás

Abstract In order to control the clonal population's fitness to manage the expense of the resources by the community, it is not surprising that bacterial communities coordinate the formation of persister cells (bacterial subpopulations that survive stress conditions such as antibiotic or environmental threats). The development of these persister cells is linked to the activity of intercellular signaling molecules. Among them, we focus on acyl-homoserine lactone (AHL), the competence-stimulating peptide (CSP), indole (IND) and autoinducer-2 (AI-2), all involved in the quorum sensing systems activation in several pathogens. In this work, we will describe the action of these molecules related with quorum sensing systems in Gram positive *Streptococcus mutans* and *Staphylococcus aureus* and in Gram negative *Pseudomonas aeruginosa*, *Escherichia coli,* and *Acinetobacter* spp. bacteria.

Keywords Quorum sensing · Persister cells · Homoserine-lactones (HSL) · Competence-stimulating peptide (CSP) · Indole · Inhibitors

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Introduction

Persistence is defined as a dormancy state; i.e., a metabolically-inactive state in which cells cannot be affected by antibiotics. Persistence helps a sub-population to survive when in the presence of antibiotic lethal concentrations [1]. It has been shown that extracellular compounds which induce stress produce a dramatic increase in persister cells [2].

It was Hobby and collaborators who, in 1942, first described persister cells [3] during an experiment in which they found the ineffectiveness of penicillin against *Staphylococcus aureus* non metabolically active cells; the term persistence was coined in 1944 by Joseph Bigger when he was also analysing a culture of *Staphylococcus aureus* which had been treated with penicillin [4]. But it was not until the 1990s, when scientists discovered that biofilm formation by pathogens was related to chronic infections [5]. In recent years, it was observed how surprisingly resistant biofilms are to antibiotics and, after a deep analysis, researchers found that there were persister cells in biofilms [6, 7]. The immune system is thought to eliminate both persister and regular cells when it identifies them, but in biofilms, cells of the immune system cannot penetrate, so persister cells survive [7]. Persister cells, unlike resistant mutants, do not have the capacity to grow in the presence of toxic compounds; instead, they only grow when antibiotics have been removed and a carbon source is present [8].

Maisonneuve and collaborators have shown that even in the persister cell sub-population there exists heterogeneity, which implies that persister cells, created by analogous molecular methods, show various antibiotic-specific persistence levels [9]. Several aspects of persister cell sub-populations, such as their size and composition, are likely controlled by stress signalling pathways, like the SOS response that includes the messenger (p)ppGpp [10]. Surprisingly, Hong et al. noticed that those cells which are more sensitive to stress are more likely to become persisters [11].

In the last few years, the quest to identify persistence-related genes has been intense. The first protein found to be related to persister formation was HipA (in 1983), a toxin from the HipBA Toxin-Antitoxin system (TA system) [12]. In ensuing years, many TA systems were found to be involved in persister cell generation; for example, it has been shown that the deletion of various particular TA loci such as *mqsR* and *mqsRA* [13, 14], *tisAB/istR* [15], as well as *yafQ* [16], reduce the persister cell formation. The most prevalent TA systems are two component systems composed of a toxin, which has the capacity to inhibit some metabolic cell process; and the antitoxin, which blocks the toxin action by degrading it or preventing its action. Depending on the type of interaction between the toxin and the antitoxin, six different TA systems have been characterised [17]. TA systems of type I as well as of type II are the most analysed ones in relation to persister cell formation, but the others could be involved as well. The activation of the TA systems by the (p)ppGpp alarmone signal through polpyphosphate and Lon protease [9] has been widely reported but is controversial [18] and recently shown to be false [19]. Therefore, for the formation of persister cells, other pathways may also be involved, such as the ones associated with the SOS response, phospholipid synthesis, folate biosynthesis,

purine metabolism, DNA repair and energy metabolism [20]. Even RpoS has been identified as a persister formation regulator [11]. Also, the SOS response has important functions in persister cell formation: first, it is a stress pathway signalling which modulates the creation of persister cells, and second, it controls DNA repair that may be required for persister resuscitation [10]. Due to the high amount of TA systems in most bacteria and the fact that any toxin appears to increment persisters, even without the presence of (p)ppGpp [21], persister cell formation may have redundant methods. It seems that persister cells act in a similar way to spores, playing a role in the dissemination and survival of the organism [7].

Persister cells are not only produced as a result of deterministic components, but also as a stochastic phenomenon. Bacteria use stochastic processes when they are in an adverse growth situation with more than one solution. Stochasticity has also been established as a property of different family gene expression which participates in host-parasite interactions. There are various amounts of environmental signals in different cells of clonal populations which could modulate the level of persistence in growing cultures [8].

Quorum Sensing Signals

Quorum-sensing (QS) is a way of communication between bacteria, based on the generation as well as secretion of small molecules, which are known as autoinducers. Bacteria have the capacity to detect and respond to the environmental autoinducers when they reach a threshold concentration [22]. It is important to take into account the criteria that must be satisfied for a compound to be considered a QS signal: (i) the compound must be generated during a specific moment, (ii) the signal must amass outside the cell and be detected by specific receptor, (iii) the molecule must produce a concreted reaction, finally (iv) the signal has to produce a further response than it would be needed for the metabolization or detoxification of the compound [23]. Below we describe the main QS systems, then summarize how they are related to persister formation.

(a) Homoserine-Lactones (HSL)

The *Vibrio fischeri* bioluminescence is a widely studied example in which the regulatory control system of this phenotype is established by the genes *luxI* and *luxR*. This system controls the quantity of the dissolvable signal *N*-(3-oxohexanoyl)-*L*-homoserine lactone (OHHL), known as AI-1 [24]. LuxI enzyme produces OHHL signal which gets out from the cell, and the LuxR receptor is the response regulator that recognizes the OHHL signal that is internalized; the LuxR receptor bound to the OHHL signal then serves to induce the QS-related genes, which include enhanced production of OHHL signal. Homologues of LuxR receptor have been found in other bacteria which indicates the QS system exists in a wide spectrum of bacteria and the OHHL signal is only one AHL member [25]. Basically, gene expression and cellular differentiation are controlled based on this intercellular

communication via the concentration of small molecules; this communication confers benefits in terms of hosts colonization, defense against competitors, response to environmental conditions, cellular differentiation, and evolution [26].

(b) Competence-Stimulating Peptide (CSP)

Streptococcus spp. CSP acts as a classical QS signal, which works as a signal in response to stressful environments [27]. Competence-stimulating peptide is generated via ribosomes as a precursor of a peptide which depends on the transporter ComAB (an ATP-binding one) to be secreted. The rest of the genes needed to synthesize CSP are in a cluster: (i) *comC* produces CSP precursor; (ii) *comD* codes for a membrane-bound histidine kinase sensor; (iii) and *comE* codes for a response regulator [28]. The CSP pheromone is recognized by a two-component regulatory system consisting of a membrane-associated, histidine kinase sensor protein, which senses a specific environmental condition, and a cytoplasmic response regulator, which enables the cell to respond via regulation of gene expression when this condition varies. ComD is the sensor which binds extracellular CSP and initiates a cascade of phosphorylations which activates the sigma factor ComX. Leung et al. observed that in streptococci, a sub-population of the culture has a stress-response via QS system. They hypothesized that under stress and using exogenous CSP, bacteria of the culture can experience a different transcriptional profile [1]. Moreover, this author published a work in 2015 about the affect of the LexA regulator during the generation of persister cells in *Streptococcus mutants* due to the QS peptide pheromone [29]. Finally, we must highlight an interesting review where two distinct CSP-induced phenotypes are discussed, the "suicide" and dormancy of bacteria, and the subjacent mechanisms by which *S. mutants* uses the same Quorum Sensing signal to manage both opposite phenotypes [30].

The *S mutans* QS system is shown in the Fig. 1.

(c) Indole

Indole has been identified as a QS signal by Lee et al. [23, 31]. In addition, indole is an interkingdom signal which is produced by bacteria in the gastrointestinal tract and protects them from pathogens [32, 33]. Indole has been related to spore formation, biofilm production [34], plasmid stabilization, persister cells development, virulence [35] and acid resistance [36]. Besides, indole can affect membrane stress and oxidative stress responses and contribute to an increase in Minimal Inhibitory Concentrations (MIC) by stimulating the efflux pumps [37, 38]. Lee et al. showed that SdiA is an indole signalling regulator [34], which is affected by the temperature being more active at 30 °C [31]. They discovered that SdiA is required by indole for the biofilm formation inhibition, among other functions in *E. coli*, as well as indole has more significant functions between 25 and 30 °C than at 37 °C [31]. Moreover, in the pathogen *Vibrio anguillarum*, RpoS and indole signalling control the virulence factors [39].

Remarkably, there are studies which suggest a relationship between indole-mediated and AHL-mediated signalling. It was determined that in the presence of indole, there is a reduction in the biofilm formation. Moreover, indole produced by

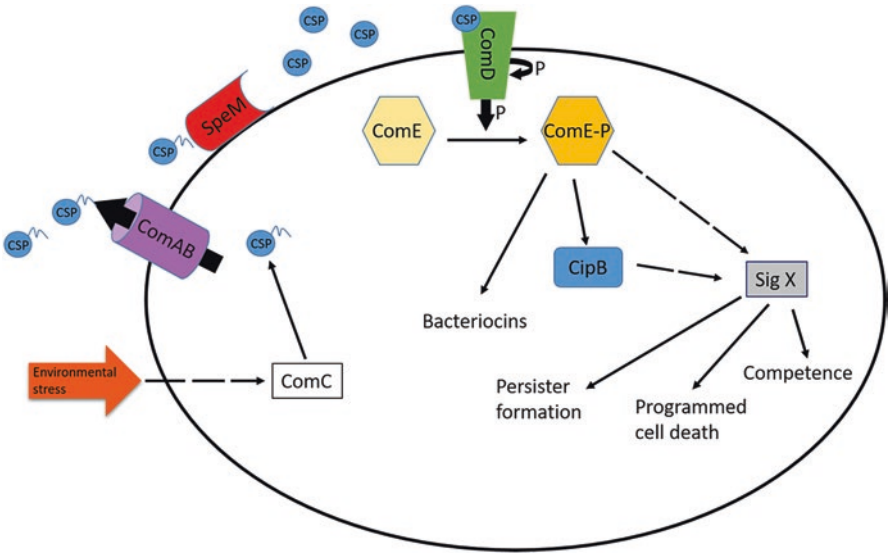

Fig. 1 The *S. mutans* CSP-ComDE QS system. In the presence of environmental stress, *comC* is induced and produces the pre-CSP protein, which is recognized by ComAB (a specific ABC transporter). Once in the extracellular environment, pre-CSP is recognized by SepM extracellular protease, which eliminates the 3′-tail of pre-CSP converting it into functional CSP. When the levels of CSP reach a sill, it links with the ComD receptor, which is auto-phosphorylated. ComD~P produces phosphorylates ComE. ComE~P activate the expression of bacteriocin genes which results in production of CipB. ComE~P and CipB also have the capacity to indirectly activate *sigX*, but the mechanism is unknown. SigX activates genes related to competence, persistence and cell death

Escherichia coli can have a negative effect in QS-related virulence factors of *P. aeruginosa* [35, 40]. In addition, it was discovered that high concentrations of indole (1 mM) (a physiologically-relevant concentration that can be found in the mammalian intestine, which usually has an indole concentration range between ≈300 μM and 1 mM [41]) inhibit LuxR detection of AHL in *E. coli, S. typhimurium,* and other strains [42]. It was also noticed that indole can affect the folding of QS regulators, producing their rapid degradation [43].

Interestingly, a recent study analysed the expression of a Quorum Quenching enzyme (MomL), which is a novel AHL-degrading enzyme identified in marine bacteria. They found that indole produced a stress-response and regulated the expression of MomL, producing a competitive advantage over other bacteria [44].

(d) AI-2

The cyclic borate diester known as the Autoinducer-2 (AI-2) [45] derived from (*S*)-4,5-dihydroxy-2,3-pentanedione that may be converted into a family of compounds [46] used for species-nonspecific signaling [47]. In *E. coli,* AI-2 is created by LuxS enzyme [48], exported from the cell by TqsA protein [49] recognized and

imported by LsrABCD protein. It has been demonstrated that AI-2 controls the population-dependent behavior for example biofilm formation [50].

Quorum Sensing and Persistence

Kwan et al. detected that cyclic adenosine monophosphate (c-AMP) has a role in persistence as related to the QS compound indole. They observed that the phosphodiesterase DosP increases persistence by decreasing the tryptophanase activity (tryptophanase converts tryptophan to indole) by reducing cAMP, which resulted in a reduction in the indole levels. This mechanism is controled by the elimination of intracellular cAMP by DosP protein. The activity of DosP protein depends on oxygen concentration, which could explain why persister cells are found between cells in the outer level of biofilms, given the higher exposition to air [51]. Corroborating that indole reduces persistence, in 2015, Hu et al. reported that the toxin YafQ has the capacity to reduce the indole production by reducing the levels of RpoS (which has been related with indole production) and tryptophanase. The authors detected that YafQ toxin increases the number of persister cells by decreasing tryptophanase levels which results in reduced indole levels [52].

Moreover, there are many indole derivatives which reduce persister cells. 5-iodoindole, a halogenated indole, has shown an extraordinary capacity to reduce persister survival and is an effective inhibitor of the generation of biofilm in *S. aureus* and *E. coli* [53]. Although antibiotics such as ampicillin or rifampicin produce the development of persister cells in these two bacteria, 5-iodoindole has the capacity to inhibit not only persister cell formation, but also biofilm production, and even has antimicrobial activity against both microorganisms. However, it did not have activity against *P. aeruginosa*. Researchers concluded that halogenated indoles, specially 5-iodoindole, can kill persisters in stationary phase of *S.aureus* and *E.coli*, prevent its formation and inhibit biofilm production [53]. Therefore, indole reduces persister cell formation as shown by two independent mechanisms, contrary to what was previously published [37].

In addition to the QS compound indole reducing persistence, several other studies show inhibiting QS prevents the development of persister cells. In 2012, Pan et al., analysed the use of (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one, a derivative from natural furanones of the algae *Delisea puchra* that is known to prevent both AI-1-based and AI-2 based QS [54], to sensitize *P. aeruginosa* cells to antibiotics [55, 56] as well as to sensitize *E. coli* [57]. These results make sense given that the QS signal 3-oxo-C12-HSL increases *P. aeruginosa* persister formation. Mökerand and collaborators, [58] demonstrated that *Pseudomonas aeruginosa* increase the number of its persister cells in the presence of 3-oxo-C12-HSL or Pyocyanin (PYO) molecules during exponential growth. They suggested that additional secreted molecules could act, alone or together, with PYO in the persister cell number regulation during the log-phase. They also observed that high concentrations of both 3-oxo-C12-HSL and PYO molecules had to be added on their own, to

detect persister-promoting activities. Nevertheless, in synergy, lower concentrations of both compounds suffice to increase the number of persister cells. These researchers recorded that the increase in persistence was a response determinated by 3-oxo-C12-HSL and PYO molecules which is not related to bactericidal antibiotics, growth arrest, oxidative stress or environmental stress. Interestingly, it was noticed that these molecules improve persister creation in *P. aeruginosa,* and inhibit other bacteria, [58].

In addition, for the PQS QS system of *P. aeruginosa,* Allegretta et al. [59] found that inhibitors of the PQS transcriptional regulator MvfR reduce persistence. Also, Que et al. [60] found that persister formation is a consequence of cell density as mediated by QS. They postulated that 2-AA (2-amino-acetophenone), a PQS precursor, is not only involved in biofilm-related tolerance, but also in persister cell formation.

Similar to AHLs molecules in Gram negative systems [58], for the Gram positive *S. mutans,* the CSP pheromone molecules increases persistence, perhaps by activating bacteriocin Mutacin V which causes a drop in ATP [1]; reduction in ATP has been shown in multiple strains to increase persistence [15, 61–63]. Perryan and collaborators, noticed that in the presence CSP, pheromone and Mutacin V are activated to cause *S. mutans* autolysis. Furthermore, they identified that the main factor in CSP-induced degradation was Mutacin V [27]. In contrast, it could be that Mutacin V regulates control of the transcription of persister genes [1].

In contrast to the results with CSP molecules, the Agr QS system of *Staphylococcus aureus* increases persistence via its regulation of phenol-soluble modulins [64].

Conclusion

Persister cells are an urgent health problem which has been related to chronic infections, for example tuberculosis and cystic fibrosis, although persisters are being linked to other infections, too. Currently, many researchers are trying to find new therapies against this type of cells, but the therapies are still far from practical use in the clinic. In this work, we considered persister cells from a different point of view; i.e., we considered how QS signals influence persister cell formation. It is clear that indole, its derivatives, and compounds that mask AHL-based signalling have the potential to reduce persistence. However, more research about the mechanism of persistence (e.g., how persisters form and wake) is needed to obtain new efficient treatments against them.

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Microbial Social Interactions in Biofilm

Jin Zhou and Zhong-hua Cai

Abstract Biofilm is a kind of biological structure with high complexity and high-self-organizing characteristic. It consists of many kinds of microorganisms and biological matrixes (polysaccharide, protein, fatty acid, etc.), with special structure and ecological function. It plays an important role in microbial colonization, niche construction and environmental adaptation. Many microbial behaviors, such as adventitious infection, toxin production, drug resistance, biofouling, mesh blocking, etc., are related to biofilm. The generation of these events is inundated with diverse microbial behaviors, including signal communication, cooperation/competition, labor division, "bacterial intelligence" under stress condition, etc. These features show more and more sociological characteristics of the biofilm, which provides a new perspective to know biofilm better. Therefore, based on the structure of biofilm, we took the communication signals, collaboration, and intelligent resistance (responsed to the ecological stress, co-evolution) inside biofilm as the core to elaborate its social characteristics, in order to better understand the social interactions in bioflim.

Keywords Biofilm · Social interactions · Microbial signals

Introduction

In the natural environment, the microorganisms will colonize on one cattier (particle, colloid or host) via free collision and random selection, and take this as an ecological niche to excrete polysaccharides, proteins, lipids and nucleic acids, and then form the extracellular polymeric substances (EPS). These EPSs will pack various microorganisms and matrixes inside in order, and then form a membranoid substance with certain structure [1], which is usually called biofilm. It is a kind of

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film compound with some coordination ability, function and high orderliness. Unlike the free microorganisms, the various microorganisms in the film have diverse communication, cooperation and competition, and are inundated with multiple nonlinear interactions, which make the biofilm present a series of biological characteristics [2]. This structure with diverse compositions and malleable function can provide a certain niche for the microorganisms and help them to take their ecological actions and adapt to the changeable environment better [3]. Generally, the biofilm formation has three advantages: (1) Plumping up to resist the adverse environment (antibiotic or stress from the host); (2) Obtaining the nutritional ingredient that is difficult to acquire individually; (3) Getting new genetic information by means of gene flow [4]. The researches indicate that more than 90% of environmental microorganisms live in the biofilm. In the past 20 years, the researchers have conducted a lot of studies on the formation process, regulation mechanism, influence factor and comprehensive application of the biofilm, and obtained a lot of progress.

With the further research, the focus has been on the biofilm characteristics, such as the communication mechanism, features of the interaction between the microorganisms, network relationship, intelligent behavior, etc. Under this background, the new crossing theory-social-microbiology is proposed [5]. This theory believes that a social behavior is implemented by a certain individual (donor) and influence other individuals (receptor). According to the types, it can be classified into four classes: self-interest, altruistic, win-win and double-lose [6]. Some researchers think the biofilm is similar to a human community, where the extracellular matrixes (proteins, lipids) are material basis, EPSs constitute the house frame, bacteria are residents, and the mix of multiple species forms the microbial community [7–9]. Bacteria are distributed in the biofilm in colony form. EPSs are in charge of the processes like bacterial density, water channel, ion distribution and signal communication in the biofilm, and further impact the material transport and energy exchange in the biofilm. This kind of "resident-house-community" structure regulates the inner social relations and maintains the stability and ecological function of biofilm in a certain way.

This article focuses on the micro-ecological process of the biofilm, refers to the latest reports and references, and explains its sociological characteristics based on the 3D structure, communication language, interactive relationship (cooperation, competition and labor division) and intelligent anti-stress behavior (environmental stress, co-evolution) of the biofilm, to know the micro-scale characteristics of sociomicrobiology from a macroscopic perspective.

Biofilm's Structure

The microorganisms mainly exist in the environment in the form of biofilm. Almost all the bacteria (gram positive or negative microbes, pathogenic bacterium or nonpathogenic bacterium) can form the biofilm. *Escherichia coli*, *Pseudomonas*

aeruginosa, *Vibrio*, *Streptococcus*, *Pseudomonas* and *Mycobacterium* are common microorganisms that form the biofilm. The biofilm formation process is dynamic and can be affected by multiple factors, such as external condition, strain specialty, genetic background and signal transmission [10–13]. It can be divided into four phases generally: Free collision (reversible contact and irreversible contact), colony formation, film maturity and aging and detachment [14]. The detailed dynamic process includes: (1) Adhering to the carrier surface by utilizing the extracellular organelles and outer membrane proteins, such as flagellum, pilus and hypha [15, 16], and enhancing the adhesion between the cell and the carrier by the EPSs excreted from the cell [17]; (2) The cells adhering to the carrier surface divided into small colonies, and during this process, the colonies growing obviously and EPSs increasing significantly, and forming a layer of hydrogel covering over the cell surface [18]; (3) With the growth of the adhered colonies and regularization of the EPS sequence, the mature film with 3D structure forming on the carrier surface, and now, the colonies enable to resist certain mechanical shearing force and prevent falling off from the carrier surface; (4) Aging phase; some outer cells falling off or flowing away from the biofilm, this process benefits the reproduction of the biofilm and the update of the colonies, and the detached cells can also re-select carrier in the new environment to build new biofilm.

On the structure, the biofilm is a mini-environment that constituted by microbial cells and extracellular matrixes. A microbial cell is a collection of multiple species, and mainly consists of bacteria (proteus, bacteroides, and firmicutes, etc.) and includes a small amount of archaea (methane archaea, thermophilic bacteria, and halophilic bacteria) and tiny eukaryote (algae, worms, and copepods). These species constitute the basic unit of the biofilm-residents, colonize in the mini-environment in a way, and form a big well-organized, multi-nationality family. The other major component-extracellular matrixes are mainly EPS with certain mechanical stability, which can support and protect the microbial cells, just like a pair of scaffolding. Meanwhile, the extracellular matrixes are also the places where various "residents" make food and exchange material, honored as "food processing station", and constitute the material basis for film-like structures. Different residents take up a certain niche in the form of the colony, and process and exchange materials based on their own features, form the multi-level, structural and diverse coexistence environment, and constitute the basic 3D frame of the biofilm. Though this 3D structure is a mini ecological niche, it has an important ecological function. It can against the outside attack or other adverse factors, and assist the original adhesion, aggregation, water preservation, nutrient absorption, energy storage and enzymatic reaction in the bacteria. Figuratively speaking, the biofilm is a flourishing microorganism community. In the microbial biosphere, it provides an "infrastructure" for the rest and reproduction of the multiple species [19].

On the chemical composition, EPSs are the main compounds in biofilm. The chemical compositions of the EPSs are mainly carbohydrates, proteins, lipids, nucleic acids, peptides and low molecular compounds, which are from the extracellular secretions, metabolites, special substances or fragments in the environment, etc. [20]. Some extracellular genomic DNA (eDNA), and multivalent cations [21],

broadly defined, belong to EPS. Recently, hydrophobic lipids and amphiphilic surfactant molecules are also classified into the components of EPS. They can change the surface tension on the vapor-water interface and further control the gas exchange rate between the water phase and atmosphere [22], therefore, it can assist the bacteria to adhere to some hydrophobic surfaces such as polytetrafluoroethylene and polystyrene, and assist them to take ecological actions. For example, in the extracellular substances excreted by the *Serratia marcescens*, the surfactin, viscosin and emulsan can disperse the hydrophobic substances on the surface, and then benefit the ingestion by the bacteria. Rhamnolipid excreted by the *Pseudomonas aeruginosa* serves as surfactant, and participates in the early aggregation and migration, internal transport and late dissipative process of the biofilm [23] Besides, EPSs include some diffusible soluble molecules, such as c-di-GMP, AHLs and polysaccharide & DNA connector. These molecules can enter and exit the biofilm freely, serve as free messengers or communication tools, and play an important role in the biofilm development, maintenance, anti-stress and information communication. They are important factors to promote the biofilm's complexity, functional diversity and circulation [24–27].

It is noteworthy that the biofilm has a specific chemical composition and 3D structure, therefore, it has certain stability and strong plasticity. Its stability benefits from its hydration basis and composition of diverse species. The moisture content takes up 97% of the biofilm weight. It can solidify in the film and also can flow as a solvent. This specialty provides an extremely hydrous environment for the existence of the bacteria and the presentation of the polymers in the biofilm, and makes the dry rate of the biofilm much lower than the surrounding environment to resist the outside volatility. Most of the EPSs are hygroscopic, they don't have to react with the water, and they can retain the water by means of entropy driven and maintain the stability of the biofilm structure indirectly. Its plasticity benefits from the diverse species in the film and the variable capability of EPS. Some EPSs like lipopolysaccharide have a rapid response capability to environment, and their components are significantly different as the nutrition, temperature, flow field and microorganism species in the environment change [28]. In addition, some high molecule proteins and low molecule signifiers can regulate the ratio and quantity according to the different adherent or surrounding conditions, to adapt to the living environment better [17, 29].

Quorum Sensing and Biofilm

The biofilm formation is regulated by many chemical signals, of which, the quorum sensing (QS) signal is the most common one. It is a kind of mechanism that senses and regulated the microflora density by chemical signals, and plays an important role in regulating the cell density, toxin production, bioluminescence, microbiological activity and secretion of the secondary metabolite [30]. In the gram negative bacteria (such as *Pseudomonas aeruginosa* PAO1), there are three known sets of homoserine-lactone depended QS systems: *rhlI/R*, *lasl/R* and *pqs*. The signal

molecules are 3-oxo-C12 -HSL, C_4-HSL and PQS, respectively. The three sets of system perform different functions: *rhlI/R* mediating the random collision, staying and leaving of the bacteria; *lasI/R* regulating the structural differences of the biofilm; *PQS* signal monitoring the production of the toxic factors. However, each function isn't isolated completely. In some situations, they will co-regulate the growth, maturity and disintegration of the biofilm. Compared with gram negative bacteria, the QS system of the gram positive bacteria (such as *Staphylococcus epidermidis*) is a kind of bi-component sensing oligopeptide protein. The bacteria use the bi-component protein to sense the stimulation from the outside environment, and regulate the gene expression through the phosphorylation-dephosphorylation mechanism. It includes two series-wound sets of QS systems, SQS1 and SQS2. The former includes self-induced factor, RNA-*RAP*, and its target molecule, *TRAP*; the later includes the generation of the agr system, can induce the molecular peptide – receptor protein, *AgrC* and the toxin production, and can regulate the production of RNA. When *RAP* is activated or *TRAP* is phosphorylated, the formation of the biofilm is started, the toxin is produced and pathogenicity is generated.

Though there is a difference between the gram negative bacteria and gram positive bacteria in signal regulation mechanism, diverse behaviors appear in both biofilms, and certain socialization characteristics loom up through these behaviors, such as leaving and staying behavior, competition specialty and environment adoption [31–40]. In the early stage of the biofilm formation, the random collision and surface sense is the first step of absorption behavior, and also the original step of biofilm formation. It matters with the bacterium states from actively free state to adhesion to the film. At first, it was thought that the process from free state to adhesion was random, and the bacteria would grow and increase naturally, and finally stack together, after adhesion on the surface. However, many experiments indicate the phenotypes of the bacteria in the biofilm have changed [41]. Whiteky et al. [42] detected the gene expression in the biofilm of *Pseudomonas aeruginosa* using the microarray method. It has been found that though most of the gene expression level isn't changed basically, but about 1% of gene expression rises up or decreases obviously. Meanwhile, the ratio of the dry EPS weight in the biofilm rises up significantly. It can be seen that the capability to excrete polysaccharide and extracellular protein of the bacteria in the biofilm is better than that of the free bacteria. The further researches find that up to 12% gene of the 1% gene above is related to QS, which indicates the sensing behavior experienced the regulation of QS system.

In the interaction, the regulation process of QS is mostly related to EPS secretion. When the density of *Pseudomonas aeruginosa* (PAO1) is intense, EPS secretion will be increased significantly, which is related to the competitive advantages of PAO1. The competition between the different strains in the biofilm is morefierce than that under free state, and EPS secretion will help to increase the competitive advantages [37]. In contrast, no or less EPS secretion under low density or free state is to save energy, which sufficiently reflects the living strategy of PAO1 under different densities [38]. However, EPS secretion is regulated by the signal molecules, C_4-HSL, C_{12}-HSL and C_8-O-HSL, which indicates the close correlation between biofilm and QS system [38–40]. Wang et al. [43] transferred the *aiiA* gene derived

from *Bacillus thuringiensis*, which can degrade QS signal, and found the biofilm quality and structure formed by PAO1 are inhibited obviously. Tan et al. [44] reported that in the research of transformation between the dynamic flocculent and granular biofilms in the sewage disposal tank, it was found that the QS strain and signal density are strongly related to the transformation of the biofilm structure. Increase of QS signal substance will accelerate the grouping of the biofilm, which is consistent with the research on PAO1. However, the strange thing is that the researchers also found some phenomenons which are inconsistent with the PAO1 living condition, which means when the cell density in the biofilm reaches a certain limit, EPS secretion will be inhibited or reduced under the regulation of QS. For example, in the enteropathogenic bacterium – *V. cholerae*, when its density in the film reaches a certain threshold, EPS secretion will be reduced, and then due to lack of EPS protection, part of the biofilm will separate from the matrix and seek for new habitat outside. After the adhesion to a new carrier, the flagella on the strain will degenerate, and EPS secretion will be started to form new biofilm. When the colony density reaches the upper limit, EPS secretion will be reduced again, some colonies will separate from the film and become free again, and then go into the next circulation [12, 45]. Though it seems like the contradiction between EPS and bacterium density in *V. cholerae* and *Pseudomonas aeruginosa* is inconsistent, the stronger living competition is finally obtained through the signal regulation.

In addition, QS signal plays an important role in regulating the transfer and evolution of the biofilm, for example, AHLs molecules widely regulate the biofilm spread of *Vibrio* sp., *Serratia* sp. and *Rhodobacter* sp. [46–49] and the Type VI secretion system of *Burkholderia thailandensis* [50]. The new discovered decanoic acid signal molecules (cis-11-methyl-2-dodecenoic acid and cis-2-decenoic acid) are considered as a kind of high-effective regulator for biofilm spread, which can effectively control the biofilm spread of *Xanthomonas campestris* [51].

Similar with QS molecule, c-di-GMP - another signal regulator, is a kind of circular dinucleotide, and often called as the second messenger [52], it has a dual-effect on regulating the formation and spread of the biofilm, it can activate the *Lux* gene family in the formation period and make the AHL lytic enzyme in the detachment period highly express [53, 54]. In the research on *Acetobacter* sp., the mucopolysaccharide and mucoprotein secretion in this strain is significantly related to the c-di-GMP signal molecule. And the c-di-GMP signal molecule expressed by this strain is regulated by two functional proteins – *GGEDF* and *EAL*. The former can promote the synthesis of c-di-GMP, while the later can degrade c-di-GMP [55]. The following researches on the gene level further proved that the above c-di-GMP is of certain universality on regulating the polysaccharide secretion, and related to several uncertain signal regulation systems [56].

It can be seen that the signal regulation mechanism participates in different biofilm formation phases (adhesion, maturity, aggregation and dispersion), and also regulates its socialized behaviors. Therefore, the signal system is very essential to the biofilm formation. However, it's necessary to note that though the dynamic process of the biofilm and the colony behavior are regulated by the signal, different

biofilms have different material bases, strain colony and environment, so the features showed out are different.

Besides the chemical compounds (such as QS and c-di-GMP) in the representative of the chemical signals, new electric/ionic signals are also discovered [57–59]. Li and his co-workers proved that in the self-regulation of the biofilm, the potassium channel can form a current signal by changing the film potential, to regulate the self-metabolism of the biofilm. This pattern greatly improves the remote metabolism interdependence of the biofilm [60]. After that, Humphries et al. [61] further researched on how the bacteria inside and outside the bioflim communicate through the electric signal. The results indicated that the bacteria colonies in the biofilm use the ion channel to communicate with the bacteria outside in the form of periodic electric signal, and then attract the specific bacteria to change the current biofilm structure. This is an "intelligent" evolution of biofilm, which means immigrating foreign species or gene from the environment. Though this will impact the current original biofilm and have certain risk, it will benefit the evolution and diversity of the biofilm in a long term. It is a kind of "risk financing". Therefore, the biofilm not only regulates its self-behaviors by signals, but also remotely influences the environment outside by electric signals.

Interactions in Biofilm

Cooperation and competition are two main types of interaction in biofilms, and they can take place in the same species and between different species. Generally, it is considered that the cooperation between the microorganisms is a favorable behavior to the recipient bacteria in the interaction, while competition is an adverse behavior to the recipient bacteria. Madsen et al. [62] proposed a definition mode: if the quantity of the biofilm is higher than the sum of biofilm of each single bacterium, it is a synergistic effect; if the quantity of the biofilm is lower than the biofilm quantity formed by the bacteria with the minimum biofilm formation capability, it is a competitive effect. In the biofilm formation process, cooperation and competition are a pair of association factors. They have certain differences in different space-time conditions, but under a dynamic balance. Behind the phenomenon of which, certain social relation is followed. The final purpose is to maintain the formation of the biofilm and the circulation of the living history.

The phenomenon of cooperation was first discovered in single bacterium biofilm. Taking *Pseudomonas putida* SB5 and *Chryseobacterium* sp. SB9 for example, and comparing the single cultivation and mixed cultivation, it is found that the biofilm quantity is increased significantly after mixed cultivation, which indicates there is a synergistic effect between them. The reason is that the metabolites from each serve as "public goods" that can be used by the other [26, 63]. After that, this phenomenon is gradually discovered in biofilms of many species. Each two of the 13 strains separated from the environment are cultivated in mixture. It is found that the biofilms of multiple species have a special property that is different from the single

strain, and there is a synergistic effect between different strains [64]. Ren et al. [65] detected the soil-bacterium biofilm formation process by using four-strain mixed cultivation. The results indicate the biofilm quantity formed in the four-strain mixed culture is higher than that in the single strain culture, of which, the synergistic effect is most significant in the mixed culture of *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus*. This indicates the synergistic effect widely exists in the biofilm formation process in many species. When summarizing the causes, McGlynn et al. [66] pointed out the material exchange (electron acceptor or donor, etc.) and metabolism codependence are the main causes for cooperation. The nitrifying process is a typical example. In this process, ammonia oxidizing bacteria converse the ammonium salt into nitrite, and then nitrite is oxidized by nitrite oxidizing bacteria. *Nitrospira moscoviensis* can synthesize ammonia to supply the ammonia oxidizing bacteria, and the metabolites of each other are exchanged to realize the cooperation and commensalism [67].

Compared to cooperation, competition is also a common ecological behavior in the residents inside the biofilm. Because it is limited by the supply of space, nutrition, oxygen and light. A typical example is the microflora evolvement process in sewage treatment. In the anaerobic biofilm composed of sulfate-reducing bacteria (SRB) and methanogenic archaea (MA), SRB and MA compete for matrixes, such as organic matters and acetates. In the early period, SRB is dominant; when the biofilm is gradually mature, the release rate of CH_4 is increased significantly, and then MA becomes dominant [68]. Recently, Nadell et al. [69] verified that the space competition exists during the biofilm formation, taking *Pseudomonas aeruginosa* for example. The space competition is related to hydration structure and matrix permutation of the bacteria. And it proved the capability differences of occupying space under different environments. In addition, besides competing for nutrition and space, they can also compete by excreting antibiotics, toxins, surfactants to kill or inhibit the accompanying bacteria. The VI secretion system (T6SS) of *P. aeruginosa* can transfer the toxic protein into the neighboring cells without T6SS, and then inhibit the growth of the surrounding cells [70, 71]. Some biocontrol bacteria like *Bacillus* can excrete the antibiotic substance – 2,4-diacetylphloroglucinol to kill the phyllosphere bacteria [72]. When concluding the ecological meanings of competition, Sun et al. [73] think competition is an internal contradiction inside the biofilm, from the sociological perspective. It is based on the diversity of the biofilm residents, which means there are many strains of different natures in small mixed-living ecological niche, such as pioneer, speculator, selfishness and retainer. Therefore, the biofilm is inundated with unbalanced cooperation and competition behaviors.

It should be pointed out that cooperation and competition are a pair of characters which should co-exist in the biofilm, but the roles are different according to the environment and species. When the bacteria in the biofilm are from the same environment (non-heterogeneous background), the strains inside the biofilm are likely to cooperate. However, if they come from different environments (heterogeneous background), the competition degree will be enhanced [74]. On the other hand, the species type and genetic relationship will also affect this pair of gaming rules. If the members inside the biofilm has the same genotype in the locus which decides the

specialty of a certain colony, then it will benefit cooperation; otherwise, competition will likely take place [75]. In addition, the spatial arrangement of the biofilm also participates in it. Under the isolation state, if the neighbouring cells come from the same colony, the local cooperation will like take place. And if they are mixed heavily, then the cooperation will be weakened in most of the time [70]. For the reason why the double roles take place alternately in the biofilm, the metabolism co-dependence theory proposed by Zelezniak et al. is widely accepted [76], in which it revealed that the metabolism correction is the key to balance competition and cooperation, in the protein and molecular level. However, the reports for the explains from the sociological perspective should be enhanced. The author predicted that this dependent mode of mutual achievement and compromise may have a game rule like bacterial intelligence besides meeting the environment adoption, and the molecular mechanism behind it is worthy of further investigation.

Ecological Stresses in Biofilm

The formation of biofilm faces many natural selections and ecological stresses. To establish its own ecological niche, the colony will come over many challenges to finish the ecological process. The most prominent environmental stress is reflected in: (1) Contradiction of individual increase and lack of nutrition; (2) Attack from the outside conditions (mechanical damage, antibiotic, oxidative stress), and (3) Two-way pressure inside and outside the biofilm.

The first kind of stress (individual increase and lack of nutrition) is the commonest stressed performance of biofilm. On one hand, the accumulation of organism quantity benefits the biofilm growth, on the other hand, it leads to the relative lack of nutriment; Meanwhile, the reduction of conduction electron and the accumulation of metabolic toxin are easily caused in this situation. How to coordinate is the primary issue that should be resolved by the residents in the biofilm. The current researches indicate that the biofilm develops two kinds of capability to handle with this contradiction: first, outward spread; second, intermittent growth. Outward spread is a common regulation behavior. Most of the gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus mutans* and *Pseudomonas aeruginosa*) and few of the gram positive bacteria (*Enterococcus* and *Staphylococcus*) follow this mode. Taking *Pseudomonas aeruginosa* for example, when the carbon source, phosphates, iron and oxygen is insufficient or the metabolic inhibitor – carbonyl cyanide chloro phenylhydrazone (CCCP) is increased during the growth [77–81], the colony will activate the flagella (*pf4*) to regulate the protein and induce the biofilm to spread outward, to relieve the lack of space and energy. For the bacteria without motion capability, for example, *Xanthomonas* sp. may depend on the interaction with the accompanying bacterium – *Paenibacillus vortex,* and use it to complete the chemotactic migration together. This kind of behavior like “free ride” easily takes place on the carrier surface (such as phyllosphere, rhizosphere environments) [82]. Compared with spread, intermittent growth is a more intelligent

behavior. Liu et al. [60] used the microfluidic chip method to observe the biofilm formation process of *Bacillus subtilis*: the growth of the cells in the biofilm depends on the C sources transported by the outside cells, and the growth of the outside cells can benefit from the N sources provided by the inside cells; To achieve win-win situation, the inside and outside cells adopt the oscillation to grow intermittently, to ensure the alternate energy supply and smooth biofilm maturity. This process is regulated by both *Roc* and *GDH* genes, the former is the N regulate factor and the later is the C regulate member.

Among the outside condition changes, resistance to the mechanical damages is the earliest reported type. In extreme deep-sea environment (hypersaline, high pressure), the film structures and microbial mats represented by Firmicutes, *Proteusbacillus vulgaris* and few archaea are commonly seen. To maintain the osmotic balance and resist the outside high-pressure environment, these bacteria activate the secretion of the backbone substance - EPS, to tolerate the mechanical stress. Bolhuis et al. [83] compared three environments of microbial mats: coastal, hot spring and hypersaline conditions, it is found that the cyanobacteria is the dominant component in this three mats, and the excreted mucopolysaccharide is obviously higher than that in the surrounding environment, to form a frame to protect the film structure. Wong et al. [84] further included the mechanism of maintaining the microbial mat structure under hypersaline condition, and the Na^+-K^+ efflux system induced by ion gene of proteusbacillus vulgaris is the main cause.

Similar with resistance to the mechanical damage, resistance to antibiotic is another anti-stress behavior of biofilm, especially common in pathogenic bacteria. In this process, the film-forming bacteria will form an intense protection layer under the regulation of QS signal, lead to drug permeability obstruction, inhibition of active transport mechanism, change of drug acting target and generation of drug-inactivating enzymes, and then generate the drug tolerance or resistance. This is a living strategy of pathogenic bacteria, to protect the individuals in the colony by the film. Taking *Streptococcus* for example, there are surface bacteria and deep bacteria around its film. The surface bacteria can easily obtain the nutrition and oxygen, and the metabolites are also easy to excrete, therefore, the physiological metabolism of the surface bacteria is more active and the bacteria division is faster; the deep bacteria are living in deep biofilm. Due to lack of nutrition and oxygen, the metabolism is slow, and they are usually under dormant state, and the bacteria division is also slow [85]. When encountering antibiotics, the deep bacteria will accept the signal from the surface bacteria and automatically become tight. It's hard for the drug to permeate, and then it will gain some time to maintain the biofilm and generate the drug tolerance by the deep bacteria. So far, there are mainly three kinds of theories about the generation mechanism of the drug tolerance: permeation limit effect, nutrition limit theory and gene phenotype mechanism [86]. Recently, a new mechanism for drug tolerance is proposed, namely genetic information drift and conversion. Hughes and Webber [87] proved that the enteric pathogenic bacteria resist the attack from the harmful material in the environment (such as phages, toxins and antibiotics) through frequent DNA exchanges. Besides the drift the drug-tolerant gene, quorum sensing (QS) regulation also participates in the control of plasmids

and integrases, and it helps to improve the transmission efficiency of the plasmid and the expression of the integrase. Plasmids and integrases are closely related to the transmission of drug-tolerant genes, therefore, QS regulation may play an important regulation role in the transmission and drift of drug-tolerant genes [88–90]. This high-efficient gene-level drift is not only an inducible adaptive capacity of the bacteria in a short time, but also affects the development and evolution of other species.

Resistance to oxidative stress is the third mode of biofilm against the environment outside, and this mainly targets at reactive oxygen species (ROS). In the biofilm formation process, on one hand, oxygen is needed for respiration, on the other hand, plenty of reactive oxygen is generated. Reactive oxygen is toxic to the cells, inhibits the development and maturity of the biofilm, and should be removed. Some researches reported that the biofilm has three ways to remove ROS: ROS scavenger, generation of exopolysaccharides (EPS) and heterogenization of the biofilm. In the first mode, the active component is mainly *OxyR*, which exists in most of the gram negative bacteria and removes the residual ROS after activating the related genes, such as *katA*. In the *Pseudomonas aeruginosa*, *OxyR* is combined with the *Pf4* promoter, to exclude the extra ROS after reserving the O_2 required in respiration [48]. When the amount of ROS is beyond the handling capability of the *OxyR*, it will react with *bdlA* molecule, to accelerate the biofilm separation to reduce the accumulation of ROS. The generation of polysaccharide EPS is the second way to remove ROS. The researches found that when *Azotobacter vinelandii* and *B. subtilis* expose to the oxygen stress condition, exopolysaccharides (ESP) will increase significantly, which is considered as an adaptive method to relieve oxygen stress [91]. Under the stress condition, alginate (a kind of EPS) of in *A. vinelandii* will be excreted substantially, which is related to remove the hydroxyl radical (•OH) [92]. Compared with EPS, the heterogenization of biofilm is an active behavior against ROS [93]. There are different bacterium individuals in the biofilm. Each individual has different sensing and removal capability to ROS. Under the exogenous stress, these members will change their previous states to switch phenotype, to ensure to maximumly remove the intracellular ROS. When *E. coli* is exposed to the Fe environment, it will cause significant oxygen stress. Then the rugose will increase, the bacterium individual will change matrix-arrangement into non-matrix arrangement under the regulation of the phenotype switch gene – *SoxRS*, to respond to the stress of oxygen radical [94]. Some oxidative stresses can increase the mutation probability of some strains. When *Staphylococcus aureus* exposes to H_2O_2 and oxygen, the mutation probability of the DNA double-strand break repair system will increase in a small part of the individuals, in order to improve the expression of the hydrogen peroxidase and then degrade the extra ROS [95]. Gambino and Cappitelli [91] pointed out in conclusion of the response mechanism of biofilm to oxidative stress that the bacteria will response somehow to this situation, some may be harmful to the individual, but favorable to the whole, this may prove the previous saying – the biofilm is a kind of intelligent structure, sacrificing the “individual family” while catering “the big family” [96].

As an open system, the biofilm formation not only relies on the non-linear interaction between the inner microorganisms, but also depends on the mutual communication between the microorganism and the outside environment. Taking the oral environment as an example, the nutriment in the oral cavity is of great significance in the bioflim formation of plaque bacteria. On one hand, the salivary gland provides multiple nutriments, such as proteins, sugars, peptides and minerals. On the other hand, the salivary gland also excretes some antibiotic substances, for example histatins, it has a strong inhibition effect on both *Streptococcus mutans* and *Candida albicans* [97]. To survive in the environment with antibiotic substance, the microorganisms develop a set of protection mechanisms against the defensive system of the host, for example, the periodontal pathogen – *Treponema denticola* has a high tolerance to β-v-defensins generated by oral epithelial cells [98]. In addition, some nutriments in the oral cavity (such as complex glycoproteins) are hard to be used under the action of a single bacterium strain, and only can be degraded under the cooperation of multiple bacterium strains. For example, the sialomucin – MUC5B can't be used under the environment of single *Streptococcus* or *Gordonia Streptococcus*. It only can be degraded by sufficient hydrolytic enzymes, under the co-existence of the two bacteria and other bacteria [99, 100]. It can be seen that the communication and cooperation between the biofilms of different species plays an important role in the processing and utilization of the nutriment.

Seen from the long-term co-existence and evolution progress, the behavior of the biofilm under the stress condition is also a coevolution, besides an adaption to the environment stress [101]. Ehrlich and Raven pointed out that: a certain specialty of one species is changed under the influence of another species, and the new specialty of the later also promotes the evolution of the former, which is the original theory of coevolution [102], and emphasizes on the synchronism and feedback of the genetic change. In recent years, coevolution is an important crossing field in ecology. Many ecological phenomenons only can be well explained under the guidance of evolution theory. For biofilm, due to the diversity and variability of the species, the coevolution in biofilm involves in more species, with higher possibility and faster rate. To better understand the coevolution in biofilm, it should be analyzed from the whole and individual perspectives. Taking the decomposition and spread of the biofilm for example, from the whole perspective [103] the decomposition and spread of the biofilm benefit the biofilm re-generation, because it gains the nutrition for the following growth. Though one or several kinds of microorganisms which has separated from the matrix temporarily will reduce the biological diversity of the biofilm itself, more repair and supplement will be obtained later, the increase of its diversity not only comes from the natural mutation, but also from the selection and evolution of the colony [92]. From the individual perspective [104] spread makes the bacteria experience a natural selection process, and makes the strains with closer genetic relation collect in hereditary character, to gradually meet or match up to the spread requirement of the mother biofilms [32]. Gyllenberg et al. [105] further explained coevolution from the perspective of a mathematical model. This model sets a hypothetic limited environment, without spread and with the natural death of species, the development of the biofilm will finally collapse. However, in present of spread, the

biofilm will live in an open space, with a possibility of unlimited circulation. This model proved that the spread behavior is an inevitable specialty for the sustainable development of biofilm, and it also avoids the risk of biofilm destruction under occasional extremely adverse environment [105]. Compared with the active behavior of decomposition and spread, the anti-stress situation is a passive factor to biofilm evolution, and it is more complex and variable. Taking the resistance to antibiotic for example, the main expression of the drug resistance of the biofilm is the inhibition to antibiotics and the bacteria generating antibiotic. To obtain this ability, the bacteria should accumulate the tolerance ability through spontaneous mutation and level transfer of resistance gene, and form a stable and heritable mutant during the continuous generation processes [106, 107]. Though some certain theoretical consensuses have been achieved on the above active and passive evolution processes, some problems are still to be resolved, such as, whether the new biofilm would pillage the nutrition from the surrounding mother biofilms. The component and nature differences between the cultured biofilm and natural bioflim; and whether the evolution rule is of universality. The answers to these questions should be implemented by means of the microbiome program, such as the Omic methods and big database [108].

Conclusion

The microorganism has the structural and functional diversity. The expression of its ecological behavior relies on the colony, with the typical characteristic of not fighting alone. And the biofilm provides a platform for the presentation of this characteristic. Tough the biofilm ecological niche is tiny, but it is an active platform. In this microbial castle, there are complex interrelationships, networked mutual feature and diverse colony behavior. The organized activities of these structures have some certain characteristics of socialization, reflects the coordination and division, cooperation-competition, self-regulation functions similar with human society, and maintains the balance and stability of the community [109]. Though the previous researches explained the biofilm formation process, regulation mechanism and circulation and evolution of the biofilm in the cellular and molecular levels, the understanding to the labor division, self organization and regulation and intelligent control of the biofilm from the social perspective is still an open topic. Because the interactions between the microorganisms in the biofilm are complex and diverse, and affected by many non-linear factors. The further researches should explore more genetic foundations behind the microbial behaviors, link the microbial behavior in microcosm and the sociological characteristics in macrocosm, and investigate the ecological meanings behind. We believe that understanding the spacetime development, action mechanism and molecule mechanism of the biofilm from the sociological perspective is a new direction in the future researches. It can not only help to explain the biological behavior from the sociological perspective, but also help to start understanding the biofilm nature from the viewpoint of behavioral philosophy.

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Part II
Functional Aspects of Quorum Sensing in Microbes

Quorum Sensing Regulated Swarming Motility and Migratory Behavior in Bacteria

Pallaval Veera Bramhachari, N. M. Yugandhar, A. M. V. N. Prathyusha, G. Mohana Sheela, Jalaja Naravula, and Nagam Venkateswarlu

Abstract Bacteria produce and sense chemical signal molecules, communicate with closet neighbors. Chemical signals are diverse and comprise cyclic and linear peptides, short and long chain γ-quinolones, N-acyl-homoserine lactones (AHL) and unsaturated fatty acids. These signaling molecules are collectively called auto-inducers. Certain signals are readily diffusible small molecules, while others are hydrophobic and can be vesicle or membrane -associated. Several chemical signals are vastly genus or species specific, while LuxS gene product furanosyl borate diester, a more universally synthesized and recognized molecule. It is assumed that bacteria use these auto inducer molecules not only to identify their neighbors and cell density, but also to govern some aspects of their environment, such as confinement and diffusion. In a broad sense, quorum sensing allows harmonization of cell density wide activities, together with virulence factor production, biofilm dynamics, bioluminescence and swarming motility on surfaces. Swarming motility is a flagella-driven movement of bacterial cells through it can spread as a biofilm over a surface. Different chemical signals produced either by bacteria (AHL) may persuade the QS regulated swarming activities in bacteria. This review emphasizes the

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role of AHL and other low-molecular-mass signal molecules involvement of in swarming motility of bacteria.

Keywords Quorum sensing · AHL · Swarming motility · Quorum-sensing controlled gene expression systems

Introduction

Swarming motility is a quick and synchronized movement of a individuals of bacteria through solid or semi-solid surfaces, and is an exemplar of bacterial multicellularity and swarm behavior [29]. Bacterial cells distinguish into a specific state (swarmer cells) which is morphological delineated by hyperflagellation and cell elongation [22, 29]. Swarmer cells require augmented synthesis of some extracellular wetting agents which reduce surface friction and facilitate smooth relocation bacterial cells on viscous surfaces [29]. Swarming warrants intercellular communications, secretion of surfactants and inflates in flagellar numbers. The ecology of swarming is yet mysterious, but often linked with pathogenesis. Swarmer cells also benefit from improved antibiotics resistance and eukaryotic engulfment with gaining better nutrition and obtain aggressive help from secreted surfactants. They often involve the chemotaxis sensory transduction system for tasks that are distinct to chemotaxis [39]. Swarmers display high speed twirls, vortexes and frequently tacits to drive microbial community development at a cost of cell growth. Hence, swarming is divergent from flagella dependent swarming so as to represent individual cell movement in a liquid/solid medium with minor agar concentrations. In order to swarm bacterial cells translocates by extracellular slime (a blend of carbohydrates, proteins, peptides, surfactants, etc.) through which they can disperse a biofilm over a surface.

Swarming bacteria are divided into two groups based on ability of flagellar propulsion to surface motility: robust swarmers, (swarm across a solid agar surface) and temperate swarmers, (swarm only on a softer agar surface). Robust swarmers comprise polarly flagellated bacteria, such as *Rhodospirillum, Azospirillum*, *Vibrio* and *Proteus* species. Temperate swarmers comprise *E. coli, Pseudomonas*, *Rhizobium*, *Bacillus*, *Serratia*, *Salmonella* and *Yersinia* species. Conversely, swarming ability is heightened by lipopeptide and rhamnolipid surfactants secreted by the temperate swarmers *Bacillus*, *Serratia*, *Rhizobium, Pseudomonas* and species [11, 24, 29, 39, 66, 88]. Swarming and swimming motility along with extracellular enzyme activities, i.e. nuclease, protease, lipase and haemolysin, are other behaviors that may extensively add to bacterial pathogenesis [31]. This multifaceted multicellular behavior needs the combination of physical and chemical signs, this leads to physiological and morphological characterization of bacteria (normal cell) into swarmer cells. Swarming motility was first documented by Jorgen Henrichse and has been typically studied in genus *Salmonella, Serratia, Bacillus, Aeromonas, Pseudomonas, Escherichia, Yersinia, Vibrio* and *Proteus*. However swarming ability is prevalent in various genera of Gram-negative and positive flagellated bacteria and is characteristically evaluated

on a solidified agar medium [65]. Swarming motility can also endow with an aggressive advantage in search for nutrient-rich environments and it can be pretentious by bacterial population size, water content and nutrient composition [64].

Swarming motility is a multicellular cooperative way of flagella dependent motility on surface. Biofilm formation forms a sessile bacterial community surrounded by their own extracellular polymeric substances (EPS) matrix. Cell density is a highly indispensable and essential factor to initiate and uphold the swarming process. Therefore, not unexpected that swarming in several bacteria, is coupled to quorum sensing (QS) [12]. QS within bacterial populations can promote pathogenesis, cellular dissemination or dispersal, symbiosis, DNA transfer, microbial biofilm development, in addition to production of antibiotics and other secondary metabolites [74]. QS mechanism also allows bacteria to coordinate swarming, biofilm formation, stress resistance and production of toxins and secondary metabolites. QS is a biochemical communication, that relies on production, secretion and detection of auto inducer (AI) signals to regulate gene expression in response to changes in population density [25]. These molecules are the mediators of QS. This sensing is natural among bacteria and helps to retain the bacteria in a good location. However this assists the bacterial swarm from overcrowding and avoids from several toxic substances. QS permits bacterial colony to control their gene expression in harmony, which is imperative for moving out set of behaviors such as bioluminescence production, biofilm formation, virulence factor and genetic exchange etc. [58]. Nonetheless many bacterial species rely on QS to control important cellular processes which are essential for surveillance, endurance and acclimatization to their changing environments [5]. By monitoring the accumulation of specific AIs, bacteria can even track shifts in population density and species complexity in the vicinity and quickly respond as a group for that reason [59, 86].

Nonetheless, reviews on QS regulated swarming are yet scanty, while summary of QS behavior in a various range of bacteria is documented. The current review introduces swarming motility phenomenon from a practical perspective, then describes cellular necessities and phenotypes associated with swarming in diverse model organisms. This review also covers current advances in swarming and relation between swarming motility and biofilm formation in well studied model strain mentioned below.

Pseudomonas *sps.*

In *P. aeruginosa* production of biosurfactants, rhamnolipids is regulated by QS by N-acyl-homoserine lactones (AHL). QS monitor swarming motility in *P. aeruginosa* is nutritionally conditional. QS mutants are defective in swarming behavior when supplemented with succinate as their sole carbon source (but not on glutamate) [72]. *P. aeruginosa* is achieved several types of motility, including twitching, swimming, and swarming motility. In contrast, *P. aeruginosa* is capable of

swarming with a single polar flagellum, although cells with two flagella have been observed in swarms [69]. *P. aeruginosa* swarming motility is also dependent upon the secreted surfactants 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA), mono-rhamnolipids, and di-rhamnolipid [8, 69]. Rhamnolipids can act as secreted virulence factors [37] and several studies suggest that swarming motility in *P.aeruginosa* may be correlated with increased virulence. The rhamnolipid biosurfactants which are thought to be one of the virulence factors in *Pseudomonas*, act as a surface-active agent (swarming modulating factor) [15, 42]. Overhage et al. [65] revealed a wide range of genes viz. chemosensory, secretion; regulatory, hypothetical and metabolic genes contribute to swarming motility of *P. aeruginosa,* by screening a transposon mutant library. In a study that investigated the changes in gene expression that occur in swarming cells relative to swimming cells, swarming cells up regulated expression of genes encoding the type III secretion system and secreted virulence factors, including exoenzymes ExoT, ExoY and ExoS; secreted proteases and elastases; and the biosynthetic genes for the production of pyocyanin and the siderophores pyoverdine and pyochelin [64, 88]. Over past two decades, regulated synthesis of rhamnolipids *via* QS and their role in swarming motility, virulence have been broadly illustrated in *P. aeruginosa* [1, 17].

Coordination of biosurfactant synthesis, swarming motility and biofilm matrix (EPS) production has been well established in *P.aeruginosa*. Recently Wang et al. [83] demonstrated that elevated levels of Psl and/or Pel slashed swarming motility of *P. aeruginosa* but had petite effect on twitching and swimming. The decreased motility was due to low rhamnolipid synthesis with no relation to the transcription of *rhlAB*, two key genes viz. rhamnolipid-negative *rhlR* and *rhlAB* mutants produced more Psl, whereas EPS-deficient strains demonstrated a hyper swarming phenotype. This implies that QS signals might control EPS biosynthesis indirectly in bacterial populations. Motile and ubiquitous *P.aeruginosa* can quickly colonize surfaces and form biofilms in numerous environments. Mattingly et al. [53] reported that maintenance of intracellular c-di-GMP levels is essential to stimulate flagellar motility or biofilm development is conditionally controlled by diverse phosphodiesterases (DipA) harmony to nutrient cues. Notably, DipA demonstrated additional effects on minimal medium harmonized with glutamate/glucose, while swarming in rich medium conditions occurs below elevated levels of c-di-GMP [53].

Rhizobium *sps.*

In *R. etli,* AHL molecules are long-chain fatty acid moieties have a dual role in swarming [11]. The *cinIR* operon is a QS system that is tangled in synthesis of AHL ligands and is necessary for swarming behavior. *cinIR* operon is auto regulated, which leads to elevated levels of expression and AHLs production.

Serratia *sps.*

The velocity with which *S. liquefaciens* swarming colony inhabits the surface of suitable solid substratum was regulated by modulating *flhD* master operon expression. However in liquid medium, the expression of *flhD* operon resulted in hyperflagellated, multinucleate and filamentous cells that were impractical to differentiate from swarm cells. Thus, expression of the *flhD* master operon seems to play an essential role in the progression of swarmer cell differentiation [18]. The *SwrIR* genes of QS system in *S. marcescens* MG1 was discovered from liquefied plant tissue to control some phenotypes for instance biofilm formation, swarming motility, exoenzymes and carbapenem production, which cause pathogenesis of the host [79]. Swarming motility of soil pathogens is essential for survival while it is beneficial for drive movement of pathogens to a sustainable environment. Many plant-pathogenic bacteria depend on QS to induce disease. However interaction of *P.heterophylla* with QS and quorum quenching *S. marcescens* interceded by root exudates in a monoculture system. Interestingly *aiiA* gene encode for lactonase in *S. marcescens* caused in pathogenicity reduction, implying its toxicity on seedlings was QS-regulated. However surplus lactonase in *S. marcescens* lead to reduction in antibacterial substances, exoenzymes, and swarming motility, is expected to cause to pathogenesis on the seedlings [89]. Strikingly, *S. marcescens* uses two-component system (TCS), which stereotypically contains a sensor kinase and a specific cognate response regulator, to correctly respond to environmental fluctuations. Interestingly the TCS QseBC, which was seen in numerous human pathogens tangled in flagellar biosynthesis and virulence regulation, was evidenced cross-talk with non-cognate response regulator RssAB [66]. Fascinatingly in a recent report, Wei et al. [85] demonstrated that the phosphorylated QseB suppressed *flhDC* expression, decreasing rate of swarming migration with meek influence on migration initiation. However QseC dephosphorylates RssB and deactivates RssAB signaling in swarming lag. Interestingly, antagonistic activities of few probiotic bacteria *L.acidophilus* ATCC 4356 and their metabolites are capable to impede virulence factors such as antibiotic resistance and swarming motility and may be employed as alternatives to antibiotics. Moreover, culture supernatant of strain ATCC 4356 with concentrations greater than 2%, was demonstrated with a significant influence on the swarming ability of *S. marcescens* ATCC 13880 was repressed [78].

Bacillus *sps.*

The swarming motility gene *swrA* in *B. subtilis* has high reversibility [38]. Thus, one probable mechanism for regulation of swarming motility is genetic as phase variation swiches the *swrA* reading frame linking the functional and non-functional alleles [38]. The regulation of flagellar biosynthesis in *B.subtilis* is still scantily known, however the putative candidate for master regulator of flagellar gene

expression is the protein SwrA. Unlike FlhDC, SwrA of *B.subtilis* is not obligatory essential for flagellum synthesis, as *swrA* mutants swim but do not swarm. It yet not clear whether a putative swarming elongation factor gene efp (yqhU) and its translation of transcripts are involved in swarming? [38].

Proteus *sps.*

Peptides/amino acids are proposed to have a signaling function in *P. mirabilis* [22]. Interestingly, it has been reported that externally supplemented fatty acids modulated various bacterial activities including virulence, cell growth, differentiation and motility. Straight-chain saturated fatty acids (SCFAs) suppress the production of hemolysin and swarming motility in *P. mirabilis* and *S. marcescens* [46, 48]. Swarmer cell elongation and subsequent swarm motility of *P. mirabilis* requires conserved ECA biosynthesis gene *rffG*. Strikingly the *rffG* gene encodes a protein homologous to dTDP-glucose 4,6 dehydratase protein of *E.coli,* which contributes to ECA biosynthesis and is required for production of large lipopolysaccharide-linked moieties necessary for wild-type cell envelope integrity. The absence of *rffG* gene induced several stress-responsive pathways include those controlled by transcriptional regulators RpoS, CaiF, and RcsB [50].

Vibrio *sps.*

V.parahaemolyticus possesses two types of flagella: numerous lateral flagella and a single polar flagellum. Lateral flagella are induced under viscous environments and promote swarming motility on surfaces, while polar flagellum is expressed continuously and propels swimming motility. The polar flagellum is powered by sodium motive force, whereas the lateral flagella are powered by proton motive force, hence considered as model organism for studying robust swarming motility and biofilm formation. QS regulation of swarming and virulence in *V. parahaemolyticus* commences a reversible phase transition among the opaque (OP) and translucent (TR) cell types. The OP cell type has sticky surface colonies and yields elevated amounts of capsular polysaccharide (CPS), while TR cell type yields very little CPS and are extremely motile on surfaces [19]. The transcriptional regulator *OpaR,* positively regulates expression of *cps* genes and lateral flagella genes negatively, which monitors the switch amongst OP and TR cell types [27, 36]. However *OpaR* homologs are reported in all *Vibrio* species that are still unexplored. It is notable that OpaR regulator acts as terminal output regulator of QS cascade in *Vibrio* species. At LCD *opaR* expression is silenced by small regulatory RNAs (*Qrrs*) which are prompted

by phosphorylated LuxO protein [20]. If cell density increases LuxO is dephosphorylated and OpaR is expressed as reviewed in [56]. In *V. parahaemolyticus*, OpaR acts as a negative regulator of swarming while LuxR homolog of *V. harveyi*, is involved in switching of colony morphology (opaque–translucent and vice versa). *OpaR*-dependent expression of *cpsA* locus, mediates phase transition in *V. parahaemolyticus*. In addition, OpaR suppresses the *laf* gene expression and subsequently hinders swarming behavior [36] while enhances biofilm formation, cell–cell adhesion, and controls type-III secretion [32].

Salmonella *sps.*

Swarming cells of *S. typhimurium* have different global gene expression paralleled to swimmer cells and have been reported to characterize a different physiological state [40, 82]. At phenotypical level, *S. typhimurium* swarmer cells display an enhanced antibiotic resistance. In contrary to most flagellar genes in *S. typhimurium*, genes implicated in LPS synthesis, virulence and iron acquisition are induced during swarming [82].

Burkholderia *sps.*

Plant pathogenic bacterium *Burkholderia glumae* uses an AHL-mediated QS system to regulate protein secretion, oxalate production and major virulence determinants such as toxoflavin and flagella. Nickzad and group [61] detected *rhlA*- mutant that controls swarming motility behavior through positive regulation of rhamnolipids through QS mechanism. In few swarming bacteria, for instance in *P.aeruginosa* and *B. thailandensis*, the social motility behavior is dependent on cell-to-cell signalling and promotes the production of rhamnolipids [16, 77]. *B. glumae* was shown to produce rhamnolipids and carries a *rhl* gene homologous to rhamnolipid biosynthesis in *B. pseudomallei, B. thailandensis* and *P. aeruginosa* [10].

Motility is essential for virulence and there are plethoras of reports that non-motile strains of phytopathogenic bacteria are avirulent [75]; additionally rhamnolipids bestow to the virulence of *P. aeruginosa* and *B. pseudomallei* respectively [30, 91]. Numerous factors controlled by QS in *B. glumae* are virulence determinants (e.g. toxoflavin, flagella). Therefore, rhamnolipids promote swarming motility in *B. glumae* which may contribute to virulence. It has been documented that when environmental conditions (e.g. nutrient availability) are becoming adverse for bacteria, swarming motility helps their dispersal to discover new niches [76].

Aeromonas *sps.*

Ten clusters of chemotaxis genes have been documented in genome of *A. hydrophila* ATCC 7966^T, which includes two gene-system homologs to *P. aeruginosa* PAO1 gene clusters I and V [87] important for chemotactic motility [52]. The VgrG proteins correspond to type VI secretion system (T6SS) components and effectors of *A. hydrophila* strain SSU and promote biofilm formation. Their function is not fully established but may occur during attachment step due to VgrG3 that enhances swimming motility [70].

Yersinia *sps.*

QS in *Y.enterocolitica* regulates swimming and swarming motility. The LuxI homologue YenI guides the production of N-3-(oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) and N-hexanoylhomoserine lactone (C6-HSL). Reverse transcription-PCR shown that mutation of yenI had no effect on yenR, flhDC (motility master regulator) or fliA (flagellar sigma factor) expression, while fleB (flagellin structural gene) was down-regulated [2].

Azospirillum *sps.*

A.brasilense is an essential PGPR bacterium that needs numerous critical steps for root colonization, biofilm, EPS synthesis and cell motility. Interestingly this strain doesn't carry a *luxI* gene, however it has several *luxR* solos that might identify AHL molecules. By external supplementation of AHLs, biofilm, EPS production and cell motility (swimming and swarming) were controlled in strain Ab-V5 [24].

Complex Relation Between Swarming and Biofilm Formation

The relation among motility and biofilm development has a tendency to be complex for the reason that both processes may entail analogous components at some stages. For instance, during reversible attachment, initiation of biofilm formation frequently needs flagella, and motility on a surface might be essential for biofilm architecture. Nevertheless, motility helps in bacterial release from mature biofilms [7, 41]. Yet, bacteria may opt among motility (swarming) and biofilm formation under certain conditions. It seems that intracellular messenger c-di-GMP, produced by diguanylate cyclases functions as a secondary messenger in reaction to extracellular signals and regulates multicellular behavior, motility and virulence in numerous diverse

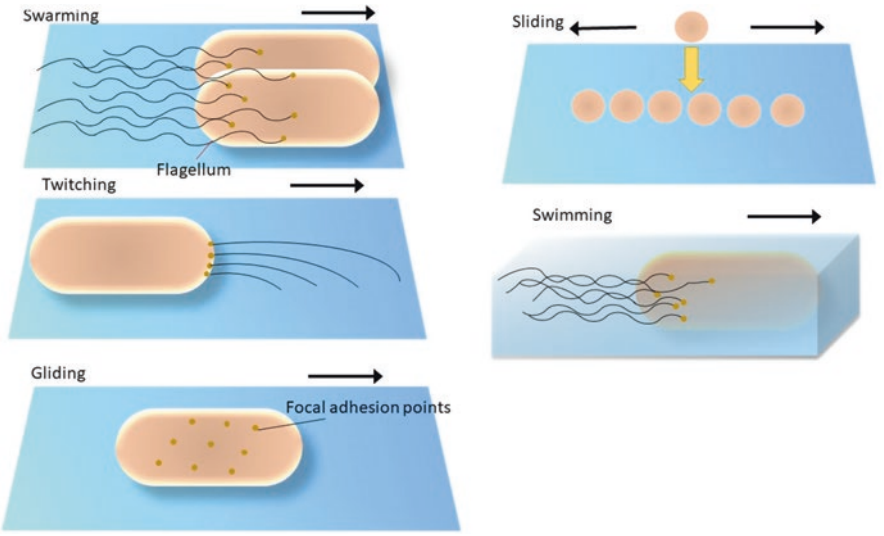

Fig. 1 Demonstration of different stages of multicellular swarming behavior

bacteria (Fig. 1). Generally, elevated levels of c-di-GMP associate with augmented sessility, reduced motility and virulence. *BifA*, an intracellular messenger, Cyclic-Di-GMP phosphodiesterase, inversely controls the surface related behaviors such as biofilm formation and swarming motility in *P. aeruginosa PA14* [43]. A study demonstrated early stages of biofilm formation that is involved to regulate flagellar reversals and synthesis of EPS as potential factors in formation of a sturdy union with swarming motility and substratum. The same research group evidenced that *SadC* an inner membrane-localized diguanylate cyclase, controls certain cellular functions viz. biofilm formation and swarming motility via intonation of EPS production and flagellar function. Mutation/deletion of *sadC* gene effects in a strain to be defective in biofilm formation and a hyper warmer, while multicopies of this gene promotes sessility [54].

Bond Between Swarming with Virulence in QS Bacteria

Hyper flagellation contributes to rapid contamination of host tissues, bond between swarming and virulence is not clearly established in majority of swarming bacterial pathogens. Nevertheless, swarm cell differentiation is frequently complemented by expression of virulence determinants, which might help bacteria in inhabiting new environment. Biofilm formation and EPS production depict an essential part in *P.mirabilis* infection. Despite, sensing external messengers, *P.mirabilis* endures a multicellular behavior which is controlled through virulence factors expression. Fatty acids were shown to function as environmental cues to control swarming and

virulence in *P.mirabilis*. *RsbA* gene which encodes a histidine-containing phosphotransmitter of bacterial two-component signaling system, acts as a repressor [48].

Strikingly the virulence proteins such as metalloprotease, urease, haemolysin and flagella were shown to up regulate during swarming in *P. mirabilis*, and phospholipase action is prompted in *S. liquefaciens* [6, 23]. At phenotypical stage, *S. typhimurium* swarmer cells elicit heightened antibiotic resistance. In contrary to flagellar genes in *S. typhimurium*, genes involved in virulence, LPS synthesis and iron acquisition are prompted in swarming [82]. In *P. aeruginosa*, several virulence-related genes are upregulated in swarmer cells. In addition, two virulence genes (*lasB* and *pvdQ*) appear to be essential for swarming. The coordinated regulation of swarming and virulence is also accomplished by c-di-GMP signaling [64].

Regulation of Swarming Behavior and Virulence in QS Bacteria

RsmA was reported to regulate swarming motility and expression of virulence genes in many *Erwinia* sp. [58]. However, in *E. coli*, *CsrA*, a homologue of *RsmA*, positively controls swarming motility and expression of *flhDC* gene [84]. Swarming in *P.mirabilis* includes differentiation of exemplary short vegetative rods into filamentous hyper-flagellated swarm cells that endure cycles of rapid and synchronized population migrations across surfaces and demonstrate elevated levels of virulence gene expression. Role of *RsmA* in swarming motility and expression of virulence factors (haemolysin, protease, urease and flagellin) in *P.mirabilis* was examined [49]. Numerous factors have been reported to control swarming [12, 81]. For instance, social motility in *B. cenocepacia* H111 is under the regulators of *CepRI* and *RpfRF* QS systems [14, 33] However, in *B. glumae* it has recently reported that QS regulates swarming by controlling rhamnolipid biosynthesis under nutrient starved conditions [60, 61].

In *P. aeruginosa* swarming was reported to be regulated at post-transcriptional level through RsmAB system and c-di-GMP levels [3, 44]. In *P. aeruginosa*, the GacS/GacA two-component system controls a master switch between two growth states, one characterized by virulence factor production and swarming motility, the other defined by sessile growth as a biofilm [47, 80]. Activation of *RsmA, RsmY* and *RsmZ* signaling cascade down regulates swarming, type II secretion, type III secretion, T4P, exotoxin A, and lipase expression and up regulates Pel and Psl expression, promoted formation of biofilm [26]. Thus GacS/GacA system reciprocally regulates swarming motility and biofilm formation. In addition, GacS/GacA regulatory system coordinately regulates swarming motility, type II secretion, type III secretion, and virulence factors ToxA and LipA secreted by type II secretion system [26, 80]. The small RNA *ErsA* of *P.aeruginosa* contributed to biofilm formation and motility by post-transcriptional modulation of *AmrZ* which is a transcriptional regulator

regulon. Conversely, *AmrZ* knock-out mutant strain was evidenced with twitching motilities and enhanced swarming [21]. NtrC-dependent control of EPS synthesis, motility and for first time that it is reported that swarming ability of *B. cenocepacia* H111 is regulated by NtrC and by σ^{54} [51]. A distinctive role for ArcA and ArcB homologues in swarming motility was explored in *S. marcescens* FS14 by inserting in-frame deletion mutations in *arcA, arcB* and *arcAB* genes. Strikingly, only ArcB affects swarming motility of FS14, but ArcA does not [90]. In a recent report *SwrD (YlzI)* was shown to promote swarming in *B.subtilis* by boosting power to flagellar motors. *swrD,* gene is located within 32 gene *fla-che* operon committed to flagellar biosynthesis and chemotaxis, when mutated stopped swarming motility [28]. Strikingly the deletion of *DegU* or *DegS-DegU* impeded swarming motility, biofilm development, sporulation and binary toxin synthesis by controlling related genes while phosphorylation was essential for *DegU* function. Transcriptional regulator DegU is essential for multicellular function in *L. sphaericus*. Given the fact, swarming seems to be a highly synchronized form of motility including many cellular activities. Conversely, molecular mechanisms elemental for swarming remain largely undiscovered.

Inhibition of Swarming Motility and Biofilm Formation

12-methyltetradecanoic acid (anteiso-C15:0) (branched-chain fatty acid) was shown to possess reduced flagella mediated swimming motility and totally restrained surface motility in *P aeruginosa* PAO1 [35]. Along with motility suppression, anteiso-C15:0 also triggered 31% suppression of biofilm development by PAO1, signifying that BCFA might affect multiple cellular activities (Fig. 1). Swarming motility by *Photorhabdus* temperate, an insect pathogen and nematode symbiont is affected in different environments and uses same flagella as that used in swimming motility as detected by immunoblotting experiments [55]. The swarming motility of *P. aeruginosa* PAO1is blocked by cranberry proanthocyanidins and other tannin-containing materials. 3-Phenyllactic acid produced by *Lactobacillus* antagonistically attaches to QS receptors RhlR and PqsR with a greater affinity than their cognate ligands N-butyryl-l-homoserine lactone (C4–HSL) and 2-heptyl-3,4-dihydroxyquinoline [63] which inhibits expression of virulence factors such as pyocyanin, protease and rhamnolipids that are essential for biofilm formation of *P. aeruginosa* PAO1. In addition to swarming motility, other important criterion for formation of biofilm in *P. aeruginosa* PAO1, was also inhibited by PLA [9]. An effect of on some selected QS-regulatory genes viz. *lasI*, *lasR*, *rhlI* and *rhlR* at transcriptional level was investigated using *P. aeruginosa* PAO1. It reduced the expression of QS, virulence and biofilm formation pyocyanin, elastase, protease, rhamnolipid, hemolysin and swarming motility by extracts of *Andrographis paniculata* in strain PA01 [4].

A small synthetic cationic peptide 1037 inhibited biofilm formation in Gram-negative pathogens viz. *P. aeruginosa* and *B.cenocepacia* and Gram-positive *L.monocytogenes* by decreasing swimming and swarming motilities while,

activating twitching motility, and repressing expression of diverse genes employed in biofilm formation [13]. Stilbenoids, identified an important phytoalexins in plants, and were famous for their positive effects on cardiovascular, neurological and hepatic systems. Among them few molecules viz. resveratrol, piceatannol and oxyresveratrol showed decreased synthesis of pyocyanin and swarming motility of *P. aeruginosa* PAO1 [71]. Additionally Zingerone, was reported to have lowered biofilm forming capacity, decrease swimming, swarming and twitching phenotypes and production of virulence factors including rhamnolipid, elastase, protease, pyocyanin, cell free and cell bound hemolysin signifying anti-virulent property attributing to attenuation of virulence of *P. aeruginosa* PAO1 [45]. In another study, Petroselinic acid (PSA) efficiently blocked biofilm-related phenomena such as EPS, hydrophobicity production, swimming, and swarming motility without influencing bacterial growth of environmental pathogen *S.marcescens.* Therefore it could be envisaged that PSA could be considered as an antipathogenic drug for treatment of QS-mediated infections caused by *S. marcescens* [68].

Recently a study evaluated anti-QS activity and anti-biofilm activity of *A.tsaoko* extracts inhibited swarming motility in food borne pathogens viz. *Staphylococcus aureus, S. typhimurium* and *P. aeruginosa* which causes extreme problems in food safety, biofilm related infectious diseases etc. Interestingly, *A. tsaoko* extracts can provide value to food products and medicine by regulating pathogenesis [67]. Microbial control and QS inhibition by phenolic compounds of acerola (*M. emarginata*) was recently demonstrated in QS controlled phenotypes such as violacein production in *C. violaceum*, swarming motility in *A. hydrophila* and more importantly, biofilm formation in these bacteria and also in *S. marcencens* [62]. Anti-QS and anti-biofilm activity of *D.tsuruhatensis* extract obtained by attenuating QS regulating virulence factor. These extracts also showed inhibitory effect on swimming and swarming motility and regulatory effect on *P. aeruginosa* virulence factors for instance pyocyanin, rhamnolipid, elastase, and protease [73]. Leaf extracts of *Mangifera indica L.* inhibited QS controlled synthesis of virulence factors and biofilm in *P. aeruginosa* PAO1 Interestingly, the QS inhibitory activity is also demonstrated by reduction in elastase, total protease, pyocyanin, chitinase, exopolysaccharide production and swarming motility in PAO1 strain [34].

Future Perspectives and Conclusions

Numerous bacteria that swarm on surfaces signify that swarming is an essential way for surface colonization in natural environments. Despite the fact that swarming motility is a different social behavior, but many questions still remain unanswered. Recently, it was demonstrated that swarming motility includes a broad range of cellular activities; conversely, molecular mechanisms essential for swarming movement hover unfamiliar. The research on swarming motility promises to acquiesce novel insights into physiology of multicellular behavior in microbes. Specific novel swarming genes anticipate the innovation. New biochemical mechanisms are

considered necessary to unite swarming phenotypes to other, better implicit cell physiologies. Swarming is closely linked to biofilm formation, QS virulence and pathogenesis in several bacteria. A full understanding of these parameters guiding the swarming process is incredibly fundamental. Eventually, regulation over these processes will be expedited by the knowledge of key environmental, intracellular messages along with regulatory mechanisms guide to swarming behavior. The discovery of QS signals and mechanisms is an essential challenge for future research.

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Bacterial Quorum Sensing in Pathogenic Relationships: Relevance to Complex Signalling Networks and Prospective Applications

K. V. Deepika and Pallaval Veera Bramhachari

Abstract Quorum sensing (QS) is of at most significance in bacterial pathogenic relationships as it permits bacteria to coordinate gene expression of local populations therefore work in harmony. Bacterial growth and virulence usually related to the cooperative release of extracellular factors liberated due to QS. QS enables pathogenic bacteria to control genes promoting invasion, defence and spread of diseases which invariably affect human and animal health besides agricultural productivity. Apparently several bacterial pathogens use QS to regulate premature occurrence of virulence factors to protect themselves from host defence systems. More over emergence of bacterial strains with multiple drug resistance increased the need to develop modern approaches to control bacterial diseases. Since bacterial pathogenesis depends on QS regulatory systems, intervention with QS serves as a novel approach for the therapeutic or prophylactic control of infection. In this review, paradigms of pathogenic relationships, focusing on gram positive and gram negative model microorganisms were elucidated. Thereafter, attention is drawn on the exploitation of QS in antimicrobial therapy and biological control.

Keywords Bacterial pathogens · Quorum sensing · Antimicrobial therapy · Biological control · Multiple-drug-resistance

Introduction

Quorum sensing (QS) signifies the modulation of gene expression in reaction to extracellular autoinducer (AI) molecules secreted by bacteria, once the threshold concentration reached, whole bacterial population modify gene expression, which has implications in microbial pathogenesis, biofilm growth or pathogenic microbial relationships with higher organisms. QS regulatory systems make certain that these traits are only exhibited on the high population density of bacterial pathogens adequate to overpower the host organism [14]. In addition, QS confers bacterial

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pathogens a method to reduce resistance strategies of host organism by interrupting the release of cell destructive virulence factors as far as enough bacterial density has reached to cause infectivity.

It is evident that a considerable part of genome of bacteria (4–10%) along with proteome (≥20%) may perhaps be regulated by QS. It indicates QS mechanism exploited by bacterial pathogens modulates virulence factor secretion as well as acclimatizes to the metabolic requirements of population [3, 12]. These QS systems controls various roles in Gram negative and positive bacteria including pathogenesis, symbiosis, dissemination or dispersal, DNA transfer, virulence factor production, bioluminescence, motility prototypes, exopolysaccharide secretion, antifungal/antibiotic synthesis, other secondary metabolites, competence, exit from dormancy, cross-signalling between different strains and species etc. [13, 59].

Bacteria exploiting QS can measure their neighbouring cell mass by sensing the production of small signal molecules. Increasing population density results in elevated levels of signal molecules intern resulting in coordinated gene expression (Table 1). Many gram negative bacteria exploit N-acyl homoserine lactone (AHL) as QS signal molecules. Various pathogenic bacteria mediate successful colonization of the host through appearance of virulence factors and secondary metabolic compounds produced due to high levels of AHL in elevated population densities. The key method of QS includes the interface of auto inducer (AI) with a transcriptional regulator directly or by stimulation of a sensor kinase [6]. Gram positive and negative bacteria exploit species specific AI's for stimulation of QS system. Various QS signalling molecules work as neighbouring sensors to contact bacterial population. The above mentioned signalling molecules along with their receptors were generally grouped into three main classes: N-acyl homoserine lactones (AHLs), characterized by variation in length and oxidation position of acyl side chain and synthesized by Gram negative bacteria; oligopeptides/autoinducing peptides (AIP), comprising 5–34 amino acids commonly utilized by Gram positive bacteria; autoinducer-2 (AI-2) which is a ribose derived compound (4,5-dihydroxy-2,3-pentanedione) used equally by Gram positive and negative bacteria for interspecies interaction [53, 63].

In this chapter, we highlight the QS regulation in Gram-positive and Gram-negative model organisms to present a perception of how QS governed traits assigned to bacterial pathogenesis/secondary metabolite production. Additionally, we discuss the biological relevance of bacterial QS in antimicrobial therapy and biological control.

QS in Gram Negative Pathogenic Bacteria

Gram negative bacteria employ short signal molecules passing through cytoplasmic membrane and combine with regulatory proteins in the cell. They use N-acyl homoserine lactones (acyl HLs, AHLs) as signalling molecules. AHL molecules are produced by AHL synthase encoded by LuxI homolog. Small side-chain AHLs can

Table 1 Quorum sensing signal molecules and QS regulated behaviours in pathogenic bacteria

S.no	Pathogenic bacteria	Disease	Signal molecule	Reference
1	*Yersinia ruckeri*	Enteric red mouth disease	3-oxo-C8-HSL	Kastbjerg et al. [25]
2	*Aeromonas salmonicida*	Fish furunculosis	C4-HSL, C6-HSL	Schwentei et al. [55]
3	*Edwardsiella tarda*	Fish pathogen, exophthalmia, etechial hemorrhage in fin and skin, rectal hernia	C4-HSL, C6-HSL, 3-oxo-C6-HSL	Romero et al. [54]
4	*Pseudomonas fluorescence*	Strong spoilage activity in fish and stored food	C10-HSL, C6-HSL	Li et al. [35]
5	*Aeromonas hydrophila*	Tail rot, fin rot, hemorrhagic septicemia	C4-HSL, C5-HSL,C6-HSL	Patel et al. [47]
6	*Agrobacterium tumefaciens*	Crown gall disease	3-Oxo-C8-HSL	Zhang et al. [65]
7	*Erwinia carotovora subsp. carotovora*	Soft-rot diseases	3-Oxo-C6-HSL	Andersson et al. [1]
8	*Burkholderia cepacia*	Cystic fibrosis	C8-HSL	Lewenza et al. [34]
9	*Staphylococcus aureus*	Folliculitis, impetigo, cellulitis, infective endocarditis, septic arthritis, osteomyelitis	Auto inducing peptide (AIP-1)	Kong et al. [29]
10	*Streptococcus pneumoniae*	Pneumonia, septic shock, necrotizing fasciitis	Competence stimulating peptide (CSP)	Cvitkovitch et al. [7]
11	*Bacilli*	Anthrax, diarrhoea, food poisoning	Competence and sporulation stimulation factor (CSF)	Fujiya et al. [15]
12	*Enterococcus faecalis*	Bacteremia, endocarditis, urinary tract infections, endophthalmitis	GBAP	Arias et al. [4]
13	*Clostridia*	Neurotoxic, cytopathic, enterotoxic diseases	AIPs	Darkoh and Asiedu [8]

circulate profusely through cell membranes but lengthy side-chain AHLs require dynamic efflux to separate from the membrane [48]. After attaining certain level in the extracellular medium, AHL's are recognized by cytoplasmic proteins of LuxR family to control the downstream processes [45]. Thus, appearance of specific activities like pathogenic factors necessary for disease incidence is activated at elevated levels of population in a synchronized mode.

Gram negative facultative anaerobes like *Aeromonas* sp. have wide pathogenic traits, infecting both humans and animals. Among the members of *Aeromonas*, *Aeromonas salmonicida* pose a potential threat to aquaculture [38] and *A. hydrophila* is reported as a gastrointestinal pathogen of humans. In addition *A. hydrophila*

contributes to extra intestinal infections such as septicaemia, peritonitis, osteomyelitis and soft tissue infections [62].

Aeromonas species produce diverse extracellular compounds giving rise to pathological process of disease. The virulence factors produced by *Aeromonas* sp. include adhesins (e.g. pili), S layers, exotoxins for example hemolysins, enterotoxin, a range of exoenzymes capable of digesting cell components including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase and protease. These virulence factors cause diseases in fish and humans [21, 22]. The expression of virulence factors is related to elevated levels of cells in the late stationary phase consequently serves as apparent phenotypic entities to be regulated by QS [26].

The function of AHL linked QS system depending on LuxRI homolog AsaIR and AhyRI in *A. salmonicida* and *A. hydrophila* was previously reported which makes it probable that appearance of a range of virulence traits of *Aeromonas* sp. could be regulated by QS. The main signalling factor produced by AhyI locus in *A. hydrophila* is N-(butanoyl)-L-homoserine lactone (BHL) [23]. QS systems reported in gram negative bacteria are mainly three types each one comprising a sensor–autoinducer pair, type 1, type 2 and type 3 autoinducer (AI) systems.

Aeromonas AI-1 QS system is schematically shown in Fig. 1. AhyI and AsaI are LuxI-type enzymes in *A. hydrophila* and *A. salmonicida* respectively which synthetizes AHL molecules. According to Garde et al. [16], there are two phases in *Aeromonas* AI-1 QS system. Owing to the slow decay of AI-1receptor (AhyR), it either triggers the QS loop of initial AI-1 producer bacterial cell during exponential growth (Fig. 1a) or of other bacterial cells during the stationary phase (Fig. 1b). Auto induction takes place in log phase in which the enzyme (E) AhyI produces AI-1signal molecules of AHL from S-adenosyl-methionine (SAM) and acyl–acyl carrier proteins (acyl) [46, 58]. AhyR protein serves as the sensor (S) of AI-1system and is triggered by AHL molecules [58]. After activation, Ahy R act as transcriptional regulator for ahy RI locus surrounding AhyI and AhyR coding genes and contribute in auto amplification loop [16, 27]. The transcription of ahy RI locus also probably increased by second messenger c-di-GMP, AI-2 synthase LuxS or by AI-3 transcriptional regulatory protein QseB [30]. Bacterial membranes enable the AHL molecules to diffuse liberally and assemble in the extracellular environment [16]. During stationary phase, intercellular stimulation takes place above the threshold of AHL molecules equivalent to elevated population density (Fig. 1b) [16]. AhyR, after activation by AHL molecules acts as transcriptional regulating factor for a number of genes related to virulence and biofilm development.

Only AI- 1 system was comprehensively illustrated in *Aeromonas*. Once the AI-1 systems were triggered, they intern regulate fitness and virulence in *Aeromonas*. On the whole, AI-1 system stimulation in *Aeromonas* is linked to the development of biofilm maturation [26, 36] and virulence. Since it was shown that several virulence determinants were excessively secreted at elevated population density in log phase, population density might be a requirement for pathogenic behaviour in aeromonas and virulence occurrence [2]. It signifies the crucial role of QS in the way of infectivity by *Aeromonas* sp.

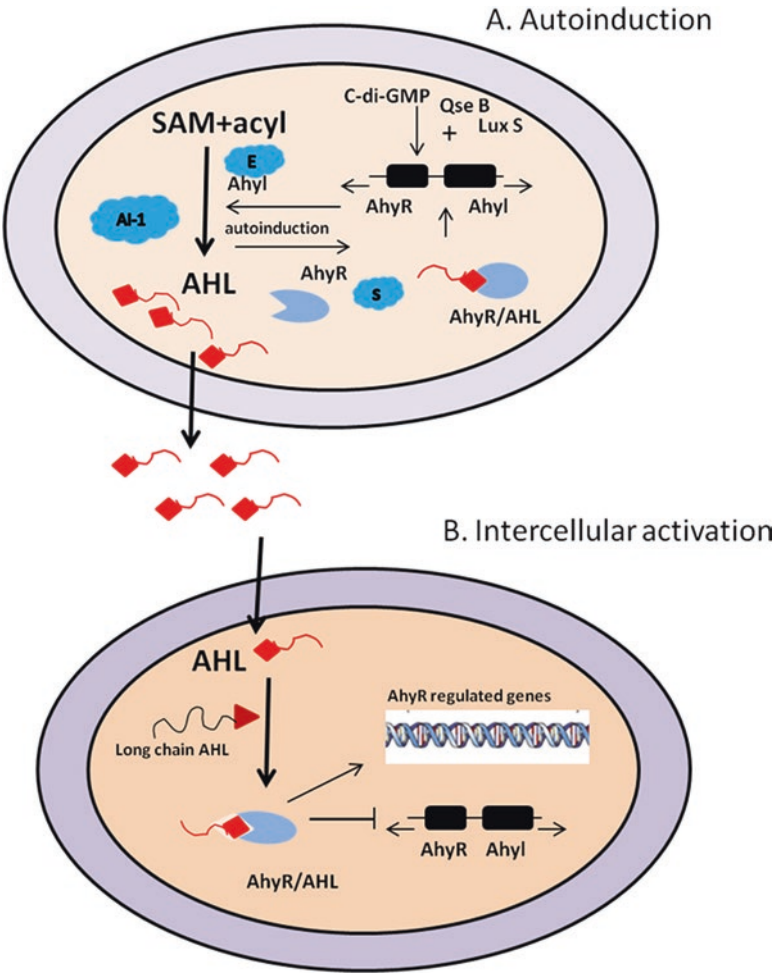

Fig. 1 Schematic illustration of AI-1 quorum sensing system in *Aeromonas*

Quorum Sensing in Gram Positive Pathogenic Bacteria

Many gram positive bacteria exploit QS systems albeit the signal molecules are different from the gram negative counterparts. Hitherto AHL producing gram positive bacteria were not reported. Gram positive QS systems generally utilize short post translationally modified peptide signal molecules. The detection of peptide autoinducer was facilitated by the use of two component adapter response proteins and this two component signalling was regulated by phosphorylation/ dephosphorylation cascade. Peptide signals were identified by two component sensor kinases. QS helps to control the increase of bacterial competence in several gram positive

microbes like *Bacillus subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis* and virulence in *Staphylococcus aureus* [28].

Enterococcus faecalis is a gram positive commensal in intestine of humans and other animals. Occasionally it can act as opportunistic pathogen and cause infections such as bacteremia, endocarditis, urinary tract infection and endophtalmitis. A number of virulence factors were reported in *E. faecalis* including cytolysin, aggregation substance (Agg), Enterococcal surface protein (ESP) and two extracellular protease, gelatinase and serine protease. In particular, gelatinase and serine protease are encoded in the gelE-sprE operon and their appearance is positively controlled by QS. *Enterococcal* QS is under the regulation of cyclic peptide pheromone in addition to the *staphylococcal* agr QS [17, 51]. The gelE and sprE are together situated next to the fsr genes and are synchronized by a common promoter [51].

The gelE gene codes for gelatinase polypeptide comprising 318 amino acids and has a molecular mass of 34.5 kDa [37]. Gelatinase acts as a virulence factor in *E. Faecalis* since it is an extracellular metalloprotease which liquefies gelatine and collagen. Moreover, it destructs a variety of substrates produced by host such as fibrinogen, fibrin, endothelin 1, bradykinin, LL-37, complement components C3, C3a and C5a [44, 61]. The sprE gene codes for a serine protease called glutamyl endopeptidase I having a mass of 25 kDa and serves as a virulence factor for *E. faecalis* [50]. Gelatinase confers pathogenecity in several animal models [49, 50], plants [21] and insects [60]. However, the main pathway of virulence in the above hosts is not fully elucidated.

In *E. faecalis*, Gelatinase biosynthesis activating pheromone (GBAP) is a characteristic autoinducing peptide (AIP) molecule that regulates the occurrence of two pathogenicity linked extracellular proteases [40]. GBAP is peptide in nature comprising 11 amino acids with a lactone ring. GBAP linked QS system refers to a cognate QS system, which is often programmed in the genomes of Firmicutes like *Staphylococci, Clostridia* and *Listeria* as well as *Enterococci* [64]. *E. faecalis* fsr locus encodes a two component regulatory system which detects the cell density and regulates virulence [50]. The fsr locus measure about 2.8 kb and contains four genes: fsrA, fsrB, fsrD and fsrC [41]. The fsrA gene encodes the FsrA protein, related to the LytTR family of DNA-binding domains [10]. Remarkably, fsrA transcription is linked to a constitutive promoter hence it is independent of Fsr QS system [51]. The fsrB gene encodes a transmembrane protein, FsrB, which belongs to the accessory gene regulator protein B (AgrB) family. FsrB processes a propeptide, FsrD (encoded by fsrD), to generate GBAP which is further pushed out of the cell [41] (Fig. 2). The fourth gene fsrC encodes the transmembrane histidine protein kinase FsrC which is the sensor transmitter of fsr operon [40]. The two virulence associated extracellular proteases GelE and SprE were apparently related to adhesion/biofilm growth and many host proteins which may possibly implicated in immune system. All these events imply that fsr QS system is firmly correlated with virulence of *E. faecalis* [57].

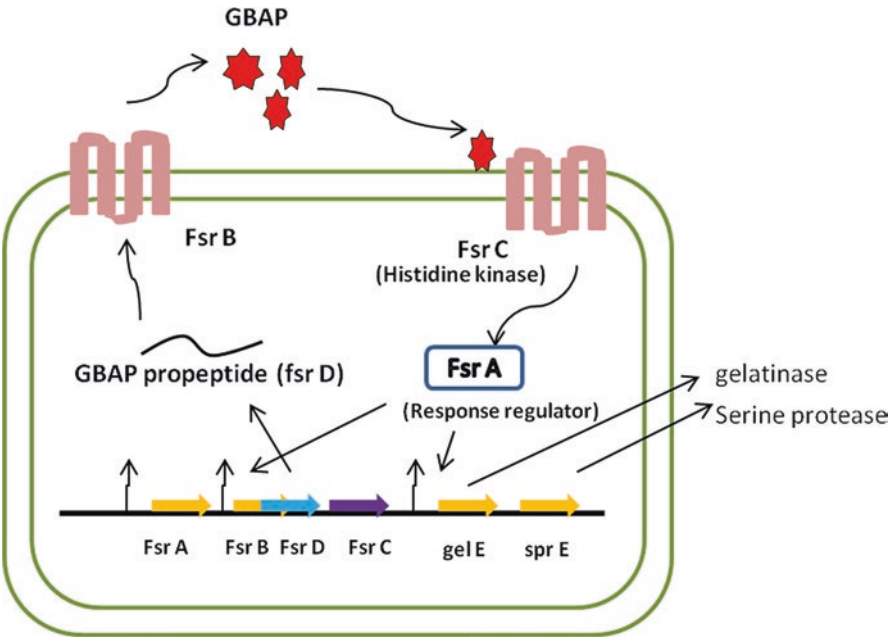

Fig. 2 The fsr quorum-sensing system in *Enterococcus faecalis*

Quorum Sensing in Antimicrobial Therapy

QS emerged as a main target for therapeutic interference of bacterial contaminations consequently quorum sensing inhibitors (QSIs) might be exploited as unique antimicrobial drugs. Therefore new methods targeting at interference with these systems might be beneficial for regulating virulence and colonisation in their hosts. Different QS prevention approaches such as (1) Blocking QS signal biosynthesis (2) Degradation of QS signal (3) Blockage of receptor for inhibition of QS signal detection (4) Interruption of efflux pumps target QS components. QSIs can be acquired from natural resources like plants and fungi or prepared as synthetic molecules. Irrespective of their origin, QSIs function by targeting either production of the signal molecules or triggering the QS regulon by targeting gene expression.

In view of the fact that AHL's main role in microbial communication process which directs to biofilm formation, enzymes capable of AHL degradation became promising candidates for QS inhibition. These enzymes can inactivate the synthesized AHL signals. In addition to these enzymes, much attention in QS inhibitors is given to the structural similarity of signal molecules, which block the signal receptor proteins consequently preventing modulation of specific gene expression [19]. Compounds capable of inhibiting the biosynthesis of fatty acids, biosynthesis of

SAM, protein synthesis or efflux pumps might be functional at the initial stage of signal generation of QS therefore act as QSI. Substrate counterparts like butyryl-S-adenosylmethionine, holo-Acyl Carrier Protein, sinefungin and L/D-S-adenosylhomocysteine were reported to hinder the AHL synthesis in vitro [52].

AHL molecules can be inactivated or completely destroyed by means of chemical degradation, enzymatic degradation of AHL molecules. Many bacterial pathogens were reported to produce AHL degrading enzymes including *A. tumefaciens, Klebsiella pneumoniae* and *P. aeruginosa* [20, 65]. Enzymes like AHL-lactonase, AHL-acylase and paraoxonase take significant part in the degradation of AHL and the above enzymatic degradation of QS is characteristically denoted as Quorum Quenching (QQ).

Inhibition of QS signal recognition by receptor interference involves inhibition of AHL molecules non enzymatically. Structural Analogues of AHL's were produced to obstruct the receptor. These structural counterparts were synthesized by modification in the acyl side chain or in the lactone ring or in both the above components of AHL molecule. A marine red algae, *Delisea pulchra* synthesizes halogenated furanone compounds which are structural analogues of AHL molecules and function as competitors for QS and hinder bacterial colonization, swarming behaviour and biofilm development [18]. Furanones act by displacing AHLs from their receptors [66].

Quorum Sensing in Biological Control

Plant pathogenic bacteria exploit complicated regulation systems to coordinate the infectivity process and promote definite virulence factors once entered in the host plant. Apart from the insight of plant signal molecules or availability of nutrients, QS plays vital role in the beginning of pathogenic cycle. As a result, QQ approaches are treated as promising substitutes to the use of pesticides [39]. The participation of QS in the modulation of early stages of microbial attachment and biofilm development lead to the investigation of strategies which can regulate host colonisation by interference or blockage of QS systems. Bacteria capable of producing AHLs induce several immune responses possibly will unlock novel prospectives in the prevention of plant pathogenic infections. The possibility to obstruct with microbial QS system using analogues or enzymatic destruction of QS compounds by rhizosphere microorganisms present further promising approach to conflict with pathogenic invasion because aforesaid strategies reduce the virulence by bacterial pathogens. Several rhizospheric bacteria were endowed with quorum quenching properties including *Bacilli* [11] producing AHL lactonases thus serve as potential candidates for pathogen eradication in crop fields. For this reason, QS mechanisms were proved as novel strategies against diverse plant pathogenic infections [32, 33].

Based on the pathogenic bacteria, diverse QS signalling compounds are synthesized however an enzyme esterase secreted by soil bacterium *Ideonella* sp. 0- 0013 degrades 3-OH-PAME from *R. solanacearum*. The enzyme CarAB (a carbamoyl phosphate synthetase) synthesized by several *Pseudomonas* spp. impair DSF signals. Lactonases or acylases are synthesized by several microbes to diminish AHL signals [42, 56].

Bacterial diseases encompass a major limitation to the growth of aquaculture [9]. At this instance, QQ approaches are beneficial for preventing bacterial diseases in the field of aquaculture. AHL-degrading enzymes were considered for disrupting QS of fish pathogens. *Bacillus* sp. strain AI96 produced AHL-lactonase which decreased the *A. hydrophila* contamination in zebrafish. QQ enzymes could be employed as a blend with prebiotics, probiotics, immunostimulants and vaccines to prevent and protect fish from wide range of pathogens [5].

Biofouling is one of the major issues for objects in contact with seawater for example boats, fish nets, or pipelines [31]. In the pursuit of non toxic alternatives, QQ enzymes were preferred for impeding biofouling or eradicating biofilms. Extremophilic QQ enzymes might serve as promising substitutes since they are generally robust and perhaps functional in non conventional environments. Moreover QQ enzymes signify an eco friendly solution compared with QS inhibitors as they can be infused into paints or coatings. Many marine microbes were reported to disrupt biofilms through the production of lytic enzymes able to degrade parts of biofilm. For instance, a marine *Bacillus* sp. produced 4-phenylbutanoic acid capable of strongly inhibiting the formation of biofilm by marine bacteria as well as by human pathogens [43]. In another study, α-amylase from a marine isolate of *B. subtilis* inhibited *Vibrio cholera* biofilms by 20–80% [24].

Future Perspectives and Concluding Remarks

The perceptive of bacterial cell communication with each other has many significant applications for the control of microbial pathogens and for the development of antibiotics, secondary metabolites and other products of high commercial value from bacteria. In view of the fact that several plant and animal pathogens utilize QS to control virulence and pathogenicity, various approaches anticipated to obstruct with their signalling networks may present prospective applications. The interruption of signalling networks present an opportunity to avert bacteria from reacting to the QS signals thus prevents the expression of virulence factors. In addition, studies are warranted on exploration of novel resources to acquire quorum quenching molecules to offer substitutes for developing antivirulence therapy against microbial pathogens. Bioprospecting of such molecules from extremophilic microbes may assist in accomplishing success in abolishing multidrug resistant microbial pathogens.

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Cellular Signaling in Bacterial Biofilms

Abhik Saha, Kriti Arora, Andaleeb Sajid, and Gunjan Arora

Abstract Development to sedentary community-based complex growth from planktonic growth is one of the most interesting feature of bacterial lifestyle, which is discussed in this chapter. Bacteria have smaller genomes and use simple signaling schemes for such complex transformation. However, diverse bacteria send either specific or shared signals to their neighbors and form complex biofilms in environment and in human host. The interaction between host immunity and antigens associated with bacterial biofilm has been elaborated. The signaling machinery help bacteria modulate transcription, protein synthesis and post-translational modifications that regulate motility and promote adhesion. In this context, the role of Ser/Thr protein kinases such as PrkC is discussed in detail. Biofilms protect the bacteria in harsh conditions, from environmental stress, host immunity and antibiotics, and also alter the virulence patterns. This is one of the most effective way to manipulate host defenses which can lead to development of therapeutic candidates that can alter bacterial signaling machinery and can help in utilizing biofilm formation for medical and industrial proposes.

Keywords Bacterial biofilms · Cellular signalling · Host defence · Virulence

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Introduction to Bacterial Biofilms

Bacterial biofilms are complex assemblies of heterogeneous (species-specific) bacterial cells, which come together to form an organized higher-order mass enclosed in an extracellular (often carbohydrate-rich) matrix. The recalcitrance of biofilms to physical and chemical methods of disinfection and sterilization allows these bacteria to not only survive harsh environmental habitats in the soil and water eco-systems but also accounts for its colonization in the human host, posing a challenge to the food industry, aquaculture and medicine. Many bacterial species are well-known for biofilm formation including Gram-negative (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*) as well as actinobacteria such as mycobacteria. Biofilm formation by mycobacteria is a major health concern due to emergence of drug resistant tuberculosis. In this chapter, we conglomerate the work done in the field of bacterial biofilm with respect to its assembly, structure and its involvement in host-pathogen interaction, leading to the complexities in the immune response towards the biofilm. We provide an overview on its structural features and factors that influence its formation. The chapter also focuses on the interplay of different signaling molecules during the event of biofilm formation, which helps in understanding the mechanism of biofilm formation.

Biofilm Assembly and Structure

A series of events lead to the development of a biofilm. It starts with the interaction of bacteria with surface, followed by proliferation and generation of a mature biofilm structure and lastly detachment. Transport of bacteria passively (e.g. *Staphylococci*) or actively (e.g. *P. aeruginosa*) initiates the development of biofilms. Attachment to the surface is mediated by protein–protein interactions for biotic surfaces, while in case of abiotic surface, attachment depends on the surface hydrophobicity [95]. Following attachment of the primary colonizers, the development of micro-colonies is initiated. The progressive growth of the micro-colonies leads to their enlargement in size and cover the surface through formation of a layer. At this stage, the cells are enclosed in a covering of mushroom-shaped matrix towers. Upon formation of a multilayer, the biofilm matures and forms a macro-colony. Nutrients and signaling molecules are distributed by the surrounding water channels. As adverse conditions arrive, biofilm forming cells can detach individually or in clumps [149]. Biofilms present in nature are mostly polymicrobial with cells displaying different phenotypes.

Maturation depends upon cell to cell disruptive factors, like surfactants. The extracellular matrix helps in the formation of the multi-layered biofilm by mediating adhesion between cells [125]. The most commonly found exopolysaccharide is

Polysaccharide Intercellular Adhesin, PIA (PNAG), a homopolymer of beta-1,6-linked GlcNAc residues [38]. PIA/PNAG, being partially deacetylated, have a positive charge that helps in the interaction with the negatively charged matrix components such as Teichoic acid [219]. Along with EPS, proteins like Aap (Accumulation-associated protein), Bap (Biofilm-associated protein) and Embp (Extracellular matrix binding protein), help in the establishment of the biofilm [18, 32]. DNA released from dying cells also aids in biofilm formation [222]. DNA being polyanionic, interacts with the oppositely charged moieties in the matrix network.

Resistance Development Against Antimicrobial Agents

The composition of biofilm and its physio-chemical properties provide a built-in resistance to antimicrobial agents. In order to be effective against the biofilm, the antimicrobial compound should diffuse through the biofilm matrix and reach the bacterial cell. The major hindrance in this activity is caused by the EPS present in the matrix. EPS significantly reduces the transport of the compound to the biofilm core. For example, reduced rate of ciprofloxacin penetration in *P. aeruginosa* biofilms was reported by Suci et al. [200]. It was also observed that free planktonic bacteria were highly susceptible to antibiotic than in biofilm [86]. Bacterial cells associated with biofilm grow slowly as compared to their planktonic counterparts resulting in a slow rate of uptake of these antimicrobial molecules. Availability of oxygen and the gradients in pH also lay an impact in the efficacy of the antibiotic negatively [218]. The presence of constitutively expressed multi-drug efflux pumps in biofilms further helps the bacteria in escaping the effects of antibiotics.

Involvement of Biofilms in Host-Pathogen Interaction

The immune system could be defined as the conglomeration of cells, tissues and molecules that help in protection from a wide range of pathogenic microbes and toxins in our environment. This defensive ability consists of two distinct reactions: innate immunity and adaptive immunity [91]. The innate immune system comprises of cells and proteins that are essential in the protection against the invading pathogen [92]. The adaptive immune system provides long term reliable and often sterile protection against repeated assaults of the pathogen. The constituents of the adaptive immune system are dormant but when activated, they proliferate and create potent mechanisms for neutralizing or eliminating the microbes. The adaptive immune response could be categorized into two types: one that involves antibodies (humoral) and other that requires the participation of T-lymphocytes (cell mediated) [103].

Importance of TLRs (Toll-Like Receptors) and Myd88 (Myeloid Differentiation Factor 88) Dependent Pathways

In an early immune response to an invading pathogen, recognition of an endogenic threat is mediated by TLRs. TLRs are proteins expressed on the transmembrane region of various immune and non-immune cells. TLRs could be detected on the cell surface and in the endosomal compartments (Fig. 1) [185]. They belong to a family of protein receptors (PRRs) that identify certain conserved patterns on the pathogen [48, 206]. PRRs have been classified into three families: (1) Endocytic receptors such as CD14, SR (scavenger receptors) and CLR (C-type lectin receptors), (2) Soluble PRRs such as ficolins, pentraxins and collectins, which mediate complement system activation by opsonization of ligands by phagocytes, and (3) TLRs and Nod-like receptors that are associated with the activation of inflammatory signaling. A total of 13 TLRs in humans and 10 in mouse have been discovered [185].

Upon escaping the first line of defense (skin/intestinal mucosa) the microbes are recognized by TLRs, which in turn initiate the immune responses. Many studies have reported that *S. aureus* cell wall components like peptidoglycan (PGN), lipoteichoic acid (LTA) and lipoproteins behave as potential PAMP (Pathogen-associated molecular patterns) motifs which in turn are recognized by host TLR2 [54, 82]. In Staphylococcal biofilms, the presence of PGN is observed in growing as well as in the dead bacteria, thus acting both as PAMP and DAMP (Damage-associated molecular patterns). As the bacteria are phagocytosed and digested, the presence of unmethylated CpG motifs in bacterial DNA are recognized by TLR9 [43].

Structurally, TLRs share three conserved features, namely, a transmembrane domain, LRR (leucine rich sensing) domain and Toll IL-1 receptor (TIR) cytoplasmic domain [24]. The TIR domains are a part of IL-1R family proteins that initiate intracellular signaling. One of the important adapter protein belonging to this family is myeloid differentiation factor 88 (MyD88). In the absence of TLR2 or TLR9 pathways, IL-1β aids in the containment of *S. aureus* biofilm. This is achieved

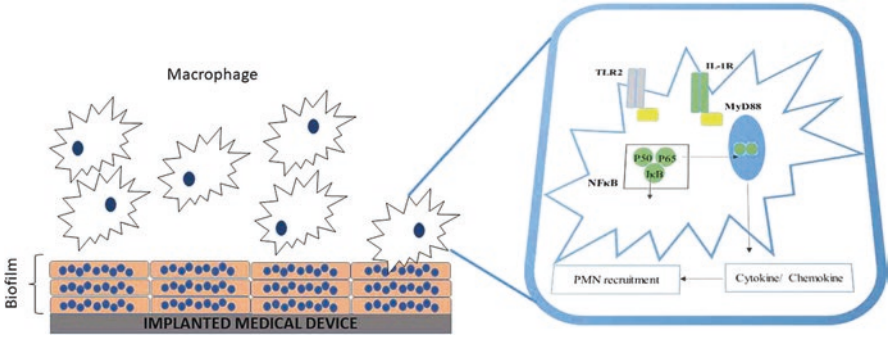

Fig. 1 Innate immune system: representation of innate immune system components involved with biofilm formation

through MyD88 [212]. Bacterial burden increases in MyD88 knockout mice as compared to WT animals. In addition to this, the *S. aureus* titers are elevated significantly in the visceral organs of MyD88 KO mice, indicating that MyD88s function in containing the bacterial load at the site of biofilm infection [80]. TLR signaling also in turn activates various transcription factors like NFκB and AP1, which eventually causes release of certain inflammatory cytokines and chemokines (Fig. 1) [213].

Innate Cellular Immunity

P. aeruginosa and *Staphylococcus epidermidis* are the two most extensively studied organisms for innate immune responses towards biofilms [21, 84]. White blood cells comprise around 40–75% of neutrophils and are a critical part of the immune system. They are short-lived, highly phagocytic and mobile, as they can enter remote regions of a tissue. Together with basophils and eosinophils, neutrophils constitute the polymorphonuclear cells family (PMNs). Neutrophils are recruited first at the site of infection where they can phagocytose the pathogen. Inside the phagosome, as neutrophils hide the microbe, the combined effort of reactive oxygen species (ROS), antimicrobial peptides and proteolytic enzymes leads to the degradation of the microbial target [62]. PMNs also secrete cytokines such as CXCL2, CXCL1 and CCL3 which help in initiating an inflammatory response (proinflammatory) [118, 135]. It has been reported that the rate of ROS production is reduced in PMNs when they phagocytose bacteria associated with the biofilm as compared to the planktonic bacteria [163]. Whereas, *S. aureus*/CA-MRSA possessing virulence factors like aureolysin, staphylococcal complement inhibitor etc. circumvent its killing and further sabotage the host immune system. This is achieved by compromising the viability, chemotaxis, phagocytosis, antimicrobial properties and inhibition of opsonisation [136]. The limitations with PMNs are that despite being an important antimicrobial effector molecule they have a short life span and low transcriptional capacity, so there is a huge requirement of PMNs into infection sites [58, 202]. The resident macrophages are ubiquitous and are found in all host tissues. Advantage of macrophages over PMNs is that they endure for a longer period and are critical in the recruitment and activation of other immune cells [192].

Biofilm formation leads to the infiltration of neutrophils and other immunocompetent cells. But as the infection persists due to inability of the cells to remove the bacteria, destructive inflammatory processes are observed. This is due to failed attempts of the infiltrating leukocytes, which leads to the release of bactericidal and cytotoxic components leading to extended tissue damage. The very same phenomenon is termed as “frustrated phagocytosis” and it leads to degradation of tissue and promotes a proinflammatory environment that facilitates more leukocytes migration to the site of inflammation [196]. The necrotic or lysed leukocytes are not cleared from the inflamed site and leads to the aggravation of the situation. Hence, the clearance of the dead leukocytes is absolutely necessary to limit an inflammatory process [180]. This indicates an interesting fact that in biofilm

infections, neutrophils might even promote biofilm formation, which again would aggravate the inflammatory response.

It was found that upon stimulation with IFN-γ/LPS, activation of macrophages differ qualitatively. M1 macrophages predominantly produce toxic nitric oxide (NO), whereas M2 macrophages are more focused towards the trophic polyamines synthesis [137]. The M1 stimuli direct the macrophage towards the release of prototypic inflammatory responses and markers, while the M2 group of stimuli directs towards an anti-prototypic inflammatory responses and markers [131]. Generally, in a typical response to bacterial infections, macrophages are polarised towards the activation of proinflammatory M1 genes. It is found that *S. aureus* biofilms can divert the host immune response from a Th1 background to a Th2 background, which favours the persistence of the bacteria, by affecting various enzyme activities of macrophage that help in M1 polarization [79].

Günther and colleagues demonstrated that macrophages display a limited ability to phagocytose *S. aureus* biofilms as compared to the PMNs [75]. In contrast, macrophages can phagocytose bacteria that are mechanically removed or are planktonic, indicating two possibilities. Either the large size of the biofilms compared to the macrophage restricts them to phagocytose or they are unable to opsonize the biofilm [212]. This suggests that biofilms are able to change the macrophage activation state and down-regulate their phagocytic potential.

Adaptive Immunity

Adaptive immune responses are recruited after the first line of defense (innate immune system) is breached during an infection. They are also involved with the formation of memory responses. The system is categorically divided into B cell (antibody mediated) and T cell (cell-mediated) immune responses. In *S. aureus*/MRSA infection, different antibodies against toxins, cell wall proteins and virulence factors are generated [85]. Nevertheless, these antibodies are inefficient in providing a proper immunity as it has been found that when used as a vaccine they elicit a restricted immune response in clinical trials [190]. Currently, vaccines are being targeted towards *S. aureus* virulence factors. For example, antibodies are targeted towards the *S. aureus* toxins, α-toxin and PVL, protein A, IsdB, coagulase and von Willebrand factor binding protein [100, 102]. The biggest challenge in vaccine development is the identification of an appropriate immunodominant antigen(s) which will be able to elicit a robust antimicrobial activity. Expecting an induction of an antibody mediated immunity, studies have focused towards the development of multivalent vaccines [25]. Till date, no vaccine (quadrivalent or monovalent) has been discovered that effectively resolves *S. aureus* biofilms in vivo. For all these reasons, biofilms are hard to get-rid of by the host unless being removed physically.

Mycobacterial Biofilms

Several species of pathogenic and non-pathogenic mycobacteria are known to form biofilms [11, 112, 166, 187, 211]. Mycobacteria sp., such as *Mycobacterium smegmatis*, *Mycobacterium fortuitum*, *Mycobacterium chelonea*, *Mycobacterium ulcerans* and *Mycobacterium abscessus* as well as slow-growing members of the *Mycobacterium avium intracellulare* complex are all known to form biofilms [33, 59, 161, 171]. Until recently the existence of biofilms in *Mycobacterium tuberculosis* was unclear. However, recent work by Ojha et al. demonstrated that under special laboratory conditions (use of Sauton's minimal medium and growth for 5 weeks in parafilm-sealed culture dishes), it was possible to induce biofilm formation in *M. tuberculosis* [110, 146]. Whether this experimental phenotype is relevant to the clinical physiology of *M. tuberculosis* remains to be understood, however, the presence of increased drug-tolerant persisters (upto 10%) in the *M. tuberculosis* biofilm suggests that such a physiology, if induced in the human host, could promote survival despite chemotherapy [146].

Key Structural Features of Mycobacterial Biofilms

Mycobacterial biofilms, just as any other microbial biofilms, help in pathogenesis, attachment to substratum and procurement of nutrients [5, 76, 174]. Like conventional bacterial biofilms, mycobacterial biofilms are also derived from planktonically-growing cells which get organized into early biofilm-like masses followed by development into a matrix-encapsulated fully-developed mature biofilm [171]. The biofilm matrix comprises of short chain mycolic acids (C_{56}–C_{68}) in *M. smegmatis* [171]. In *M. tuberculosis*, the biofilm matrix has been found to contain free methoxy- and alpha-mycolates as its major component and a minor amount of keto-mycolates [146]. A more unconventional biofilm structure was observed in *M. ulcerans* where the extracellular matrix was found to surround only the outermost layer of the cells rather than encasing the entire bacterial mass and it was also found to be vesicular in nature [130]. However, like conventional biofilms, this extracellular matrix imparted tolerance to concentrations of rifampicin up to ten-times the minimum inhibitory concentration [130].

Clinical Significance of Mycobacterial Biofilms

The clinical relevance of biofilms stems from their antibiotic-refractory nature and from the contribution of these structures to pathogenesis. In many mycobacterial species, the significance of biofilms is only now beginning to be better understood.

M. ulcerans is the etiologic agent of Buruli ulcer, an ulcerative skin disease that is widespread in tropical countries. It has been shown to colonize the salivary glands in aquatic insect, *Naucoris cimicoides* under laboratory conditions and it is thought that the transmission of *M. ulcerans* in its natural habitat occurs through the bite of the infected insect [129]. *M. ulcerans* establishes itself in the salivary glands of the insect vector and from there spreads to its raptorial legs where it encases the legs in a biofilm-like material. Interestingly, the establishment of *M. ulcerans* in the insect is dependent on the production of the polyketide mycolactone, a major constituent of the biofilm vesicles. Mycolactone is highly cytotoxic to phagocytic cells and is the only known virulence factor of *M. ulcerans* [130].

M. avium is an opportunistic pathogen which commonly causes lung infections in immuno-compromised patients. Disseminated *M. avium* infections can also occur. The primary source of *M. avium* exposure (as with many other non-tuberculous mycobacteria) is environment. The main reservoirs are water distribution systems (pipes, faucets, shower heads etc.) and hospital equipment such as catheters [63]. *M. avium* was shown to form biofilms on polyvinyl chloride catheters under laboratory conditions where the cells in the biofilm reached densities of up to 6×10^4 CFU/cm^2 in 4 weeks and were uninhibited by concentrations of clarithromycin and rifamycin which were sufficient to kill majority of planktonically growing *M. avium* cells [63].

M. abscessus is also an environmental mycobacterium which causes opportunistic pulmonary infections in patients with compromised lung function due to a previous pulmonary infection such as tuberculosis. It is naturally resistant to a variety of antibiotics and presents with a disease which is difficult to diagnose [115]. Confocal laser scanning microscopy confirmed the presence of *M. abscessus* biofilm in four patients with *M. abscessus* disease [162] providing the first evidence for in vivo formation of biofilm in *M. abscessus* infection. Subsequently scanning electron microscopy provided the first evidence for the presence of matrix-encapsulated bacteria in cavity walls in the lung of a patient suffering from chronic obstructive pulmonary disease. Further the bacterial burden in 0.5 gm of a lung cavity showed 7×10^5 CFU to be present in the biofilm alone [64].

Molecular Mechanisms Influencing Mycobacterial Biofilm Formation

The phenotype of biofilms has been found to be influenced by diverse genes and metabolites in mycobacteria, some of which are listed below:

Glycopeptidolipids (GPLs): The earliest indication of metabolites that regulate biofilm formation in mycobacteria was from studies that showed the requirement of GPLs in *M. smegmatis* biofilm formation [167]. GPLs constitute the outermost capsular layer of the *M. smegmatis* cell-membrane envelope and are associated

with smooth colony morphology, sliding motility and biofilm formation. Mutations in *tmptC* (a transporter) and *mps* (involved in GPL biosynthesis) genes abolished production of GPLs completely and in turn led to a complete loss of biofilm formation by *M. smegmatis* on polyvinyl chloride surface [168]. A partial biofilm phenotype was also seen where acetylation of GPLs was abolished by a transposon insertion in the *atf1* gene [167] lead to incomplete formation of the biofilm.

GroEL1: Recent work on *M. smegmatis* biofilms demonstrated the involvement of the chaperone protein, GroEL1 in formation of mature biofilms in laboratory cultures [8, 147]. The molecular basis for GroEL1-mediated regulation of biofilm formation was the association of GroEL1 and KasA. KasA is an enzyme that belongs to the fatty acid synthase II (FAS-II) machinery of mycolic acid biosynthesis. It has been suggested that the association of GroEL1 with KasA influences the change in the mycolate profile, leading to maturation of the biofilm. In the GroEL1-deficient strain, biofilm formation is initiated but does not reach maturity. The major effect of the mutation was seen on the chain length of the mycolates with a significant decrease in short-chain (C_{56}–C_{68}) mycolates obtained from the immature biofilms of the mutant but not the wild-type.

Lsr2: It is a DNA-binding protein which is a major regulator of metabolism in mycobacteria. Several studies have shown that disruption of *lsr2* function in *M. smegmatis* leads to abolition of biofilm formation. Although the molecular basis is not fully understood, it has been suggested that this phenotype is due to alteration of the lipid profile of *M. smegmatis* [17, 31, 35]. Further, the identification of two new species of mycolated diacylglycerols (MDAGs) have been suggested to be the molecular basis for the Lsr2-mediated control of biofilm formation in *M. smegmatis* [31].

Signal Transduction Events During Biofilm Formation

Intracellular Signaling

The bacterial cells form biofilms in response to certain stimuli from the environment. Surface proteins on bacterial cells react to these stimuli by passing along a signal in the form of protein modifications (post-translational modifications, PTMs). These modifications affect the protein activity that in turn affects biofilm formation. These intracellular signaling pathways can either induce biofilm formation or inhibit it, depending on the stimulus [126, 144, 228, 229]. In bacteria, there are two basic types of phosphorylation signaling modules- His/Asp phosphorylation (Two-Component System, TCS) and Ser/Thr/Tyr phosphorylation.

Two-Component Systems and Biofilms

The two-component signaling is mediated by sensor Histidine kinases, which receive a signal from the environment and phosphorylate its response regulator. The response regulator can further transmit the signal to appropriate substrate protein or may itself regulate the pathway leading to altered response by the bacteria [122, 159]. A large number of bacteria employ their TCS to sense the need for biofilm formation. In fact, some bacteria have dedicated multiple TCS for this purpose, which sense different environmental cues and activate/deactivate biofilm machinery.

E. coli: A number of TCS are involved in biofilm formation by affecting formation of organelles involved in bacterial motility, such as Flagella, Curli and other motor proteins. The most common example include the osmotic sensor EnvZ (sensor kinase)/OmpR (response regulator) TCS, which affect *E. coli* biofilm formation altering the motility by altered expression of flagellar, fimbria and curli proteins [156, 160, 182, 189]. In contrast to EnvZ/OmpR, the QseC/QseB TCS negatively affects biofilm formation through decreased formation of flagella and curli in pathogenic *E. coli* [107]. Additionally, other TCS in *E. coli* like RcsCDB [72, 179], CpxA/CpxR [51, 114] and BarA/UvrY [178] also affect biofilm formation through altering the bacterial motility and adhesion.

Staphylococcus sp.: In *S. aureus* and *S. epidermidis*, there are two TCS- SrrA/SrrB and SaeR/SaeS- that work exclusive of each other to govern the biofilm formation under hypoxic conditions during infections. The phenomenon called as fermentative biofilm formation is regulated through these TCS via altering the activities of AtlA (murein hydrolase) and FnBPA (fibronectin-binding protein A) [39, 121, 132, 133]. The LytS/LytR TCS was also shown to regulate biofilm in *S. aureus* and controlling the metabolic activity during that stage via lrgAB [116, 188]. *S. epidermidis* has the ability to form biofilms even on medicals devices. This makes it a critical pathogen and generates the need to treat the biofilms. *S. epidermis* requires ArlR/ArlS TCS for its biofilm formation via regulation of icaADBC [226].

Streptococcus sp.: In *Streptococcus* species, multiple TCS are involved in biofilm formation. *Streptococcus gordonii*, a commensal in human oral biofilms, requires inactivation of SdbA (a thiol-disulfide oxidoreductase) for biofilm formation. SdbA helps in disulphide bond formation in secreted proteins. In *sdbA* deletion mutant, there is enhanced biofilm formation, which is dependent on the TCS CiaRH that controls the Com system and production of competence stimulating peptide (CSP) [41]. *Streptococcus pyogenes* virulence is strongly dependent on TCS YvqE, which senses the environmental pH and regulates biofilm formation [89]. It also utilizes CovR/CovS TCS for biofilm formation as well as regulation of its pathogenesis [201]. *Streptococcus mutans* that forms dental plaque biofilm, employs the TCS VicK/VicR for biofilm formation through regulation of VicX, the third gene in the same operon [140, 183]. The VicKR TCS is also important for acid tolerance and oxidative stress [52, 199].

P. aeruginosa: It employs multiple TCS for biofilm formation, which is essential for its survival and development of antibiotic resistance during different stages of infection. Four kinases- RetS, GacS, LadS and PA1611- were reported to be involved in biofilm formation. Amongst these histidine kinases, LadS regulates TCS GacS/GacA which controls two small non-coding RNAs, RsmY and RsmZ, thus comprising a unique phosphorelay system important for biofilm [28, 141, 155]. GacS also mediates cross-talks with other TCS namely BfiSR, BfmSR, and MifSR and together these systems are involved in sensing different environmental cues leading to development of biofilm under stress conditions [154, 165, 207]. The CreBC TCS along with its inner membrane protein CreD determine the bacterial persistence and biofilm formation, specifically during antibiotic stress due to β-lactams [230]. The TCS PprA/PprB is responsible for an enhanced adhesion phenotype that is resistant to antibiotic treatment along with increased biofilm formation [42].

Other bacteria: In *Bacillus subtilis*, Spo0A is the key protein regulating biofilm formation. Its phosphorylation is mediated by multiple sensor histidine kinases named KinA-E, specifically KinC [47]. Another TCS DegS/DegU regulates biofilm formation and swarming motility through phosphorylation of DegU [105, 197]. In *Yersinia pseudotuberculosis*, which normally does not form biofilms, the strain with inactivating PhoP mutation forms robust biofilms. PhoP is a response regulator of PhoQ kinase and negatively regulates biofilm formation [203]. The TCS AdeRS in *Acinetobacter baumannii* is important for biofilm formation and adaptation to stress [44, 172]. AdeRS is also important for drug resistance via regulating the efflux pump AdeABC. QseBC is a TCS that is involved in regulation of flagella and biofilm formation in multiple bacteria, including *E. coli*, as discussed earlier. The pathogen *Francisella tularensis tularensis* that causes the Tularensis, also forms biofilms to manage its survival and tackle antibiotic resistance. *Fransicella* utilizes QseBC TCS for biofilm formation [138]. In non-typeable *Haemophilus influenzae* (NTHi), QseB/QseC TCS is involved in biofilm formation under static conditions [215]. Biofilm formation in *Salmonella enterica* serovar Typhi (S. Typhi) also requires the response regulator QseB, which is dependent on its sensor kinase QseC [94]. *S. Typhi* and *Typhimurium* also possess the TCS SsrA/SsrB which directly regulates CsgD, a major regulator of biofilm formation. SsrAB regulon is critical for switching the bacteria from virulent mode to dormant biofilm forming mode during infection [45]. Another pathogen *Vibrio cholerae* utilizes VxrA/VxrB TCS for its biofilm formation. Deletion of the response regulator VxrB leads to loss of biofilm forming ability of the bacterium while its overexpression decreases the mobility [210]. *V. cholerae* also employs another TCS CarS/CarR that confers antibiotic susceptibility and negatively regulates biofilm formation and polysaccharide production [22, 23].

In *Burkholderia dolosa*, which causes lung infections in CF patients, the TCS FixL/FixJ acts as a global regulator of virulence associated phenomena. FixL/FixJ senses oxygen availability and regulates not only the biofilm formation but also controls flagella motility and persistence [181]. *Helicobacter pylori* encodes a TCS ArsR/ArsS that responds to acidic environment within the stomach. ArsR/ArsS also regulate biofilm formation in *H. pylori* indicating its critical role [184]. Gram-negative

bacterium *Stenotrophomonas maltophilia* is infectious to immunosuppressed patients and represents major antibiotic resistant biofilm phenotype. It was found that biofilm formation in this bacterium is regulated by a TCSBfmA-BfmK, wherein BfmA regulates the transcription of *acoT* (acyl coenzyme A thioesterase) involved with biofilm [231]. The pathogen *Campylobacterjejuni*, that causes food-borne gastroenteritis, possesses an essential TCS CprR/CprS which can sense stress at surface, regulating the envelope and biofilm formation [205].

Ser/Thr/Tyr Phosphorylation and Biofilms

A number of bacteria have evolved Ser/Thr/Tyr phosphorylation system similar to eukaryotic signaling systems. Most of these bacterial kinases activate themselves by autophosphorylation, followed by substrate phosphorylation. These kinases possess Ser/Thr phosphorylation (Ser/Thr protein kinases, STPKs) and mediate autophosphorylation and phosphotransfer activities with serine or threonine as target residues [2, 15, 46, 53, 157]. Unlike TCS sensor kinases, STPKs can be either surface exposed or cytosolic and can target multiple substrates. For most of STPKs, the upstream signaling pathways are not known, although there have been several studies that show the downstream signaling events and target substrates have been identified [153, 158, 173, 186]. The deactivation of kinase phosphorylation is mediated by their cognate Ser/Thr phosphatases. The phosphatases can target multiple STPKs and their substrates, bringing them back to unphosphorylated state [73, 145, 175, 177]. Recent studies have also established the tyrosine phosphorylation in bacteria, principally mediated by the STPKs, bringing them to the rare category of dual-specificity kinases in bacteria [13, 29, 37, 113].

One of the most conserved STPKs in bacteria is PknB/Stk1 or its homologs. PknB is a surface exposed kinase with N-terminal Penicillin-binding-protein And Serine/Threonine kinase-Associated (PASTA) domains [20, 139, 152, 220]. PknB is associated with critical processes in bacterial development, cell division, adaptation and survival under stress. These processes are mediated by phosphorylation of several proteins that are PknB substrates and are involved in multiple pathways of the cells [6, 12, 30, 77, 78, 81, 117, 127, 150, 151, 175, 176, 195]. PknB has been involved in regulating growth, acid tolerance and biofilm formation in *S. mutans* [19, 87]. Carolacton, a secondary metabolite from myxobacteria, can inhibit biofilm formation in *S. mutans* and *S. pneumoniae* through inhibition of PknB [50, 169]. In *S. aureus*, PknB regulates biofilm formation through the essential glycolytic enzyme pyruvate kinase, targeting anaerobic conditions [217]. In *M. tuberculosis* however, PknJ phosphorylates pyruvate kinase [14, 193]. In another report, the catabolite control protein A (CcpA) was shown to be regulated by PknB, which helps in biofilm formation [117]. The inhibition of PknB by small molecule quinazoline compound, Inh2-B1 causes inhibition of biofilm formation in MRSA [99]. In *S. epidermidis*, an opportunistic pathogen, PknB-homolog Stk regulates stress response, particularly by biofilm formation and purine metabolism [120]. In the

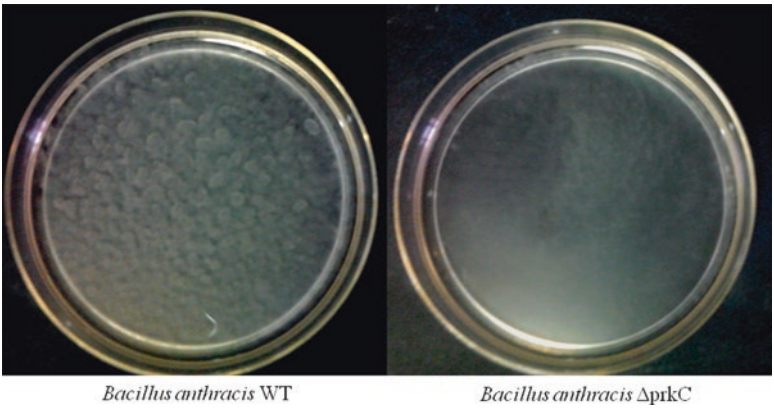

Fig. 2 *Bacillus anthracis* biofilm: *B. anthracis* (Sterne) biofilm in 3-day old static liquid culture. No biofilm was formed after deletion of STPK PrkC (Our unpublished data)

pathogenic bacterium *Bacillus anthracis*, PrkC is an infection specific kinase that phosphorylates multiple substrates like Ef-Tu, SodA2 and GroEL [12, 16]. Deletion of PrkC causes loss of biofilm formation, a process dependent on phosphorylation of chaperone GroEL (Fig. 2) [16]. Overexpression of GroEL in PrkC-deletion strain leads to restoration of biofilm forming ability partially. Previously, PrkC in *B. subtilis* was also shown to regulate bacterial development and biofilm formation [101, 123]. *B. anthracis* biofilms may alter phagocytosis in vivo and may have important implication in virulence [3, 61, 221].

Apart from PknB, there have been reports about other Ser/Thr/Tyr kinases (STPKs and BY kinases), which also regulate biofilm formation in several bacteria. The opportunistic pathogen *Burkholderia cenocepacia*, which can cause infections in CF patients, harbours tyrosine phosphorylation system with tyrosine kinases BCAM1331 and BceF being involved in biofilm formation and maturation [7, 65, 66]. In *B. subtilis*, tyrosine kinase EpsB regulates the biosynthesis of EPS [57, 69, 70]. EPS mediates a positive feedback via EpsB autophosphorylation, leading to activation of its own biosynthetic pathway. *M. tuberculosis* harbors 11 STPKs and 1 Ser/Thr phosphatase that are known to control a large number of processes. PknG, a critical STPK of *M. tuberculosis*, was shown to regulate biofilm formation during infection through alteration of redox sensing pathway [224]. PknF also regulates the colony morphology and biofilm formation in mycobacteria [71]. The BY-kinase Wzc regulates EPS production/export and capsule synthesis in bacteria such as *E. coli* [208, 209] and *Klebsiella pneumonia* [119].

Besides kinases, there a large number of protein phosphatases involved in regulation of biofilm formation [177, 223]. The PhpP of *S. pneumoniae*, which is co-transcribed with cognate kinase StkP, is involved in several important phenomena like cell division, adherence, and biofilm formation [1]. The dual-specificity phosphatase TpbA of *P. aeruginosa* regulates biofilm formation through controlling c-di-GMP and *pel* locus [214, 227]. In *Porphyromonas gingivalis*, the low-molecular weight tyrosine phosphatase negatively regulates EPS production [124].

Intercellular Signaling and Biofilms

Biofilm formation is a multistep process that requires both intra- and intercellular signaling in bacteria. For bacteria, adopting a social community lifestyle and having extracellular matrix is major step in forming a biofilm. To communicate with its community members and other eukaryotes, bacteria use a sensory method called quorum sensing [27, 164]. Quorum sensing is possibly the predominant and highly efficient signaling strategy that allows synchronization of gene expression and behavioral coordination ability [111, 191]. Quorum sensing relies on extracellular/secreted small molecular cues and specific sensory receptor pairs to probe cell density [60]. The best example of this regulatory mechanism is N-acylhomoserine lactones (AHLs) in Gram-negative bacteria [49]. Gram negative bacteria produce many AHL like "Autoinducers" while the Gram-positive bacteria generate peptide signals and many species sense a common signal viz. auto-inducer 2 (AI-2). In bacteria such as *Vibrio fischerii*, AHL is recognized by LuxR receptor and interspecies members sometimes share these sensing strategies. Such co-operative behavior provides survival advantage in difficult environments [60]. QS mediating molecules like AI-2 are important for interspecies communication and they have been recently shown to be important in inter-kingdom communication [90]. In response to bacteria, epithelial cells produce AI-2 mimic which is recognized by bacterial receptor and can activate QS pathway [90]. In animal gastrointestinal tract, host and bacteria live together and maintain a complex association in mutually beneficial manner [204]. Host response to bacterial QS was previously discussed in detail however the discovery of epithelial cell releasing AI-2 mimic indicates that QS response in vivo is influenced by both bacteria and host cell signaling [83, 109, 204].

Regulation of quorum sensing system is pretty efficient and comes at multiple levels. The most basic and well understood QS system consists of two main proteins LuxI and LuxR, which were first discovered in *Vibrio* sp. [55, 68, 143]. LuxI synthesizes autoinducer which is recognized by LuxR, consequently leading to regulation of gene expression [93]. The QS system often uses multiple signaling molecules that are recognized by many specific sensory protein regulators. Despite producing specific signals and regulatory proteins, both Gram-positive and Gram-negative bacteria show cross-talk by sensing/processing a molecule produced by different bacterium [56, 194]. Interestingly, to interfere with QS signals, microbe and eukaryotes often use QS inhibiting molecules named quorum quenchers [96, 97, 108]. Quorum quenchers hydrolyze quorum sensing molecules. They interfere with biofilm formation and bacterial virulence, thus possess immense potential as antibacterials [40, 98, 106, 108]. To gain more understanding on quorum sensing signaling molecules recently a new resource SigMol has been created that provides chemical information of signaling molecules as well as about related genes, assays and applications [164].

In *S. aureus*, the AI-2 synthase enzyme LuxS is shown to be phosphorylated by STPK Stk1 [34]. Interestingly, in LuxS mutant of *Streptococcus sanguinis*

S-adenosylhomocysteine hydrolase (*SahH)* overexpression and not AI-2 supplementation restores the biofilm formation indicating role of activated methionine cycle [170]. SahH reversibly hydrolyze S-adenosylhomocysteine (SAH), producing homocysteine and adenosine; and thereby restores the activated methionine cycle [195]. In *M. tuberculosis* SahH activity is further shown to be regulated by Stk1 homolog PknB indicating conserved role of protein kinases [36, 195].

The biofilm signaling also uses other molecules such as cyclic-Di-GMP (c-di-GMP) and indole [104, 216]. The second messenger, c-di-GMP acts as a connection between motile planktonic and sedentary biofilm forms. Another secondary messenger cAMP has negative influence on biofilm formation possibly due to inhibition of cell attachment [88, 148]. In one study, another second messenger zinc is shown to inhibit the biofilm formation in presence of poly-N-acetylglucosamine (PGA) in *Actinobacillus pleuropneumoniae*. However, in another study on *S. pneumoniae*, zinc enhances biofilm formation, indicating that zinc mediated regulation may be concentration and growth stage dependent [26, 225]. Zinc's role in biofilm formation is also shown by other studies on *P. aeruginosa*, *B. subtilis*, *Xylella fastidiosa*, *S. enteric Typhimurium***,** microlagal biofilms and anaerobic Gram-negative oral bacteria [4, 74, 128, 134, 142, 198]. The dual biofilm promoting activity of zinc is described in *S. aureus* by activating cell-cell adhesion. Zinc controls dynamics of cell wall and promotes stiffening and smoothening of*S. aureus* surface. Cell association during biofilm formation is influenced by Zinc dependent regulation of *Staphylococcus aureus* surface protein (SasG) [67].

Conclusions

Biofilms helped in our understanding of cell-cell communication in bacteria. Signaling networks such as QS have been studied till now as individual signals and relatively less is known about multiple signals being generated by bacteria and integrated by eukaryotic host in environments during different conditions. Several bacterial species coexist and bacteria may possibly sense multiple signaling molecules simultaneously both in environment and in host [93]. In simplistic example, communication by QS systems can be like neighbors sharing Wi-Fi passwords where passwords are shared by some neighbors but not others. However, in case of QS systems, exchange of signals between different partners will not restrict to bacteria but may involve host cells. It will be challenge to answer how bacteria co-ordinate different signaling machinery and produce optimum response, Further a mathematical model that consider such signaling response will be useful in development of antibacterial therapeutics and new applications such as building synthetic gene modules and networks. The use of synthetic biology approaches and genomics in microbiology is key to such pursuits [9, 10]..

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Quorum Sensing in Bacterial Pathogenesis and Virulence

Parasuraman Paramanantham, Subhaswaraj Pattnaik, and Siddhardha Busi

Abstract Quorum sensing (QS) is a widespread and highly conserved cell-to-cell communication that coordinates the social instincts of bacteria and also regulates the gene expression in an efficient manner. The QS phenomenon relies upon the production and detection of highly specific chemical signaling molecules popularly known as autoinducers. The diversity of autoinducer molecules produced by bacteria enables the bacteria to communicate within the species level as well as in between the genus level when present beyond a particular threshold level of autoinducer in the environment. Gram-positive and Gram-negative bacteria specifically synthesize different kinds of autoinducers to control the expression of different genes. Pathogenic bacterial population exploit the high throughput signaling phenomenon to regulate genes for the process of invasion, defense and more importantly to generate virulence determinants such as toxins, enzymes and ultimately leads to the formation of biofilm. Biofilm formation has been well documented in most of the pathogenic bacteria utilizing the highly synchronized QS phenomena that will give the bacterial community an impinge to confer resistance against wide array of antibiotics and other antimicrobial drugs in an efficient manner thereby possess serious threat to immunocompromised individuals. The emergence of multi drug resistant pathogens and their ability to form biofilm, infers a current need for development of alternative therapeutic strategies towards the bacterial quorum sensing phenomenon.

Keywords Quorum sensing · Biofilm · Multidrug resistance · Virulence · Pathogenicity

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Introduction

The world is facing a significant issue in antimicrobial therapeutic systems, where majority of the conventional antibiotics have lost their efficacy in treating various life-threatening diseases especially caused by bacterial pathogens. Meanwhile, as the world's population continuously increasing, there is concomitant increase in the indiscriminate use of antibiotics to treat bacterial infections and thereby bacterial pathogens have outperformed our scientific abilities to control them and associated health consequences [27]. The tendency of newly developed drugs though counteract emerging antibiotic resistance phenomenon, their short life span and inadequate effectiveness against some multidrug resistant (MDR) bacterial strains leads to severe public health issues. Besides, the indiscriminate use of antibiotics also significantly contributes to the increasing resistance shown by bacterial pathogens by exerting a continuous selective pressure [21]. Apart from that, the development of new and effective drug leads has dramatically come down over the past few years making the scenario even more complicated. In addition, the newer drugs that have been successfully developed are strictly targeted to treat only the most serious infections leaving behind other serious health issues unexplored [4]. The increased prevalence of acute and chronic bacterial infection and the concomitant inefficiency of the readily available antibiotics lead to the development of multi-drug resistance phenomenon. In this context, the development of next generation antimicrobials is targeted towards down-regulation of bacterial virulence and attenuation of pathogenic determinants production instead of targeting bacterial killing, where the probability for developing resistance can be decreased. However targeting all virulence mechanisms associated with a pathogen is not always feasible. For this reason, the next generation antimicrobial strategies are specifically targeting the specific pathways involved in the production of an array of virulence determinants [34]. The bacterial virulence and pathogenicity profile could be attributed to the highly complex, species-specific, widely conserved cell-to-cell communication mechanism termed as quorum sensing that coordinates various bacterial community activities including production of virulence phenotypes and biofilm formation [28].

A Brief Description on Quorum Sensing

Quorum sensing (QS) is a strictly density dependent bacterial cell-to-cell communication phenomenon, which involves the production of extracellular signaling molecules called autoinducers (AIs), its detection by specific cognate receptors followed by physiological response in the production of virulence factors. The autoinducers are then exchanged with the surrounding environment as per the requirement by the host bacteria and the cell density [12]. The accumulation of the signal molecules is directly proportionate with the increase in bacterial population, and when the population density exceeds a "quorate" threshold, it can induce a series of synchronized

processes resulting in expression of an array of virulent genes and biofilm formation. These autoinducers trigger the QS process by binding to specific cognate receptors, which in turn regulate the transcription of a number of genes that are involved in the cell-density-dependent behaviours such as bioluminescence, sporulation, genetic competence, conjugation, motility, antibiotic production, virulence factor secretion and biofilm formation [5, 31, 43, 56]. The underlying functions controlled by highly complex QS systems are species-specific and generally accounts for a particular species of bacteria inhabiting a given dimensional niche [36]. The production and uptake of signaling molecules during quorum sensing behaviour have two consequences such as autoinduction, which reflects the production of virulence factors. The second consequence is the process of autoregulation where uptake of signaling molecules results in concomitant increase in the production of AIs leading to a positive feedback loop and results in the production of more signalling molecules [67].

During the process of bacterial infections, bacteria have the inherent ability to communicate and behave socially like that of multicellular organisms. This particular socialistic behaviour aids benefits during host colonization process, formation of biofilms, defense against host immune system, and adaptation to changing harsh environmental conditions. Besides, quorum sensing also enables the bacteria to show immense cooperative behavior by virtue of complex coordination of the activities of individual cells. The QS system is an attractive target for antimicrobial therapy in the post antibiotic era [11, 70]. Quorum sensing is a regulatory mechanism used by many Gram negative and Gram positive bacteria to detect and respond to specific AIs which contribute an increase in microbial population density and the expression of specific virulent genes followed by biofilm formation, and maturation [13].

Brief History of QS

Quorum sensing enables the bacteria to adapt to different environmental conditions such as pH, osmolarity, population density and nutrition availability which are essential to establish pathogenesis. The presence of this chemically complex quorum sensing phenomenon was originally discovered in the marine luminescent bacteria, *Vibrio fisheri* and *Vibrio harveyi*. Quorum sensing in *V. fisheri* accounts for bioluminescence and other important traits governed by quorum sensing genes *luxI* and *luxR* [3, 60, 61]. The luciferase operon in *V. fischeri* is regulated by two specific proteins, LuxI and LuxR, responsible for the production of the autoinducer and increased transcription of the luciferase operon respectively [54]. However, in recent years extensive studies on quorum sensing has been focused towards the highly opportunistic and nosocomial pathogen, *Pseudomonas aeruginosa*; which is responsible for the majority of hospital acquired persistent bacterial infections and lungs associated diseases. Besides, the ability to form biofilm enables *P. aeruginosa* to thrive under different environmental conditions and shows significant resistance

to the readily available antibiotics and conventional antimicrobial therapies [65]. The QS network of *P. aeruginosa* is highly organized into a multi-layered hierarchy consisting of four interconnected signaling systems such as LasI/R, RhlI/R, PQS (quinolone based intercellular signalling) and IQS (integrated quorum sensing). The LasI/R system shows significant homology with *V. fischeri* prototype LuxI/R system whereas the RhlI/R system allows the bacteria to form biofilm and consequently resistant to known antibiotics. The quinolone based signalling system in *P. aeruginosa* accounts for the interconnection between the Las and Rhl system. The 4th quorum sensing sytem, IQS shows high potential in coordinating and integrating environmental stress [29]. *Aeromonas hydrophila*, the causative organism for majority of gastrointestinal and extraintestinal infections in humans also showed QS controlled socialistic behaviour regulated by two genes such as *ahyI* and *ahyR* which are homologues to *V. fisheri* quorum sensing genes *luxI* and *luxR* respectively [10].

The cascade of events during the QS phenomenon are often integrated with each other and regulated within global regulatory networks with respect to the environmental conditions. QS signal molecules, although largely considered as effectors of QS-dependent gene expression are also possesses significant impact in host–pathogen interaction [69].

Architecture of QS

During the QS process, QS signaling molecules proved to be key components. These signaling molecules are broadly divided into three groups such as *N*-acylhomoserine lactones (AHL) used as signaling molecules by proteobacteria in LuxI/LuxR-type QS system and their derivatives (AHLs, auto-inducer-1) that exist in Gram negative bacteria, autoinducer 2 (AI-2) acts as signalling molecules encoded by luxS system that exist in both Gram-positive and Gram-negative bacteria and larger oligopeptide-two-component-type QS signalling molecules observed only in Gram-positive bacteria. The third group of signaling molecules are the larger quorum-sensing oligopeptides observed especially in Gram-positive organisms [41, 72]. In Gram-negative bacteria, AHLs represent the most intensively studied family of signal molecules, which could diffuse across the bacterial cellular envelope and activate the species-specific transcriptional regulator once a threshold concentration has been reached [71]. Quorum sensing in bacteria accounts for the identification of the chemical signalling molecules (autoinducers), their respective receptors, specific target genes and their products, and the mechanism of signal transduction. The quorum sensing architectural network is basically of two types such as parallel circuits and QS circuits arranged in series. The *V. fischeri* quorum sensing network is a prototype of parallel quorum sensing circuit, which allow the entire QS network to activates or represses gene expression only when all signals are simultaneously present or absent respectively. Meanwhile, *P. aeruginosa* QS system represents the QS circuits arranged in series, which infers a series of ordered sequence of

activation and repression of genes [66]. In addition, quorum sensing architecture in *Bacillus subtilis* relies upon the multiple signals which act antagonistically instead of acting synergistically.

Intra and Inter Species QS

Quorum sensing phenomenon has the rich lineage among the diversified bacterial taxa and can occur either within a single bacterial species or among diverse species. In quorum sensing, bacteria use a wide range of molecules called AIs or pheromones for cell-cell signaling followed by production of an array of virulence factors [25]. The production, sensing and function of QS signal molecules within a single bacterial species and their role in bacterial virulence gained a considerable research interest. However, QS between different bacterial species remain under explored. For example, with respect to production of AHLs is concerned in the Gram negative bacteria, *P. aeruginosa*, *Serratia liquefaciens* and *Aeromonas hydrophila* produce N-butanoyl homoserine lactone (C4-HSL) while *Chromobacterium Violaceum* respond to even short-chain AHLs [70]. The interspecies cell-to-cell communication system may either act synergistically or act antagonistically. For example, swarming motility in *S. liquefaciens* is greatly controlled by the production of halogenated furanones and enones by seaweed which are closely related to *S. liquefaciens* AHL autoinducers thereby altering the swarming motility and making *S. liquefaciens* avirulent [35]. During quorum sensing hierarchial system, bacteria use autoinducer molecules to communicate both at intra-species and interspecies levels. There are basically two types of autoinducers such as autoinducer-1 (AI-1) system which correlates the intra-species communication phenomenon and luxS-mediated autoinducer-2 (AI-2) which addresses the inter-species signalling mechanism with special reference to bacterial virulence and pathogenicity. In addition, the AI-2 system also enables the pathogenic bacteria for colonization and infection to the host [48]. Regardless of the type of autoinducer signal used during quorum sensing behaviour, intra-species quorum sensing aids in coordinated regulation of the bacterial behaviour in a concerted manner. Likewise, regardless of the type of AI-2 used, inter-species bacterial communication aims to synchronize the specialized functions of each species in the group thereby enhancing the survival of the entire community [58]. During the inter-species bacterial communication, AI-2 is not only produced by variety of bacteria in the community but also detected by specific receptors present in many or possibly all of the bacteria present in the community. The AI-2 based inter-species signalling system greatly influence an array of niche-specific behaviours such as virulence in *Escherichia coli*, *Streptococcus pneumoniae*, *Staphylococcus pyogenes*, and *V. cholerae*; motility in *Campylobacter jejuni*; biofilm formation in *Salmonella typhi*, *S. mutans* and *Porphyromonas gingivalis*; toxins production in *Clostridium perfringens*; and bacteremic infection in *Neisseria meningitides* [15].

Virulence Profiles of QS System

Quorum sensing addresses the cell-to-cell communication of bacterial cells where the cells communicate by releasing, sensing and responding to the signal molecules and thereby regulate the highly complex cooperative social behavior and different physiological processes [38]. Quorum sensing in bacteria not only enables bacteria to show social behavior but also infers pathogenicity and influence expression of virulence by producing an array of virulence factors. The production of these virulence factors are associated with the enhanced properties of resistance to antibiotics and increased genetic competence. Among the Gram positive bacteria, in pathogens such as *Staphylococcus aureus*, *Listeria monocystogenes*, *Enterococcus faecalis*, *Clostridium perferingens*, quorum sensing network is mainly responsible for expression of virulence factors such as adhesion molecules, hemolysins, toxins, phospholipases and proteases [56]. Similarly, in Gram negative bacteria such as *P. aeruginosa*, *V. cholerae*; production of virulence factors such as pyocyanin, proteases, rhamnolipids, extracellular polysaccharides (EPS), toxins and alginates are regulated by highly complex quorum sensing system. The bacterial motility such as swimming, swarming and twitching plays a crucial role in biofilm formation which concomitantly increases the resistance to conventional antibiotics [39].

QS Mediated Virulence Factors in Bacteria

The microbial community in the most of the environment are exist either single or multiple species on abiotic or biotic surfaces. It is a known microbial behavior that they able to sense the local environment and capable of adapt to the condition by regulating their expression of specific genes. This kind of adaptation is often very common in pathogenic bacteria; they harbor on host and encounter regular metabolisms of host, also called as infection. Interestingly, bacteria in-build with highly sophisticated surveillance mechanisms to sense, process and transduce environmental information such as pH, temperature, osmolality, nutrient availability and cell population density [64]. Other than mentioned mechanism bacteria own several other signal transduction mechanisms, which coordinate various virulent traits of the bacteria such as colonization, production of toxin and tissue-degrading enzymes. Likewise, QS is a transduction mechanism practiced by most of the bacteria to coordinate their local bacterial community towards infection. The QS system architectures start drawn with small, secreted signaling molecules as called as autoinducers (AIs). From the background knowledge of QS related studies most of the AIs are belongs to one of the categories: acylated homoserine lactones (AHL) also referred as AI-1 used by Gram-negative bacteria; peptide signals are employed by Gram-positive bacteria; and autoinducer-2 (AI-2), used by both Gram-positive and Gram-negative bacteria. In addition, the existence of several other QS signaling molecules such as *Pseudomonas* quinolone signal (PQS), diffusible signal factor (DSF) and

autoinducer-3 (AI-3) are also reported in many bacterial pathogens which needs to addressed in a proper fashion [27]. In both bacterial classes, pathogens are using signaling molecules to regulate the virulence. QS system in the bacteria gets activate only once the bacterial population reach certain density, this phenomenon even facilitate the bacteria to overwhelmed from host innate immunity. Moreover, this process leads to the formation of biofilm that assist pathogenesis and resistance to antimicrobial treatments. Even though different QS mechanism were studies, the well characterized QS system are, AHLs and signaling peptides producing Gram-negative bacteria and Gram- positive bacteria respectively [9]. The following session will summarize the QS system in Gram-positive bacteria and Gram-negative bacteria with certain examples.

Peptide Based QS

Peptide based QS mechanisms was observed in Gram-positive bacteria which undergoes common principle of all QS circuits such as production, detection and response to the signaling molecules [42]. Oligopeptides (APIs) are the signaling molecule in most of the Gram-positive bacteria, which are comprehended by membrane-bound two-component signal transduction system [23]. The Oligopeptides are encoded as precursors (pro-APIs) and are widely diverse in sequence and structure. In a bacterial system, it is a common phenomenon that cell membrane is impermeable to peptides, sophisticated transport mechanism is required to secrete the signaling peptides. The mentioned transport mechanism of Gram-positive bacteria also involve in the processing of Pro-APIs and delivers with size range in 5–17 amino acid either in liner or circular structure [56]. The two-component system is basically consists of a membrane-bound histidine kinase receptor and a cognate cytoplasmic response regulator. Once the oligopiptide binds with its cognate membrane-bound histidine kinase receptor, it initiates a series of autophosphorylation events. Once the histidine kinase receptor autophosphorylate at conserved histidine residue, the phosphoryl group is then transfer to conserved aspartate residue of cognate response regulator. This phosphorylated response events coordinates the expression of QS regulon. Interestingly, each Gram-positive bacterium synthesize signaling peptides which are different from that synthesized by other Gram-positive bacteria and the cognate receptors are highly specific to the structure of signaling molecules. These behaviors of Gram-positive bacteria enlighten that QS circuits confers intra-species communication [66].

QS Circuit in *S. aureus*

Staphylococcus aureus is a fascinating example, which utilizes peptide based QS system. It is a Gram-positive bacterium acts as a causative agent for numerous acute and chronic infections. Usually it persists in the human as commensal organisms but

it turns into deadly pathogen once it harbors into host tissues. The pathogenicity of *S. aureus* is normally biphasic strategy where at a lower cell density, the bacteria involves in the process like attachment and colonization by expressing specific protein for the attachment. In contrast, at higher cell density, bacteria involves in the repression of mentioned genes expression and initiate the expression of genes responsible for the secretion of toxins, lytic enzymes and production of other virulence factors production that are presumably required for the development of pathogenicity in the bacteria. This switch in gene expression and repression circuits is coordinated by Agr QS system in *S. aureus* [66]. The mentioned Agr QS system is monitored by the extracellular concentration of a post translationally modified peptide, which is synthesized and secreted by bacteria. Normally the peptides contain seven to nine amino acids in length with the C-terminal five amino acids constrained as a cyclic thiolactone through a cysteine side-chain. When the concentration of peptides attains critical concentration, trigger the QS circuit and initiate the expression of biosynthesis pathway of their own, hence it is also called as auto-inducing peptide (API) [26].

The molecular investigations on Agr QS system emphasize that the chromosomal region contain two different transcripts such as RNAII and RNAIII. The RNAII transcript consist of four set of genes cascade namely Agr*BDCA*, which are involving in the encoding process to express the factors that required for the production of API and activate the regulator circuits. In the Agr Qs system, the mention genes Agr*BDCA* are allotted with specific work, which facilitate the successful run of Agr QS circuit. In which AgrD is acting as a precursor peptide of API, AgrB acts as a membrane endopeptidase involved in generating API, AgrC is a histidine kinase which gets activated by binding to API and AgrA is responsible for the transcriptional regulation of both transcripts RNAII and RNAIII. In case of RNAIII transcript synthesis, the regulatory RNA molecules acts as primary effector of the Agr QS circuit by upregulating extracellular virulence traits and downregulating cell surface proteins. The RNAIII transcript also involves in the production of δ-haemolysin, a small amphipatic peptide with surfactant property and aids in the biofilm development [26]. The other interesting aspect of the *S. aureus* QS circuit is cross-competition among AIP specificity. Due to the hypervariable behaviors of *agrD* and *agrB* genes, the four different types of *S. aureus* AIPs produced depending on the strains (Fig. 1). The corresponding hypervariability exists in the portion of the agrC gene encoding the sensing domain of the AIP receptor. Hence, a biosynthesized cognate AgrC sensor detects specific AIP, binding of noncognate AIP results in inhibition of QS.

QS Circuit in *B. subtilis* and *B. cereus*

The *Bacillus* sps. are group of Gram-positive bacteria consists of numerous human pathogens including *B. subtilis, B. cereus* and etc. *Bacillus* sps. are involved in the intestinal and nonintestinal infections in human. They are readily cause acute diarrheal disease due the secretion of hemolysins, phospholipases and toxins. QS in *B.*

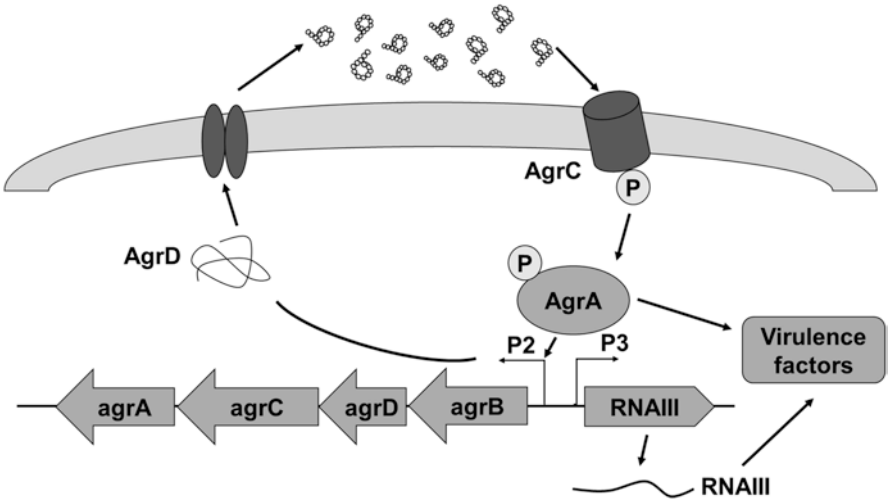

Fig. 1 QS circuit of S. aureus. The precursor *agrD* synthesized the autoinduced peptides. AgrB processes precursor to mature AIP and transport outside of the cell. AIP was detected by QS system of *S. aureus.* The membrane bounded histidine kinase, AgrC and AgrA is the response regulators. The phosphorylated AgrA activates the P2 and P3 promoters inconding the *agr* operon and RNAIII respectively. Activation of RNAIII synthesis the virulence factors

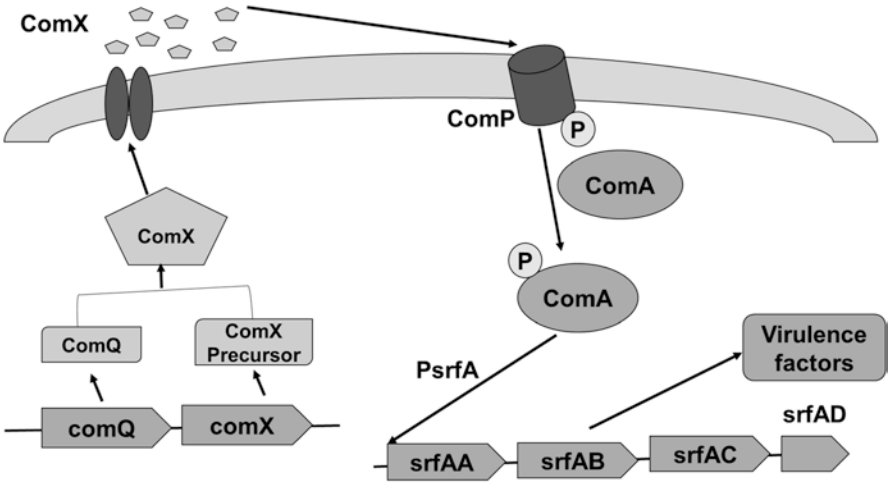

Fig. 2 QS circuit of *B. subtilis.* ComX synthesis the signaling peptide. ComP detects the signaling peptides. The transcriptional factor ComA is activated through phosphorylation. The activated ComA regulates the *srfA* cascade, which produce most of the virulence factors

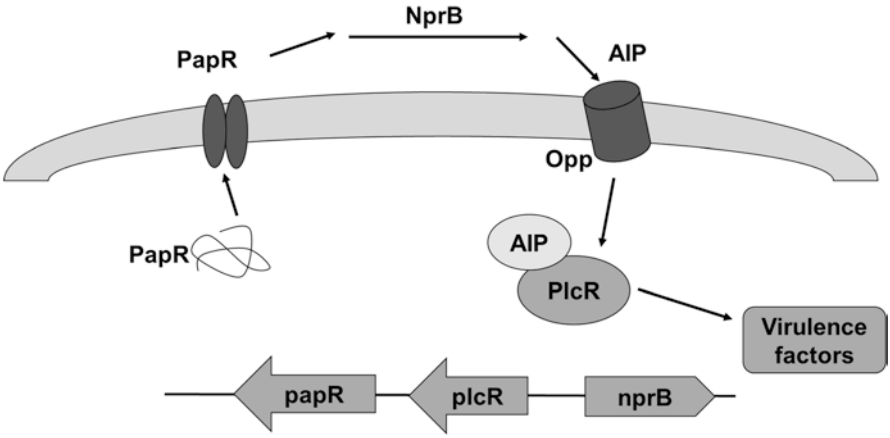

Fig. 3 QS circuit of *B. cereus.* PapR secretes precursor signaling peptides. The pro-AIP were processed and changed to matured heptapeptide by extracellular protease NprB. The mature AIP were detected by Opp receptor and transferred into the cell. The internalized AIP were bind with transcriptional factor PlcR. The activation PlcR regulates the production virulence factors

subtilis is an typical QS circuit of Gram-positive bacterium which regulate the expression of fairly 200 genes, including both extracellular and intracellular virulence factors (Fig. 2). *B. subtilis* employs four protein QS system namely ComQXPA circuit where the ComQ act as isoprenyl transferase, the ComX act as signaling peptides, the ComP as histidine kinase and ComX helps to initial synthesis of 55-residue propeptide and then post modified by the isoprenyl transferase ComQ. Once the ComX isoprenylated they secreted out of the membrane and upon reaching certain concentration, it activates autophophorylation of the membrane-bound ComP then it is further phosphorylated the ComA which act as transcriptional activator. After the activation of ComA, it regulates numerous genes expression involved in the production of virulence factors including the *srfA* operon required for the non-ribosomal synthesis of the major lipopeptides, antibiotics and surfactin [45].

In *B. cereus,* QS circuit needs the transcription factor PlcR that regulate expression of most *B. cereus* virulence factors and binds to the intracellular AIP derived from the PapR protein (Fig. 3). PapR is 48 amino acids long and once outside the bacterial cell, the PapR pro-AIP processed by the secreted neutral protease B (NprB) to deliver the active AIP. The gene, *nprB* regulates the divergently from *plcR* and *nprB* expression is activated by AIP-bound PlcR. The pro-AIP PapR was sliced into 5, 7, 8 and 11 amino acids by the action of NprB. It has been reported that only the pentapetide and heptapeptide activate PlcR activity. When AIP binds to the transcription factor PlcR and causes conformational changes in the DNA-binding domain of PlcR, which promotes PlcR oligomeriation. Once PlcR binds with PapR AIP and oligomerizes, it binds to "PlcR boxes" that regulates the production of major virulence traits including enterotoxin, hemolysis, phospholipases and protease [56].

AHL Based QS

AHL based QS system is most common type of QS circuit in the Gram-negative bacteria. This circuit is working with certain element, small diffusible signaling molecules namely AHLs, which coordinate among bacterial community to achieve better bacterial communication. The family of LuxI homologues protein synthesizes these signaling molecules, where they exploit the charged acyl carrier protein as the major contributor of acyl chain donor and S-adenosyl methionine forms the homoserine lactone moiety. The mechanisms of AHL based QS system states that LuxI protein are continuously expressed at low cell density that result with accumulation of AHL in proportion by increasing in the cell density. When the accumulation of AHL molecules reaches certain concentration also called as threshold concentration, binds to specific receptor molecules. The complex of receptor and AHL turns on several transcriptional regulator on target genes of the QS circuit [53]. The activated LuxR then binds to a specific palindromic sequence "Lux box" which upregulate the QS regulated genes. Once LuxR bounds to the lux box, arranges the RNA polymerase, resulting in enhanced production of signaling molecules and other proteins involving in the pathogenicity process. Other interesting feature of the AHL based QS system is activation QS circuit resulting in the increased expression of signaling molecules synthesizing factors, which facilitate the production of large amount of signaling molecules. These signaling molecules are acting as a positive feedback loop and significantly enhance QS mediated virulence factors. Similar kind of QS circuits were involving large number of Gram-negative bacteria, including *Pseudomonas* spp., *Escherichia coli*, *Vibrio* spp. [20].

QS Network in *Pseudomonas* spp

Pseudomonas spp. especially *P. aeruginosa* is an opportunistic Gram-negative pathogenic bacteria that involves in the numerous infections in particular chronic lung infection. The infection by *P. aeruginosa* occur with the series of events finally it leads to the establishment of a persistent infection due to the ability to develop biofilm and contributing several virulence factors such as elastase, lipase, rhamnolipid, alginate and other virulence traits. As mentioned production of most of the virulence traits of *P. aeruginosa* was coordinated by a cell density depended QS mechanisms. *P. aeruginosa* is well studied bacterium about it QS mechanisms and mediated pathogenic processes. It use at least three different QS pathways, among two are following AHL based QS network by using signaling molecules like N-(3-oxododecanoyl)-L-homoserine lactone which will binds with LasR receptor and N-butanoyl-L-homoserine binds with RhlR receptor (Fig. 4). These two AHL based QS system are interlinked with a third QS system and involves in the production third signaling molecule called as *Pseudomonas* quinolone signal (PQS) [17]. Normally, AHL based QS network is comprised of a LasI and LasR genes where LasI genes involved in the AHL synthesis process and LasR genes encodes for the

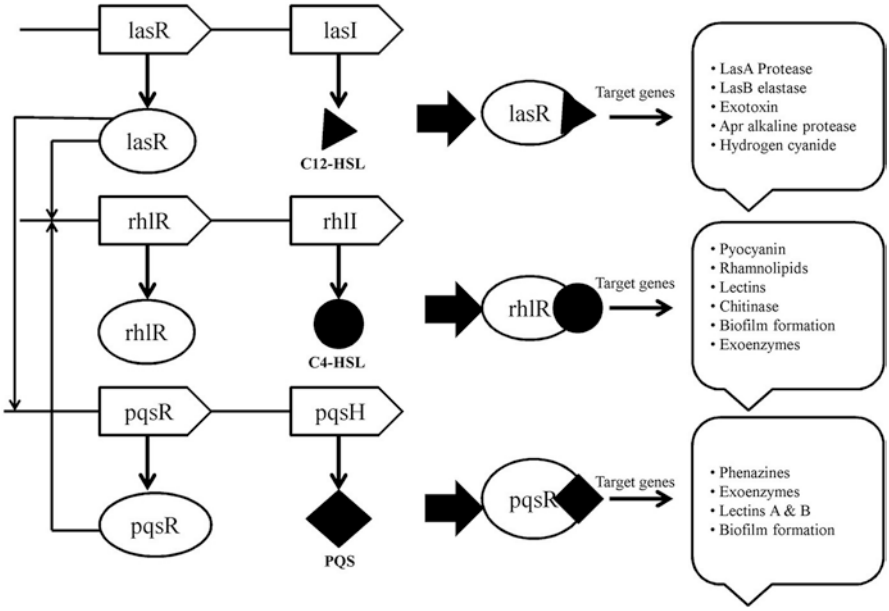

Fig. 4 Schematic representation of interconnecting quorum sensing circuit in *Pseudomonas aeruginosa*. *P. aeruginosa* uses LasI/LasR-RhlI/RhlR quorum sensing system autoinducer-sensor pairs for quorum sensing regulation of a variety of genes. The LasI protein produces the *N*-(3-oxododecanoyl)-homoserine lactones (C12-HSL), and the RhlI protein synthesizes *N*-(butryl) homoserine lactone (C4-HSL). In addition, a third regulatory link between the Las and Rhl circuits identified as quinolone signaling system, expressing 2-heptyl-3-hydroxy-4-quinolone (PQS) in presence of LasR, and PQS in turn induces transcription of *rhlI*

receptors. During the growth of the *P. aeruginosa,* the bacterial system synthesizes specific signaling molecules by LasI synthase gene. The produced signaling molecules are binds to the receptor protein, which is encoded by LasR gene and activates the AHL-responsive transcriptional activators [8]. Interestingly LasR forms multimers of protein, which is able to binds with DNA and regulates the transcription of multiple genes. The second QS network of *P. aeruginosa* framed using RhlI and RhlR proteins. The RhlI synthase produces the C_4-HSL and RhlR is the transcriptional regulator, only when there is a complex of signaling molecules and the RhlR regulate the expression of gene. Both the signaling molecules of *P. aeruginosa* were shows to be free diffusible out of bacterial cells. The third LuxR homologue termed QscR that can able to regulate the transcription of both LasI and RhlI. Still the most of the fact about the third Qs system of the *P. aeruginosa* was unknown but available data indicating that qscR is important in regulating the production of several virulence factor, nevertheless this regulations are occurs under the control of both the Las and the Rhl system [60, 61].

QS Circuit in *V. cholerae*

V. cholerae is the causative agent of the disease cholera, which is a severe diarrheal disease with ancient origin. It is Gram-negative, curved rod shaped bacteria with several serogroups, among most of them are pathogenic in nature. *V. cholerae* uses QS system to regulate their virulence factor production, biofilm formation, type VI secretion and competence development (Fig. 5). In *V. cholerae* signaling system function with two parallel QS via phosphorelay-type regulatory pathway. The CaqsA/CqsS system is one of the signaling system, which produces and detects CAI-1 (S-3-hydroxytridecan-4-one) as a QS signal used for intra-genus communication. The second signaling system namely LuxS/LuxPQ, which produces and detects AI-2 (S-TMHF-borate) as signaling molecule that even act as an interspecies signaling molecule [22]. The sensory information from both the systems was transformed through a phosphorelay mechanism which facilitates the activation of transcriptional factor called LuxO. Additionally *V. cholerae* employs

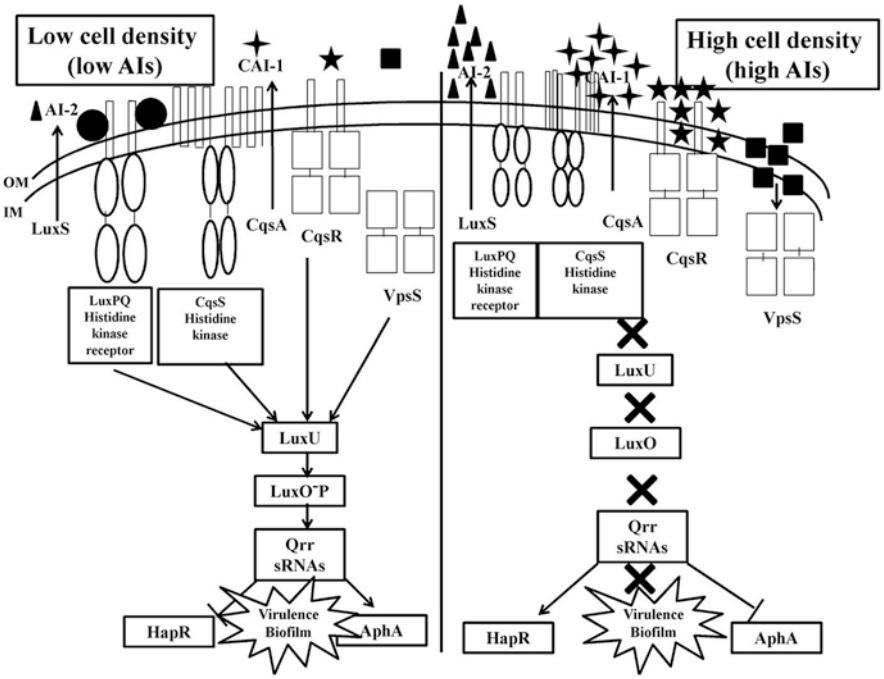

Fig. 5 Schematic representation of parallel quorum sensing system of Vibrio cholera at low and high cell density conditions. At low cell density, kinase activity of CqsS, LuxPQ, VpsS and CqsR are functional and activate response regulator, LuxO which promotes transcription of Qrr RNAs which in turn inhibits HapR thereby enhancing virulent genes and toxin production and biofilm formation. Meanwhile, at high cell density, kinase activity was collapsed due to dephosphorylation thereby LuxO was repressed and HapR become activated and virulence genes production and biofilm formation were repressed

another QS pathway involves the VarS/VarA sensor kinase/response regulator pair, this system activates three small RNAs such as CsrB, CsrC and CsrD. These sRNAs are involving in the translation of the AphA regulator and inhibit the translation of the HapR regulator which leads to the inhibition of global regulatory protein activity, CsrA, this condition mediates the activation of LuxO function independently of LuxU. These QS network will functionalized when the condition is maintained with low cell density [33]. At low cell density condition CqsS and LuxQ function as kinases and hydrolysis ATP transfer the phosphoryl group through histidine-phosphotransfer protein LuxU to global regulatory protein LuxO. The complex of LuxO and the phosphoryl group activates transcriptional genes encoding for four regulatory sRNAs namely Qrr1-4. Due to the RNA chaperone Hfq, Qrr1-4 gets activates and involving in the translation process of AphA regulator and inhibit the translation of the HapR regulator [22]. At higher cell density, the accumulation of autoinducers is increased; the kinase activities of CqsS and LuxQ are blocked due the binding of their cognate signal. This reaction consequences the reverse flow of phosphate, which facilitates dephophorylation and deactivation of LuxO. Reciprocal production of AphA and HapR at low cell density and high cell density is working has a central switch to Vibrio species to incite to their individual and group behaviors. These two transcriptional factor are involving in the regulation of expression level of more than 100 genes, among most often virulence factors triggering genes [37].

Genes Responsible for the Quorum Sensing Regulated Virulence

The quorum sensing in bacteria constitutes a complex regulatory framework which enables bacteria to make social strategies regarding the expression of a specific set of genes responsible for an array of virulent traits and determines the fate of bacterial pathogenesis [14]. The important pathogenic determinants observed during *P. aeruginosa* infection are pyocyanin encoded by *lec*A gene, biofilm enhancing exopolysaccharides encoded by *pel* gene cluster, exotoxinA encoded by *tox*A gene, alkaline phosphatase encoded by *apr*A, protease encoded by *las*A and elastase encoded by *las*B gene. These genes are expressed under the regulation of tandemly working AHL-based QS circuits responsible for biofilm formation, host tissue destruction and host immune evasion [40, 57]. Similarly, in Gram positive bacteria an array of genes and their products are directly or indirectly involved in enhancing quorum sensing assisted pathogenicity such as enhanced adhesion ability, toxin production etc. The genes and their products involved in quorum sensing associated virulence and biofilm formation are depicted in Table 1.

Table 1 Quorum sensing regulated genes and their function in developing quorum sensing associated virulence and biofilm formation in Gram positive and Gram negative bacteria

S. No.	Gene	Organism	Virulence factors	Reference
1.	RNAIII	*S. aureus*	δ – hemolysin, α – toxin, Fibronetic binding protein A & B, coagulase, Protenase and Lipase	[7, 16, 49, 56]
2.	*plcR*	*B. cereus*	Entrotoxin, hemolysins, collagenase, phospholipases, protease and toxins	[18, 19, 30, 56]
3.	*srfA*	*B. subtilis*	Exopolysaccharide, collagenase, phospholipases, haemolysins, protease and enterotoxins	[44]
4.	*phzABCDEFG, phzM*	*P. aeruginosa*	Pyocyanin production	[29]
5.	*toxA*	*P. aeruginosa*	Exotoxin production	[40]
6.	*aprA*	*P. aeruginosa*	Alkaline protease activity	[40]
7.	*lasA*	*P. aeruginosa*	Protease activity	[40]
8.	*lasB*	*P. aeruginosa*	Elastase activity	[40]
9.	*rhlAB*	*P. aeruginosa*	Rhamnolipids production	[40]
10.	*pelA*	*P. aeruginosa*	Exopolysaccharides production	[57]
11.	*lecA*	*P. aeruginosa*	Lectin production	[29]
12.	*hcnABC*	*P. aeruginosa*	Hydrogen cyanide production	[29]
13.	*ctxA, ctxB*	*V. cholerae*	Production of cholera enterotoxin	[74]
14.	*hapA*	*V. cholerae*	Encodes HA protease for establishment of new infection	[74]
15.	*flaA*	*V. cholerae*	Exopolysaccharides production	[63]

QS and Biofilm Formation

The majority of pathogenic bacteria have the inherent ability to infect their hosts by virtue of producing an array of virulence determinants which are released in response to the cellular density and the binding of the AIs to their cognate receptor proteins. This entire series of events are controlled by the highly complex and peculiar quorum sensing network [55]. Biofilms are defined as the high-density bacterial clusters with the inherent property of adhering on to the cellular surfaces and encased within a hydrated extracellular polymeric matrix. Biofilm cells share several unique properties compared to their planktonic (free-living) counterparts,

including increased tolerance to conventional antibiotics and antimicrobial therapy [68]. Bacterial biofilm confronts serious problems in the food industry as well due to the resistance shown to antimicrobial and other cleaning agents and are associated with several foodborne diseases outbreaks [32]. The majority of bacterial biofilms are the product of highly complex hierarchical quorum sensing system and are associated with food spoilage and provide uphill safety challenges in a wide range of food packaging based industries such as dairy processing, sea food processing, poultry and meat processing [50]. The quorum sensing associated biofilm formation by pathogenic bacteria on different surfaces cause enormous detrimental health hazards as well as seriously effects the medical and industrial settings. The formation of biofilms are generally the source of many life-threatening diseases such as endocarditis, cystic fibrosis, chronic prostatitis, periodontal disease, chronic urinary tract infections and other immunomodulatory diseases [73].

The biofilms formed by pathogenic bacteria communicate by highly specialized chemical signalling and encased within protective extracellular structures which impart the ability of pathogenic bacteria to survive natural host diffence system as well as antimicrobial therapy [46]. In a majority of chronic bacterial infections, extracellular polymeric matrix encased biofilms play an important role in development of ocular related infectious disease, microbial keratitis caused by *P. aeruginosa* and *S. epidermidis* [24]. The quorum sensing mediated biofilm formation, development and maturation imparts the bacteria to endure relatively harsh environmental conditions, enhanced efficacy in evading host immune system and most importantly showed predominant resistance to antibiotics [6]. The, infections resulting from quorum sensing mediated microbial biofilm formation remain a potential and irreversible threat to immunocompromised patients particularly in patients with chronic wound infections [2]. The concentration of AIs increases with the concomitant increase in the cell density and beyond a threshold concentration these autoinducers characteristically bind with their specific cognate receptor proteins thereby forming signal-receptor complexes, which regulate the expression of various genes responsible for the biofilm formation [1]. Most of the pathogenic bacteria have the inherent property of species-specific quorum sensing network which upregulates the production of biofilm to counteract the antimicrobial agents, enhances the motility which initiates the biofilm formation and production of an array of exoproteases which enable the bacteria to face harsh environmental conditions [51]. In recent years, urinary tract infections (UTI) caused by uropathogens such as *E. coli* and *P. aeruginosa* become an emerging and common infectious disease in human beings. Most of the quorum sensing associated behaviours such as production of biosurfactant and exopolysaccharides, increased motility and biofilm formation and development are generally associated in the development of acute and chronic UTIs [47].

Exploitation of Bacterial Communication System as Therapeutic Target

In the last few years, due to the increased multi drug resistance to conventional antibiotics, it has been assumed that by 2050, antimicrobial resistance (AMR) could cause ten million additional deaths annually, and a cumulative loss to the world's GDP of US$100 trillion [68]. Due to the importance and impact of QS in microbial pathogenesis and multidrug resistance phenomenon, it is imperative to understand the full scope of highly complex and synchronized QS systems. As the QS phenomenon simultaneously regulate the production of an array of virulence factors resulting in bacterial pathogenesis and biofilm formation, bacterial quorum sensing has attracted intense research interest in recent years targeting the production and detection of small molecules within and among the bacterial population [59]. In the era of antibiotic resistant and highly tolerant bacteria, which causes acute, and highly persistent infections in human being leading to serious public health issues. In this context, targeting evolutionarily conserved quorum sensing associated virulence proves to be significant alternative strategy in controlling bacterial infections and associated health consequences [62].

Besides, bacterial cell-to-cell communication system significantly enables the bacteria during host colonization, biofilm formation, and adaptation to changing environment which provide an attractive target for antimicrobial therapy. The antimicrobial therapy targeting quorum sensing focus upon inhibition of AHL molecule biosynthesis, degradation of AHL molecules by quorum quenching enzymes such as lactonases and acyclases and inactivation of AHL receptor proteins [11]. The quorum quenching enzymes and other QS-attenuation strategies do not kill pathogens but instead combat virulence factors production and thereby regulate biofilm formation which corresponds to the increased susceptibility of bacterial pathogens to the available antimicrobial drugs. Such anti-virulence strategies impart comparatively less selective pressures that lead to the development of resistant mutants than conventional antibiotics [68]. The world has reached a stage of post-antibiotic era where bacteria significantly exhibiting resistance against available antimicrobial drugs due to their indiscriminate uses. In addition, the available antibiotics more frequently have the trend to kill indigenous bacteria that are beneficial to the host. In this scenario, to cope with these limitations, the alternative approach focused on attenuating bacterial virulence rather than killing the bacterial cells by targeting the specific quorum sensing hierarchy. This new generation anti infective strategies allow widespread avenues to develop a rational preventive strategy to control bacterial virulence and biofilm formation and in the present scenario this strategy seems to be highly effective [52].

Conclusion

QS in bacteria plays pivotal roles remarkably involving in the expression of major virulence factors of the bacteria and allows symbiotic interaction between the bacteria and host which allows bacteria to exploit their host. Till date, several QS system in different microorganisms and their mediated pathogenicity were investigated. Additionally, till some more QS system and signaling molecules were yet to be discover that could facilitate the better understanding about the pathogenicity of the bacteria and enlighten the knowledge to encounters the bacterial infection in efficient manner. Interesting, counterpart of QS and pathogenicity of bacteria, host especially plants develops the QS defending mechanisms in order to overcome from the bacterial infection. In this context, natural incidence of QS inhibitors were reported which can able to degrade signaling molecules and/or signal binds receptors. In the recent year, targeting the QS gains significant attention to control the bacterial infections due to their non-target specific pressure on the pathogens, which can prevent development of anti-biotic resistance by pathogens. As is often reports with QS inhibitors but still very few effective anti-QS agent were discovered. The vast area still unexplored where high possibility to exploit an efficient anti-QS compounds which becomes remarkable drugs to treat bacterial infection and direct mainstream of therapy towards bacterial communication disruption.

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Quorum Sensing and Biofilm Formation in Pathogenic and Mutualistic Plant-Bacterial Interactions

Rajinikanth Mohan, Marie Benton, Emily Dangelmaier, Zhengqing Fu, and Akila Chandra Sekhar

Abstract Bacterial quorum sensing plays a cardinal role in determining the outcomes of plant interactions with pathogenic and mutualistic bacteria. This review dwells on the current understanding of how bacterial quorum sensing molecules, their cognate receptors and signaling pathways enable bacteria to interact with a plant host as a community. Suitable habitat niches on the plant, nutritional abundance as well as stress situations can all contribute to the formation of bacterial biofilms on plants and the abundance of nutrients such as host sugar molecules can not only serve as signals, but also as energy substrates and building blocks for biofilm formation. While biofilm formation is increasingly shown to be key to pathogenesis of bacterial plant pathogens, the modulation of plant immunity through QS signals is critical to the tolerance and establishment of mutualistic plant-microbe relationships. A new role for the versatile stress hormone salicylic acid as a possible quorum quenching molecule in plant-pathogen interactions is emerging and will be discussed. Furthermore, genetic studies coupled with -omics scale analysis of gene expression, advances in microscopy and the recent use of interdisciplinary approaches including molecular modeling and docking simulations and in silico and in vitro screening of small molecule compound libraries have provided novel insights into plant biofilm processes. The potential in targeting quorum interactions to control bacterial diseases of plants is discussed.

Keywords Quorum sensing · Plant interactions · Virulence factors · Bacterial diseases

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Introduction

Prokaryotic microbes can communicate with neighboring cells using chemical signals in a process called quorum sensing (QS) [19]. Bacteria release small, diffusible quorum sensing signals such as N-acyl homoserine lactone (AHL) or oligopeptides, the levels of which serve as a measure of population density, into their local environment [53]. When the signal concentration attains a threshold level (quorum), the bacteria in the community sense and responds to these signals. A response regulator (receptor or sensor of AHL signals) is activated by binding to AHL and triggers signaling events, post-translational modifications and alterations in gene expression that culminate in a coordinated of expression of traits in a population dependent manner [45]. This synchronizes the microbes to act in concert to develop new structures and acquire functions that they did not possess as free-living or planktonic microbes and generally results in a stronger microbial response to the environment; for instance, increased production of disease-promoting virulence factors in pathogenic microbes. The concentration of QS signals within a biofilm allows for a rapid, coordinated response among the bacteria living within the biofilms [83]. In a sense, the bacteria use chemical signals as a language to *talk* and communication through QS can transpire within a single species of bacteria as well as across different species or even kingdoms [126].

QS is a crucial regulator of many bacterial processes including virulence in pathogenic bacteria, mating, swarming motility, stress tolerance and formation of sophisticated multicellular structures called biofilms [19]. Biofilms contain a matrix of extracellular carbohydrate-rich polymer encasing a large number of microbes of the same or different genera and is generated by the communicating bacteria [20]. It is important to note that in nature, biofilms can comprise a diversity of organisms including one or more species of bacteria, yeast, filamentous fungi and possibly other microbes [82], but the focus of this review will be on bacterial biofilms.

Biofilm development occurs when bacteria attach to a surface, come together through a process known as autoaggregation and form micro-colonies that produce EPS and other biofilm material [126]. A frequent component of a biofilm is exopolysaccharide (EPS), which is not only important for biofilm formation, but also serves as a virulence factor in causing disease. The nature of the EPS differs with the bacterial species and it is typically a polymer of one or more types of sugars and often negatively charged with acidic moieties such as uronic acid and negative charge substituents including phosphates and sulfates; however, cationic EPSs also exist [17]. In a developed biofilm, EPS forms elaborate three-dimensional structures with discrete channels for the passage of water, nutrients and other diffusible material through the biofilm. Proteins, lipids and nucleic acids (extracellular DNA) form minor structural components of the biofilm. QS processes are critical in autoaggregation and subsequent production of these biofilm components and formation of the biofilm.

Biofilms can form on abiotic or biotic surfaces when a bacterium has located a nutritionally suitable substratum or as an adaptive strategy to preempt or overcome fluctuating stress conditions in the environment/host and can be important determinants of host-microbe interactions [26]. Biofilms can confer protection from

physical afflictions such as desiccation, osmotic stress, pH changes and UV radiation. As such, biofilm formation is key for the survival for microbes on external surfaces which are more directly exposed to harsh environmental conditions, such as plant leaf surfaces [83]. Biofilms also help the bacteria withstand biotic stresses from antimicrobial compounds and the host immune reaction. In addition, biofilm formation could facilitate enhanced nutrient uptake [17]. Thus, bacterial aggregation and biofilm formation, mediated through QS, confers many advantages that can help capitalize on favorable conditions as well assail stressful situations. Importantly, QS and biofilm formation confers prokaryotes with multicellular organization and behavior, that is typical of higher organisms [19, 70].

Plants are inhabited by commensal, mutualistic or symbiotic and pathogenic microbes. Many plant-interacting microbes form associations ranging from smaller microcolonies, which are simpler aggregates of bacteria, to biofilms which are relatively more complex structures, and which have been visualized recently using epifluorescence and scanning confocal microscopy [83]. Biofilms are formed on varied plant surfaces including leaves, roots, stems, vascular tissue, on the surface, within intercellular spaces and in specialized nooks such as trichomes; these plant habitats are likely sampled and explored by the bacteria and the decision to settle and associate should involve QS [26]. Plant biofilms also act as launch sites for the dissemination of propagules such as spores [83]. Thus, biofilm formation in plant-associated bacteria is critical for the bacteria not only for appropriate nutritional access and for enduring environmental variations, but also for plant colonization, survival and spread.

The formation of biofilms via quorum sensing can be detrimental to the plant, as has been shown to regulate bacterial pathogenesis in plants [70]. For example, the phytopathogenic bacterium, *Pantoea stewartii,* uses quorum sensing to activate production of the EPS, which acts as a virulence factor in maize Stewart's wilt disease [16]. Conversely, biofilm development can also have protective function as evidenced by *Pseudomonas fluorescens,* which forms a biofilm that coats plant roots and protects them from soil bacteria and fungal pathogens [84]. QS signals not only initiate the formation of biofilms, but they also control various aspects of biofilm buildup and maturation. In this review, we will focus on the broad picture of the vital role that quorum sensing plays in the construction of biofilm by bacteria in plants, with limited reference to molecular mechanisms. We will discuss the molecular dialogue of biofilm bacteria with plants and the regulation of plant physiological processes by this interaction, including symbiosis and disease.

Formation of Biofilms

The Search for a Substratum

While favorable conditions like available sugar can promote biofilm formation [15], stressful conditions like hypoxia can also trigger biofilm assembly [51]. When the population of bacteria is large enough, the concentration of QS signals secreted by

the cells proportionally rises and reach a threshold, when it signals the process of biofilm formation. This involves activation of signaling, coordinated induction of gene expression, change of bacterial structure and behavior [13, 48]. The mechanism of biofilm creation on biotic and abiotic surfaces shares certain commonalties. In both cases, bacteria discern and react to nutritional and environmental cues and may use chemotaxis to find the attachment surface. In plant-bacterial interactions, the bacteria respond to plant-produced signals, including spatial and nutritional signals [79]. Flagella and fimbriae play a pivotal role in the mobility and attachment for settlement in the formative stages of biofilm development by bacteria on host plants [41, 93, 119]. Movement through flagella is important for bacterial cells to react to signal gradients and settle down in a suitable site for biofilm formation and host plant colonization [84]. Flagellar motility ranges from free-style swimming of planktonic forms or coordinated movement called swarming in groups of bacteria [58]. These movements play important roles in various life stages of the biofilm, not only in the initial formation, but also in remodeling of the biofilm, biofilm dismantling and discharge of bacteria from the biofilm [64].

Attachment

The decision to give up motility and switch into a localized sedentary life-style is triggered by environmental cues and involves changes in bacterial gene expression, including loss of motility-related gene expression and increase in synthesis of a variety of structures to form attachment structures as has been witnessed in *E. coli* and *Pseudomonas aeruginosa* biofilms [93, 131]. Similar changes are also witnessed by treatment with sucrose, which is a plant-produced sugar and mimics plant association [116].

Bacterial cell facade structures play an imperative role in microbial attachment at a suitable location. These include flagella, matrix components such as EPS and cell wall components lipopolysaccharides (LPS) which are crucial for cell attachment [17]. Several bacteria produce exopolysaccharides that become major constituents of the extracellular biofilm matrix and play a key role in bacterial adherence to plant surfaces [114]. For example, the unipolar polysaccharide made up of N-acetylglucosamine and N-acetylgalactosamine is essential for the fixation of *Agrobacterium tumefaciens* to host plants [118, 132]. The hydrophobicity of LPS likely influences its ability to form adhesive interactions with the substratum. The importance of LPS in biofilm formation is evident in alfalfa where colonization by symbiotic bacterium, *Ensifer meliloti*, is reduced in mutants with defective LPS formation.

Protein-based extracellular projections including pili, fimbriae and adhesins are also important for attachment. Adhesins are made up of large cell-surface proteins that interact specifically with host surfaces to mediate adherence [65]. Adhesins may be part of fimbriae, which are filamentous projections that serve as attachment structures or non-fimbrial [136]. Some pathogenic bacteria deploy syringe-like

protein complexes to inject effector proteins into host cells to target host cellular processes [26]. These secretion systems, as for example, the type III secretion system (T3SS) have been demonstrated to be also vital for biofilm formation as they serve as physical anchors on host plants cells. Pili are Type IV protein assemblies that typically promote bacterial interactions, but can also generate twitching movements to promote bacterial aggregation and biofilm formation [84]. Thus, both flagella and pili are not only crucial for movement to find a suitable substratum, but also enable adhesive interactions [92]. In conclusion, a variety of carbohydrate, protein and lipid-based structures on bacterial surfaces coordinate to facilitate movement and attachment to a suitable plant surface.

Minerals can also be determinants of site selection and attachment in plant biofilm assembly. Iron is an important signal in the directed movement and the settling of bacteria for biofilm formation. This has been observed for the colonization of the vascular pathogen, *Pantoea stewartii subsp. stewartii* in corn [22], *P. syringae pv. tabaci* on tobacco leaves [117] and *E. meliloti* on alfalfa roots; siderophore mutants of some of these bacteria were defective in colonization and biofilm forming [7]. Calcium has also been implicated in biofilm formation in vitro and calcium supplementation enhanced bacterial attachment and biofilm formation in *Xylella fastidiosa*, suggesting that calcium may have a structural role in reinforcing biofilms, akin to their role in bridging pectates in plant extracellular matrix, in addition to their role in intracellular signaling [34]. Like iron and calcium, other minerals like copper appear to participate in early biofilm synthesis [26].

Once bacteria adhere to a surface, they use QS signals to communicate with bacteria in the vicinity for biofilm development. The attachment of bacteria appears to proceed through a reversible phase where bacterial structures make contacts with plant substratum through electrostatic and hydrophobic forces and an irreversible stage where the cells adhere more strongly to complete anchorage [63]. This adhesion is essential for aggregation of bacterial cells to follow [6, 35].

Autoaggregation and Biofilm Development

Once cells attach to a surface, they can interact with neighboring cells and aggregate to form a microcolony and eventually develop into a biofilm [67]. In some cases, pre-aggregated bacteria can attach to a surface to initiate biofilm formation. LPS, pili and membrane proteins can, in addition to facilitating adhesion, also promote bacterial interactions and aggregation [17]. The aggregation brings bacteria to close proximity to form microcolonies, resulting in intercellular communication through QS, which paves the way for biofilm formation [38]. This autoaggregation phase witnesses increased synthesis and secretion of the QS signaling molecules which triggers one or more signaling pathways that operate in hierarchical series or parallel circuits to stimulate gene expression changes critical for biofilm formation [125].

Auto-aggregation is greatly affected by the secretion of polymeric material such as EPS, which act as a molecule glue in the development of biofilm matrices and is

integral to the maintenance of the structure and function of the biofilm [44]. For example, Rhizobium sp. secretes galactoglucan during plant colonization. Cellulose also mediates bacterial aggregation, particularly in the root colonization of *Rhizobium* and *Agrobacterium* spp. Bacteria may also produce glycoproteins called lectins to crosslink EPS in the developing biofilm matrix, further reinforcing aggregation [80]. The surface LPS layer found on gram negative bacterial cells is important for the development of the biofilm, not only through attachment to the plant surface but for also for interaction with neighboring bacterial cells [17]. Furthermore, the biofilm formation, colonization and virulence of several pathogenic bacteria, including Pseudomonas and Xanthomonas species, was also reduced in LPS-deficient stains. Like LPS, adhesins promote both surface attachment and cell-to-cell interaction to promote biofilm formation [1,17]. Once biofilms are formed, they go through different stages including growth, maturation, ending with dismantling, whereby the bacteria become motile and explore new host surfaces [17].

General Mechanisms of Quorum Sensing (QS) and Biofilm Formation

QS signals

Gram positive bacteria such as *Bacillus* spp. and gram negative bacteria including *Pseudomonas* spp. generate quorum signals of fundamentally different nature. For this review, since plants are frequently interacted by gram negative species, we will limit our discussion of QS signaling to gram negative bacteria (although several Bacillus spp. bear great agricultural value as symbionts). In gram negative bacteria, the predominant QS signal, whose levels correlate with population density, is N-acyl homoserine lactone (AHL), whereas oligopeptides play an analogous role in gram positive bacteria [48, 129]. Some gram negative species such as Xanthomonas use, instead of the lactone ring AHLs, diffusible signaling factor (DSF) which are cis-2-unsaturated fatty acids or derivatives [12]. In addition, numerous other signaling molecules including γ-butyrolactones, furanosyl borate diester (also called autoinducer 2 or AI2), and is cis-methyl-dodecenoic acid (cis-DA) can serve as QS signals [120]. Most QS-based processes in plant bacteria are facilitated by AHL and many phyllosphere (leaf-living) bacteria reply on AHL-based QS systems [19].

QS pathways in gram negative plant bacteria are generally variants of the well-characterized *Vibrio fischeri* AHL-QS system, where quorum sensing regulates bioluminescence. An AHL molecule comprises a lactone ring conjugated to a fatty acyl chain through an amide linkage. The differences are observed in QS signals in different bacteria are mainly in the acyl carbon chain length and whether these molecules sometimes carry hydroxyl- or oxo- substitutions at carbon 3 [113]. For example, in the plant pathogenic Agrobacterium sp., 3-oxo-octanoylhomoserine lactone acts as a QS signal. Some AHLs or their derivatives are also believed to have

anti-bacterial activity by means of binding siderophores [62] and long fatty acid chains in some AHLs are observed to have a biosurfactant function during swarming motility [36].

QS Signal Synthesis and Transport

AHLs are low molecular weight, diffusible signal moieties manufactured by the AHL synthase enzymes. The enzyme forms a lactone ring with the methionine part of S-adenosyl methionine and attaches fatty acyl chain delivered by the acyl carrier protein [81, 88, 104]. The AHL may be issued into the extracellular milieu by passive diffusion or active transport using transporters or efflux pumps [61, 89]. Signals with longer fatty acyl chains are generally transported actively.

Signal Transduction and Transcription Through QS Response Regulators

AHL signals form a pool in the extracellular environment where they can bind to and activate membrane-bound AHL transcriptional-response regulator proteins to trigger QS signal transduction pathways [113]. The impact of AHLs becomes apparent when they reach a critical level and activate response regulators to coordinately stimulate quorum-related gene expression in multiple cells [49]. AHL binding brings about a conformational change in the response regulator that releases it into the cytoplasm, accessible for transcription, as observed for Agrobacterium TraR receptor [95]. The AHL ligand binding dimerizes TraR and stabilizes the DNA binding conformation and facilitates transcription [138]. Activators like the Agrobacterium TraR can stimulate transcription by direct binding to RNA Polymerase [128]. The response regulator could, in some cases like CarR of Erwinia carotovora, already exist as a dimer and ligand binding stimulates oligomerization and transcriptional function [127]. AHL binding to transcriptional repressors can also facilitate gene expression by derepression. In *Pantoea stewartii*, AHL binding to the EsaR repressor, which physically impedes RNA polymerase-mediated transcription, triggers a conformational change that releases it from DNA for transcription to ensue [121]. Similar to AHLs, the fatty acid-based DSF signals can also activate membrane proteins to trigger a signaling and transcriptional response. A DSF ligand in the plant pathogen, *Xanthomonas campestris*, was shown to activate allosteric activation and autphosphorylation of a sensor histidine kinase to initiate DSF signaling [23].

The AHL-bound response regulator transcriptional complex interacts with specific regulatory sequences (in some cases called lux boxes) in the promoters of quorum- or population-density responsive genes; however, genes without such obvious consensus regulatory sequences could also be activated in this process.

The gene expression proportionally increases with levels of AHL signals. Often, this transcriptional complex results in increased gene expression of AHL synthase, generating a positive induction loop.

Generally, one type of transcriptional response regulator typically binds to one type of AHL produced by the cognate AHL synthase, thus generating specific paired signaling system. Multiple signal-receptor pairs may operate hierarchically to promote gene expression. QS signals can also be used for communication between different bacterial species. Although the response regulators are specific for specific AHL molecules, some receptors can detect ligands that are modified or carry acyl groups of different chain lengths, raising the possibility that bacteria could also sense foreign AHLs as a means of interspecies crosstalk [113]. Besides interbacterial communication, QS signals from bacteria also crosstalk with host plant cellular processes [14, 28, 77, 89].

QS Signaling Homeostasis

To maintain homeostasis of QS signaling, AHL can be inactivated by AHL lactonases that lyse the lactone ring or by acylases that sever the fatty acid side chain (summarized in [108]). AHL degradation may serve to modulate QS signaling, to eliminate AHLs produced by competing species or even used as carbon sources.

QS Signal Amplification

Quorum signaling can be amplified by generation of small, non-protein second messenger molecules such as cyclic di-GMP (cdi-GMP). cdi-GMP is made by the enzyme diguanylate cyclase and catabolized phosphodiesterase; together these enzymes regulate cdi-GMP homeostasis and control associated processes [101, 106]. cdi-GMP can directly regulate protein activity by binding and inducing structural changes in enzymes or regulate gene expression by binding to transcription factors and riboswitches [100, 112].

QS and Biofilm Gene Expression

AHL sensing, activation of response regulators and amplification of QS signaling lead to changes to gene expression changes that can affect many processes including biofilm formation. These gene expression changes can be dramatic as observed with the human pathogen P. aeruginosa, where up to 10% of the genome is regulated by QS [73]. Many gene products that are essential for the initial cellular adhesion and aggregation are also important and expressed for the development of the biofilm.

Genome-wide transcriptional analysis of the plant-colonizing *Stenotrophomonas maltophilia* has revealed coordinate activation of genes affecting cell motility and biofilm formation through the rpf/DSF signaling system [4].

Important structural changes linked with biofilm formation include EPS production governed by cdi-GMP signaling and this can result in both enhanced cell-to-cell contacts as well improved adhesion to host surfaces. cdi-GMP signaling also promotes autoaggregation and biofilm formation as observed for *Rhizobium etli* through synthesis of the polysaccharides cellulose and alginate. Cellulose is a major constituent of plant biofilms in the widely prevalent *Pseudomonas* spp. Some bacteria like *Sinorhizobium meliloti* lack cellulose which is made of β1➔4 linked glucan chains; instead they synthesize mixed linkage glucans with both β1➔4 and β1➔3 linked glucose polymers and the pertinent synthase is directly activated by cdi-GMP binding [91]. Like EPS, expression of structural proteins upregulated by QS signaling is also important for biofilm formation. Increased expression of cdi-GMP-responsive proteins leads to enhanced accumulation of adhesins (summarized in [90]). The functions of such proteins are evident in the observation that a loss of function mutation of the transcriptional repressor praR in *Rhizobium leguminosarum* leads to increased production of adhesins and cadherins, resulting in enhanced colonization of host roots [46]. The polycyclic phenazine has been implicated as a multifunctional metabolite that may directly regulate biofilm formation through a direct structural role or an indirect signaling role in biofilm formation. Thus, QS signaling through increased intracellular cdi-GMP promotes the switch from mobile planktonic to sessile biofilm state largely by synthesizing important carbohydrate and protein structural constituents of biofilms. Like cdi-GMP, cGMP also serves as a second messenger in biofilm formation in pathogenic species like *Xanthomonas* [8]. Recently, small regulatory RNAs have been shown to regulate cellular cdi-GMP amounts and consequently biofilm formation (detailed in [26]). The various QS signals and the response systems in plant-interacting bacteria are summarized in Fig. 1.

QS and Biofilm Dispersal

During the formative phases of biofilm formation, typically higher phenotypic heterogeneity exists. During this time, QS can also promote the random exit of bacteria from biofilms as observed in *Pseudomonas putida* in vitro [25]. In this case, these cells signal themselves, rather than neighbors and QS signaling leads to the production of biosurfactants that allow these cells to slip off from the developing biofilm. In mature biofilms, if the niche is nutritionally spent or conditions become unfavorable, biofilms can also be dismantled to allow bacteria to seek out new sites for infection. This involves the action of many digestive enzymes such as endomannanase (breaking mannose polymer structures), released in concert by biofilm bacteria to promote lysis of the biofilm and release of constituent bacteria [74]. In active biofilms, cdi-GMP enables biofilm maintenance and inhibits biofilm dispersal by repressing the production enzymes like endomannanase.

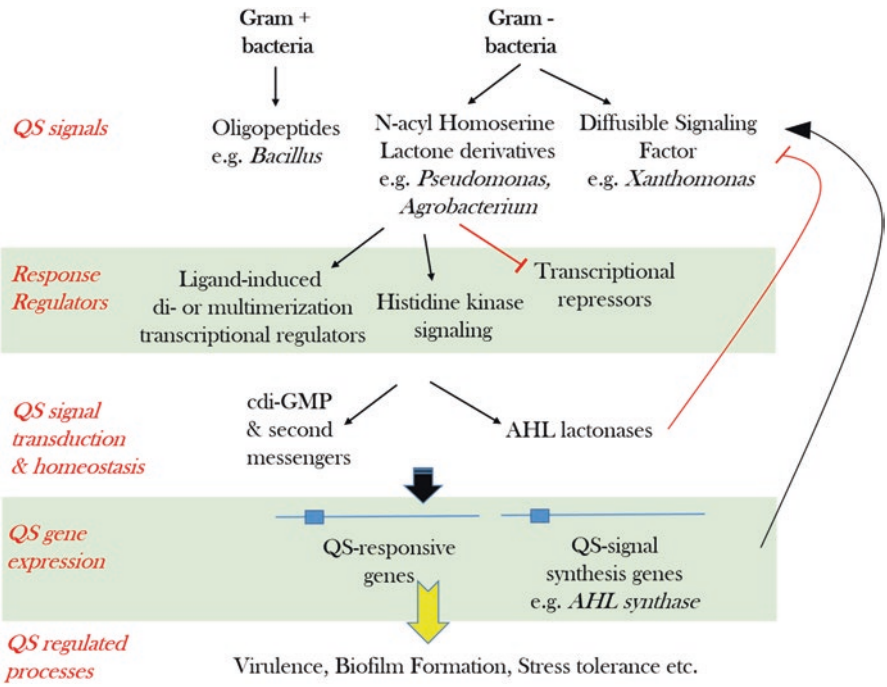

Fig. 1 Quorum sensing & signaling systems in plant-associated bacteria. Different plant-associated bacteria utilize various signals to monitor their population density. These signals may be oligopeptides, amino acid-derived N-acyl homoserine lactones (AHL) or fatty acid-derived diffusible signal factors. These signals can bind to response regulators which are frequently transcription activators, often activating them by dimerization or multimerization. The signals can also inactivate transcriptional repressors or activate histidine kinases to trigger signaling. Second messengers like cyclic di-guanosine monophosphate (GMP) can amplify QS signals, while AHL-metabolizing enzymes like AHL lactonases maintain homeostasis by degrading AHL signals. QS response regulators promote expression of QS-responsive genes that promote microbial density-regulated processes. QS signaling can also synthesize new AHLs to develop a positive amplification loop

QS and Biofilms in Plant-Pathogen Interactions

Biofilm-Forming Plant Pathogens

Many pathogens colonize different locations in the plant through biofilms. *Pseudomonas* and *Xanthomonas* spp. are common biofilm-forming foliar pathogens in cereal and vegetable crops. *Xanthomonas* spp. cause devastating diseases including blight on rice leaves and citrus canker on citrus leaves, stems and fruits. *Pseudomonas syringae* is responsible for leaf spot diseases in tomatoes, beans and other crop plants [55]. *Xylella, Ralstonia* and *Pantoea* are vascular pathogens that

can form biofilms that plug xylem vessels and block fluid transport, thus leading to wilt and blight diseases [83]. Different species of *Erwinia* can not only cause wilt by colonizing vascular tissue, but can also, like *Pectobacterium*, degrade cell walls to cause rot diseases in roots and fruits. These associations require specific molecular recognition and the host genotype can, therefore, determine biofilm occurrence; for example, the soft rot pathogen *Pectobacterium* can form biofilms in susceptible individuals, but not resistant plants [68]. In many of these diseases, biofilm not only serves to protect the pathogen, but also serves a virulence function and, as such, studying biofilm processes in these organisms in an effort to tackle their diseases is of high agricultural relevance.

Role of QS and Biofilm in Plant Pathogenesis

Biofilm formation through QS processes is important for pathogenesis of both plant and animal pathogens [35, 87]. Biofilm formation allows a pathogenic species to access plant surfaces or interiors for nutrients, while often allowing them to weather unfavorable conditions. What triggers pathogens to form biofilms? A number of plant signals are involved in regulating biofilm synthesis, but the availability of nutrients is a major factor in the decision to form biofilms. During certain bacterial infections, plant cells are lysed, releasing a plentitude of sugars and other nutrients that not only signal biofilm formation, but can themselves serve as building blocks and energy sources for biofilm formation [1, 15]. Indeed, exogenous treatment of bacteria with the plant sugar sucrose promotes biofilm production [116]. Biofilms not only protect pathogens from assault from the host immune system, but also facilitate a concentration of communication signals, leading to increased QS signaling and virulence. This may be achieved, for instance, by coordinated production, by biofilm bacteria, of enzymes that target the host immune machinery [42] or by mass-engenderment of plant cell wall decomposing enzymes [83].

Plant colonizing bacteria use QS to produce attachment and aggregation structures and secretion systems that play indispensable roles in biofilm synthesis[1]. Again, the importance of biofilm formation in pathogenesis of plant pathogens becomes immediately evident upon the observation that biofilm structural components are frequently also factors necessary for pathogenicity or virulence. LPS is essential early during infection to make host contact and for biofilm construction and virulence in various pathogenic *Pseudomonas* and *Xanthomonas* spp. [17]. EPS dictates virulence and biofilm formation in a variety of pathogens like *Pantoea* [26]. The EPSs amylovoran and levan that are abundantly secreted by *Erwinia amylovora* are virulence factors in causing blight diseases [66].

Similar to EPS, biofilm structural proteins are also important for pathogenicity. Adhesion proteins are required for seed-pathogens to be carried and to cause disease and also for bacterial attachment and aggregation to biofilms in *Xanthomonas* and *Xylella* spp. [26, 78]. Type I pili in *Acidovorax* and Type IV pili in *Xylella* also act as virulence factors and are essential for biofilm development in these and other bacte-

rial genera [10, 37, 39, 123]. T3SS is an elaborate protein-based structure formed by pathogenic bacteria to transfer effector proteins to target, perturb or hijack host biological processes [75, 96]. T3SS is a key virulence factor in *Pseudomonas syringae* infections in plants, but recently, T3SS as attachment structures, has been implicated in biofilm formation as T3SS-deficient mutants are unable to autoaggregate in *Pseudomonas* or form biofilms in *Xanthomonas citri* [18, 72, 139]. The secondary metabolite, phenazine, which is well known for a role as antimicrobial compound is also implicated in biofilm production in *Pseudomonas* sp. [76].

Biofilm formation in pathogens is crucial not only for pathogenesis, but also for survival and spread. For instance, biofilms in foliar surface pathogens, while causing a disease like citrus canker [98], can also protect resident bacteria from harsh abiotic conditions (sunlight, UV radiation, heat, wind etc) and limited surface water availability. Additionally, surface biofilms also poise the pathogens for aerosolization and dispersal to the environment and new hosts [21].

QS Signaling in Biofilm Formation in Plant Pathogens

AHL/response regulator and DSF signal/receptor system along with the second messenger cdi-GMP all play crucial roles in plant pathogenesis. Pathogenic *Pseudomonas, Pectobacterium, Ralstonia* and *Agrobacterium* spp. rely on AHL signals for QS, while *Xanthomonas, Stenotrophomonas, Burkholderia* and *Xylella* spp. utilize a DSF signal/rpf receptor system instead. These signaling systems govern biofilm generation and the subsequent development of disease [56].

cdi-GMP plays a pivotal function in biofilm construction in plant pathogens by regulating processes including EPS synthesis, type III secretion (T3SS) and reduction of motility and transition from planktonic to aggregative stage (reviewed in [26]). Promotion of EPS accumulation by cdi-GMP to promote biofilm formation and virulence is observed in many species including *Erwinia amylovora* [26, 66]. It is noteworthy that cdi-GMP not always signals to promote biofilm formation and virulence. As witnessed in certain mutants of *Xylella*, high intracellular cdi-GMP quantities inversely correlated with biofilm formation and virulence [30, 40]. Thus, in these plant-pathogen interactions, low cdi-GMP promotes mobility and virulence, indicating an adaptation of QS signaling mechanisms to suit the specific needs of pathogenesis.

Biofilms in the Evasion of Plant Immunity by Pathogens

Plants have several layers of immunity and employ signaling systems that rely on recognizing pathogen surface structures such as flagella or pathogen-secreted proteins. Consequently, plant pathogens have evolved mechanisms to evade detection by or actively suppress plant immune responses. The *Arabidopsis-Pseudomonas syringae* pathosystem model has unraveled the genetic and molecular landscape of

plant immunity. A recent study sheds light on the importance of biofilm formation and immune evasion by *Pseudomonas* sp. [43]. Perception of pathogen-associated molecular patterns (PAMPs) like flagella can set off an immune response cascade to suppress microbial growth; this form of immunity is called pattern-triggered immunity (PTI). In the pathogen counterpart, the signal cdi-GMP not only activates biofilm genesis, but also mediates evasion of PTI. When *Pseudomonas* detects a plant signal- a flavonoid, phloretin- it activates a diguanylate cyclase enzyme, Chp8, which synthesizes cdi-GMP. cdi-GMP signals a shift from motility to a sessile biofilm phase by repression of flagellin production and by promotion of EPS production. The retraction of flagella and suppression of flagellar protein synthesis confers the advantage of avoiding being detected by the PTI machinery. Thus, biofilm formation not only confers physical protection and isolation of pathogenic bacteria in biofilms, but also helps mask immunogenic structures.

Salient Role for SA in Regulating QS and Biofilms Through Quorum Quenching

Recently, the plant stress hormone, salicylic acid (SA) has been considered with growing interest in QS processes in both plant and medical contexts. Of the recent discoveries, firstly, SA has been found to possess possible antimicrobial and anti-biofilm function through a potential quorum-quenching function. Secondly, SA is an important mediator of defense responses triggered by bacterial AHL signals or biofilm structures.

Earlier studies identified SA as a modulator of virulence determinants in human pathogenic *Pseudomonas aeruginosa* [11] and this was subsequently linked with the ability of SA to suppress QS-related gene expression [134]. SA has also been established to suppress biofilm generation in *E. coli* [27]. More recently, SA was identified in a screen for AHL synthase inhibitors [29], indicating a direct role for SA in targeting QS signals and possible downstream virulence and biofilm responses.

In plants, SA suppresses QS and virulence gene expression in pathogenic *Pectobacterium* spp. [59]. In the pathogen *Agrobacterium*, plant-derived SA is believed to serve a quorum-quenching role; this is interpreted as the bacterium recognizing SA and dampening QS signals to avoid detection and response by the plant immune machinery [115]. Contrary to the repressive influence of SA on AHL QS systems, SA had an intriguing effect on the DSF signaling system in response to pathogenic *Xanthomonas* [133]. SA reduced bacterial motility and led to an increase in DSF QS signaling, EPS production and formation of biofilm-like structures (this *Xanthomonas* strain does not form classic biofilms); the decrease in motility reduced the pervasiveness of the pathogen. Thus, SA appears to modulate QS processes by regulating disparate AHL and DSF signaling and biofilm formation, while suppressing pathogen virulence.

The effect of SA on biofilm formation in plants has also been explored, albeit not in the context of QS. Older plants in Arabidopsis demonstrate a phenomenon called

age-related resistance (ARR), which is signified by buildup of SA in the apoplast or extracellular space in response to an infection; this extracellular SA is not observed in younger plants or in untreated older plants [69]. A recent study explains age-related resistance through the demonstration that SA suppressed biofilm formation in the apoplast, which was confirmed in vitro, and also displayed direct antimicrobial activity [130]. Moreover, Arabidopsis mutant lines displaying elevated SA levels showed reduced of *P. aeruginosa* biofilm formation while still showing similar population size as wild type plants [94].

Pseudomonas syringae is a hemibiotrophic pathogen which displays an earlier biotrophic phase where host cells are kept alive and a later necrotrophic phase where host cells are killed for nutrition. If an abundance of nutrients from host cell lysis in the necrotrophic phase promotes biofilm genesis [15], it is conceivable that biofilm formation could coincide with or contribute to the necrotrophic phase later in infection. The pathogens in the newly formed biofilm could further communicate through QS to aggravate the necrotrophic response. In this regard, SA could directly serve to suppress the transition of *P. syringae* from the biotrophic to necrotrophic phase by suppressing biofilm formation. Together, these results add to the endlessly versatile functions of SA in plant immunity with a possible direct antibacterial role for SA in plant-pathogen interactions and for a potential anti-biofilm role for SA as a quorum quencher in plant defense.

Systemic resistance induced by root-colonizing bacteria in an AHL-dependent manner coincided with production of SA in systemic tissues [107]. In addition, prior treatment of AHLs primed plants for enhanced resistance to future infections by reinforcing cell wall barrier structures, stomatal closure in response to pathogens and induction of accelerated defense responses [105]. Similarly, detection of bacterial glycolipids called rhamnolipids, which are known to be biofilm structural components, also elicited a strong SA-dependent defense response [103]. These results indicate duties for SA in actively suppressing microbial QS, in addition to potentiating local and systemic defense responses upon discernment of bacterial biofilm structures and QS signals.

QS and Biofilms in Plant-Mutualist Interactions

Biofilm-Forming Plant Mutualists

Mutualists are organisms that benefit and are benefited by the host they colonize. Many plants form mutualistic interactions with a diversity of bacteria. Biofilm-forming gram positive *Bacillus* spp. and gram negative fluorescent *Pseudomonas*, *Enterobacter* spp. and rhizobacteria are major partners in forming mutualistic associations with plants. These bacteria confer varied benefits to the plant, including nutrient absorption, plant growth betterment and biological control. In biological control, the mutualists confer enhanced disease resistance through

suppression of pathogen growth. Many mutualists often associate with plant roots, although leaf-associating mutualists are also known. The rhizobacterium, *Rhizobium* spp., associate with roots of legume crops and aid in the process of fixing atmospheric nitrogen. These mutualist interactions are specific and involve specific recognition events; for example, *Enterobacter* sp. colonizes rice roots but not wheat roots [2]. Thus, plant interaction with biofilm forming mutualists can be highly beneficial and influence not only plant growth and productivity, but also resistance to pathogens.

Role of QS and Biofilm in Plant Mutualism

Flagella, while generally being important for motility, are required for directed movement in forming an organized biofilm structure and for surface attachment. Flagella are important in the inceptive stages of colonization and eventual biofilm formation in rhizobacterial interactions with plant roots [47]. Similar to cases in pathogen interactions, during early microbial infection QS-induced production of LPS is vital for attachment and EPS is requisite for autoaggregation in *Ensifer (Rhizobium) meliloti* for colonization of alfalfa roots [50]. Frequently, the pathogen-produced structures engage in specific molecular interactions with cellular structures in the plant host. For instance, the EPS glucomannan produced by *Rhizobium* sp. interacts with cell-surface lectin glycoproteins in different legume hosts [71]. Sugars required for the synthesis of EPS could be derived from plant polysaccharides and these sugars can also serve as signals for biofilm formation as seen for *Bacillus* sp. [15]. Adhesins and other surface proteins were also found essential for root adherence and/or biofilm production in *Rhizobium* and *Pseudomonas* spp. [9, 54]. Similarly, in the fostering of symbiotic association between *Bacillus* sp. and host roots, the secreted protein TasA polymerizes to form long fibrous structures while being anchored to cell wall surface and mediates cell-cell interactions to promote autoaggregation [99]. Thus, biofilm structures and the QS processes that drive their synthesis are critical for the association of mutualists with plants.

QS Signaling in Biofilm Formation in Plant Mutualists

Similar to pathogenic bacteria, AHLs and cdi-GMP are critical for mutualistic association with plants. In addition, the mutualistic *Bacillus* spp. perform quorum communication through oligopeptides, rather than AHLs. These signaling processes are critical for biofilm production by mutualists. The detection of AHLs is a fundamentally important step in the process of establishing mutualistic or symbiotic relationships. cdi-GMP signaling facilitates root colonization by symbiotic or mutualistic species including those of *Pseudomonas* and *Rhizobium* [90]. Symbiotic genera like

Rhizobium showed enhanced EPS production and biofilm establishment in response to increased cellular levels cdi-GMP; this highlights the powerful role of the second messenger in biofilm creation [90].

Among the best studied molecular mechanisms of QS signaling in plant-mutualist interaction is the *Rhizobium leguminosarum*-legume system and this is discussed here as a paradigm for a general understanding of how plant-mutualist QS processes and biofilm formation translate to biologically important outcomes such as root nodulation (reviewed in [19]). Once a *Rhizobium* biofilm is formed, it creates a milieu for a concentrated exchange of QS signals to trigger nodulation. Interaction and nodule formation by the gram negative *R. leguminosarum* is dictated by an AHL signal, 3-hydroxy-C14:1-HSL, produced by the master regulator CinIR. This AHL activates AHL synthases that can generate other AHL signals, C6-HSL and C8-HSL, which have the potential to activate different target genes and proteins. Consequently, a protein called RhiR becomes activated by these new signals and stimulates transcription of rhiABC, which trigger nodulation. Thus a complex hierarchical system of signals regulates the vital nodulation process in legumes and QS and biofilm induction is paramount to this process.

Negotiation of Plant Immunity by Mutualistic Bacteria

Plant mutualists and symbionts differ from pathogens in their ability to not elicit a strong immune response. However, they also dampen plant defense responses using mechanisms surprisingly similar to those employed by pathogens [52]. Further, similar to pathogens, mutualists exhibit mechanisms for protection from the host defense machinery to ensure sustained survival as plant partners. These features are so critical that plant mutualists likely owe their survival on plants to them. This is best exemplified in the case of the root symbiont *E. meliloti*, which associates with roots of legume plants like alfalfa to form nodules for nitrogen fixation. Certain LPS mutants of *E. meliloti* are unable to colonize long-term likely due to their susceptibility to the host immunity [24]. Thus the correct structure of cell surface LPS molecules appears to be crucial for bacterial protection from host immunity as well as interaction with the host cells.

While many mutualistic species form microcolonies in colonizing plant surfaces, many go on to form functional biofilms. Biofilms containing multiple rhizobacterial species actively colonizing root surfaces and sometimes interiors. Comparative genomic analysis of different *Stenotrophomonas* species, which differentially colonize plants and animals, distinguished genes or gene groups implicated in plant colonization and biofilm genesis [5]. This study showed that symbiotic species lacked certain virulence factors and heat shock proteins essential for virulence. Thus an adaptation of bacterial cellular structures and reduction of virulence-related factors enable mutualists to negotiate plant immunity and establish long-time partnership with plants.

Biological Control Effects of Biofilm-Forming Mutualists

Biofilms of mutualists can act as physical deterrents to competing microbes and bacteria in these biofilms could release enzymes that catabolize QS signals of competing bacteria suppressing their population dependent gene expression and growth. Biofilms of mutualists can produce compounds or enzymes that can inhibit the colonization by pathogenic and other non-pathogenic bacteria and fungi by producing enzymes such as chitinases and proteases. *Pseudomonas* and *Bacillus* spp. are widely used as biocontrol bacteria through root colonization, but they can induce systemic defense responses that lead to enhanced disease resistance. Although *Bacillus* spp. typically colonize roots, foliar treatment with biocontrol *Bacillus* sp. was also effective in suppressing biofilm formation by *Xanthomonas* sp. [57]. Thus, mutualists facilitate biological control of pathogens through a variety of mechanisms.

Plant Growth Promoting Effects of Biofilm-Forming Mutualists

Biofilms formed by rhizobacteria can benefit the host plant by absorbing nutrients that are otherwise metabolically inaccessible to the plant [77]. Some stimulatory outcomes of microbial growth on plants could be directly attributed to the AHL signals that bacteria use for quorum communication. A recent study demonstrated that it may be the homoserine formed from the catabolism of bacterial AHL by a plant-derivative enzyme fatty acid amide hydrolase that may, at lower concentrations, have a growth stimulatory effect on host plants [86]. At higher levels, homoserine appears to restrain plant growth by stimulating ethylene synthesis. This shows the importance of AHLs in not only inter-bacterial communication, but also inter-kingdom crosstalk. Mutualistic biofilm bacteria also produce metabolites beneficial to plants such as spermidine. In turn, mutualists also receive numerous benefits from plant colonization. The formation of biofilms in plant roots could have protective effects for the bacteria against harsh environmental effects such as soil salinity. Root-colonizing *Stenotrophomonas* biofilms show increased salinity tolerance, likely as a result of expressing proteins that synthesize and transport osmoprotective osmolytes such as glycerol derivatives [5]. Thus, a mutually beneficial plant-mutualist interaction culminates in plant growth promotion.

Plant Responses to Bacterial QS and Biofilms

Plant Response to AHLs

Plants or animals sensing or interpreting microbial AHL or other QS signals may be akin to intelligence agencies intercepting chatter. Depending on the nature of the chatter- in this case structure, concentration or timing of production of AHL signals- plants accordingly mount a calculated response. As described earlier, AHL-induced pathways, especially from beneficial bacteria, can stimulate plant growth. However, perception of AHLs in some cases could also result in immunogenicity. In animals, AHLs could trigger an inflammatory response and production of pro-inflammatory signals such as interleukin-8 [111]. In plants, AHL signals could elicit expression of the defense gene, PR1. In the longer term, AHL sensing can result in primed (faster, stronger) defense response to pathogens- including stronger, longer MAP kinase activation, elevated production of phenolic compounds, increased cell wall fortification and defensive stomatal closure. Collectively, this AHL-induced priming translates to augmented resistance to pathogenic bacteria and fungi (reviewed in [3, 53].

Targeting of QS and Biofilm Formation by Plant Host

Many genera of plants are known to secrete substances that could affect bacterial QS positively or negatively; a few of them mimic bacterial AHL QS signals (reviewed in [19]). Some of the QS modulating plant metabolites are chemically distinct from AHLs and may represent novel compounds [14]. Plants utilize several chemicals to disrupt QS processes in colonizing bacteria to inhibit growth of pathogens and modulate that of mutualists [108]. Among the vast repertoires of plant secondary metabolites, flavonoids apparently directly target the bacterial QS receptors LasR and RhlR to suppress QS and the resulting biofilm generation and virulence in *P. aeruginosa* [85].

Rosemarinic acid (RA) produced in some plants acts as a AHL mimic and by virtue of binding to the AHL response regulator promotes QS signaling and biofilm formation [32]. As RA is exuded from plant roots in response to bacterial infection, RA is presumed to cause premature QS in invasive bacteria, this limiting their pathogenicity. Other studies show RA demonstrates antibacterial activity [122]. Treatment of *P. aeruginosa* biofilms with RA showed that they were unaffected by RA; however pretreatment with RA prevented formation of biofilms [122]. This indicates that plant resistance to biofilm formation can be pre-programmed. The lactone metabolite, coumarin is also effective against plant pathogens [97]. Volatile phenolic compounds can dampen production of AHL signals and biofilm creation by possibly targeting AHL synthase enzymes in the control of soft rot by pectin-degrading *Pectobacteria* [60]. Together, these results illustrate the variety of ways by which plants target QS signaling to control bacterial biofilm formation.

Applications and Future Perspectives

Targeting of QS and Biofilm Formation for Agriculture

Inspired by natural processes by which plant hosts target QS and biofilm processes in bacterial pathogens, similar approaches can be used to create disease resistance in plants. Biofilms have been intensely investigated in recent decades owing to the direct relevance in understanding and treating chronic infections in humans caused by biofilm-resident bacteria. In particular, focus has been on targeting *Pseudomonas aeruginosa* infections that could aggravate cystic fibrosis to lethality [110]. Similarly, targeting biofilms produced by plant bacteria is a promising strategy to control plant disease. Niclosamide, an FDA-approved drug traditionally employed as medication in tapeworm infections was evidenced to suppress biofilm formation in pathogenic *Xanthomonas* in rice [102]. In another study, thyme oil and its active ingredient thymol were found to suppress biofilm formation by triggering down-regulation of genes relevant to virulence and biofilm construction [109].

The urgent need to understand and target biofilms goes beyond crops in the field as pathogenic bacterial biofilms are also a nuisance in storage of packaged foods such as a salads [83]. Bacterial outbreaks resulting in food poisoning are recurrent and hard to treat due to formation of resilient biofilms and a better understanding of biofilm processes could help address the carriage and spread of human pathogens on plant products.

Enhancing Biological Control Through the Understanding of QS and Biofilm Formation for Plant Biotechnology Applications

Since QS can drive both virulence and biofilm formation, targeting QS processes could have the dual benefit of suppressing both pathogen virulence as well as biofilm organization. Many biofilm-producing *Pseudomonas* and *Bacillus* species are utilized as biocontrol bacteria to boost plant growth and fitness and suppress pathogenic microbes in agriculturally important plants [79]. In addition, plant growth-stimulating effects of the biocontrol bacteria are also attributed to biofilms. Understanding plant biofilms has direct relevance to plant growth, productivity and survival. Some autoaggregative phenotypes in beneficial bacteria may be desirable in terms of inoculum growth and application logistics and could possibly enhance biofilm establishment *in planta* [17].

Because different bacterial species can use distinct AHLs or analogous signals, pathogens could be targeted by specifically targeting their communication signals. These signals could be degraded using lactonases or outcompeted using inactive molecular mimics, reducing their QS signaling, virulence and biofilm formation. A non-pathogenic strain of *Burkholderia*, a bacterial endophyte, expressing an AHL

lactonase that degrades specific AHLs of pathogenic strain of *Burkholderia* diminished the occurrence of disease in rice [31]. Similarly expression of AHL lactonase in *Bacillus* targeting AHLs of pathogenic *Erwinia carotovora* reduced soft rot disease [137]. Likewise, QS signal binding proteins or receptors could also be targeted to reduce QS-dependent processes. As such, one possible broader approach is the biotization of crop plants with appropriate eco-friendly consortia of beneficial bacteria with such quorum quenching or suppressing capacities to pathogenic species, while conferring beneficial effects on plants, could be a sustainable strategy [3]. The enhanced resistance endowed by nitrogen-fixing *E. meliloti* was conferred by its QS signal, oxo-C14-HSL. Therefore, another combinatorial approach would involve combining the nutrient fixation ability of symbiotic bacteria with enhanced capability to promote disease resistance. A detailed understanding of molecular mechanisms of AHL and DSF QS systems and the cognate plant responses is imperative to bring such strategies to fruition.

Future Perspectives

Although great leaps have been made in understanding QS, biofilm formation and virulence processes, the connections between them are not always straightforward. While biofilm formation can promote virulence, motility is still essential for pervasiveness and continued pathogenesis. Indeed, the thiG protein promotes virulence of *Xanthomonas* by suppressing xanthan EPS production and bacterial aggregation; the thiG mutant displayed enhanced biofilm formation and reduced migration and reduced virulence in rice [135]. Likewise, mutants of Acidovorax, a seed-borne pathogen, with reduced twitching motility and virulence showed enhanced biofilm construction and seed attachment; in this case, biofilm formation may be interpreted as a survival adaptation due to impaired invasiveness [124]. As different structures may be important at different stages of infection and because many plant pathogens undergo multiple distinct phases during the infection cycle during which their metabolism and virulence mechanisms are different and require specialized structures, the fine-tuning mechanisms of these processes need better understanding.

Our knowledge of QS and biofilm processes has benefited from the rise of interdisciplinary approaches including protein modeling and docking simulations of compounds, coupled with in silico and in vitro screening of small molecule compound libraries [33, 60]. Combined with traditional genetic analysis using mutants, recent advances in -omics technologies and confocal imaging technology, there is great potential for rapid expansion of the knowledge in this field. The tremendous rise in microbiome studies provides us with an understanding of microbial communities and may help design ‘optimal’ communities for creating mixed-species biofilms with increased efficacy of plant growth promotion and defense.

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Microbes Living Together: Exploiting the Art for Making Biosurfactants and Biofilms

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Abstract Quorum sensing (QS), the way bacteria interact, where in accumulation of threshold autoinducer concentration due to increased bacterial number, switches on signal transduction cascade to regulate gene expression. Bacteria possess signaling and receptor molecules, such as enzymes or proteins, mostly AHLs (acyl homoserine lactone) in Gram negative bacteria, oligopeptides in Gram positive bacteria. Microbes interact inter as well as intra specially (i.e., crosstalks) through QS and participate in controlling activities, like motility, biofilm synthesis, biosurfactant production, virulence, cell differentiation, nutrient flux etc. that has considerable impact on human health, agriculture, marine and other ecosystems. To provide beneficial effects to the plants, microorganisms colonize the rhizosphere and release QS molecules that regulate the production of exopolysaccharides essential for biofilm formation. In addition, to this biosurfactants (rhamnolipids) synthesized by *Pseudomonas* spp. regulate the course of quorum sensing. Biosurfactants are reported to affect the motility, participate in signaling and biofilm formation. The present chapter will be focusing on how the social behavior of microorganisms and their signaling molecules promote biosurfactant production and biofilm formation.

Keywords Quorum sensing · Acylhomoserine lactones · Gene expression · Biosurfactant · Biofilm · Quorum sensing and swarming migration in bacteria

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Introduction

Microorganisms have developed a mechanism for intra and inter species communication, termed as Quorum Sensing (QS). It is cell density dependent gene regulation process that occurs in presence of signalling molecules viz. autoinducers, that heapup in the surrounding environment [88]. The initial research on QS is dated back to late 1960s in *Vibrio fischeri,* a marine bioluminescent bacterium and Hawaiian bobtail squid, *Euprymna scolopes,* which depends on Acyl Homoserine Lactone (AHL) synthesized by Lux regulon [31]. Detection and genetic analysis of signalling molecule, N-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) produced by *V. fischeri* was first reported in the year 1981 and then in 1983 [23, 25]. For a considerable time, they believed that AHL-based QS was confined to marine bacterium i.e., *Vibrio fischeri* and *Vibrio harveyi*. However, later studies on human pathogen, *Pseudomonas aeruginosa* also revealed similar AHL based QS system as in *V. fischeri* [27].

In nature, bacterial communities exist in diverse species with varied functionalities collectively presenting beneficial and pathogenic interactions due to successful intra- and inter- species communication. Auto inducers (AI-2) and its synthase LuxS, exist in over 40 species of Gram-negative and Gram-positive bacteria, and is universal interspecies chemical language. Bacteria communicate within the species, within the genera and also cross kingdom communication happens by process of QS. There are various traits under QS control in microorganisms and are mostly beneficial to the community as a whole. Traits that are under QS control are virulence, biofilm formation, antibiotic and biosurfactant production, in addition to other developmental features. In this chapter, we explain the significance of QS systems regulating production of biosurfactants and biofilms.

Quorum Sensing

To appreciate the bacterial cross talk or QS system, a paradigm shift of understanding bacterial world from unicellular to multicellular behaviour is important. In view of understanding the bacterial language different diffusible signalling molecules such as pheromones or autoinducers were studied and reported [68]. With the increase in population density, the biosynthesis and release of QS signals into the external environment also rise and ultimately reach a threshold concentration that activates the signal transduction cascade leading to the induction or repression of QS target genes. Mostly, ‘quorum’ (the size of the bacteria) is not considered to be constant and dependent on production and loss of signal molecules under the influence of various environmental conditions. Apart from the population threshold, QS is also explained in terms of ‘diffusion sensing’, ‘compartment sensing’ or ‘efficiency sensing’, where the signals or language of bacteria depend on the local environment and spatial distribution of cells [35, 66, 96]. QS systems facilitates

systemised population density behaviour, enhancing nutrient access and defense from the competitive microbial communities to withstand population survival threats. Henceforth, the QS system and its signalling molecules have potential use in biotechnology, pharmaceutical and agricultural industries.

Signalling Molecules

Prokaryotes and eukaryotes have varied signalling and communicating molecules. In intra-species QS, the signalling molecules reported are N- acyl-homoserine lactones, fatty acid derivatives like 3- hydroxypalmitic acid methyl ester (Gram negative bacteria), γ- butyrolactones from *Streptomyces* spp. (in Gram positive bacteria) and cis- unsaturated fatty acids (in Gram negative bacteria), cyclic peptides, dipeptides (Gram positive bacteria) and isomers of methyl- 2,3,3,4- tetrahydroxytetrahydrofuran (both Gram positive and negative bacteria) [68]. In inter-species QS, the signalling molecules reported are majorly AHL's i.e., short chain AHL like C4 HSL, N- butanoylhomoserine lactone [24, 76, 94], long chain AHL's like 3-oxo-C6 to C12-HSL [95], peptide lactones and peptide thiolactones [22]. For inter-kingdom QS signalling, the molecules produced by bacterial species in response to eukaryotes are, 3-oxo-C12-HSL [36], 2-alkyl-3-hydroxy-4-quinolone [92], autoinducer 3/epinephrine/norepinephrine whose structure is not elucidated yet and further studies are required [68].

Autoinducers in Gram Negative Bacteria

Acyl homoserine Lactone (AHL) and fatty acids and their derivatives are the most chronicled autoinducers in Gram negative bacteria. AHLs is a five-carbon molecule with two hydroxyl groups and varied side chain. This is the most common structure found in cell-cell interaction universally i.e., intra as well as interspecies, produced by Gram negative bacteria and involves biosynthesis of N-acyl-L-homoserine lactones, by AHL synthases. These AHLs are small neutral molecules, which can move in and out of cells and upon reaching certain threshold binds to specific receptors belonging to a large class of DNA-binding transcription factors called "R-proteins". This binding (complex Receptor-AHLs) regulates the expression of specific genes involved in assorted bacterial behaviour [48]. In Gram negative bacteria there are three elements: LuxI (synthase), LuxR (R proteins) and acyl-L-homoserine lactone which were first discovered in *V. fischeri* while investigating bioluminescence phenomenon [57]. This system is now the reference for cell-cell interaction based studies. AHL molecules moves through the cell membrane and forms high cell density pool. This higher molecular pool leads to binding of *Lux-R* like proteins to their connected or associated AHL molecules which further leads to transcription of specific genes. Various communicating molecules are synthesized by certain bacterial

species like AHLs by *Erwinia, Pseudomonas*; 3-OH PAME (3-hydroxy palmitic acid methyl ester) by *Ralstonia solanacearum* and α, β unsaturated fatty acid (cis-11-methyl-2-dodecanoic acid) by *Xanthomonas campestris* [30].

Autoinducers in Gram Positive Bacteria

Autoinducers in Gram positive bacteria are ring like structure constituting of amino acids, in which carboxyl termini is covalently bonded to amino termini of other amino acid and generates cyclic structures. Gram-positive bacteria primarily utilise altered oligopeptides as autoinducers [(autoinducer peptides, (AIP) or QS peptides] [62]. These oligopepides are genetically encoded and are synthesized ribosomally within the cell and each species of bacteria is capable of producing a peptide signal with a unique sequence. A membrane associated ATP binding cassette (ABC) transporter facilitates the secretion of the AIP. Due to increase in bacterial population the AIP concentration increases and after reaching threshold, the AIP binds to membrane-bound receptor [41, 89]. Since the bacterial species are different, the nomenclature of the QS mechanisms could be diverse too, due to the diverse genes and receptors. For example, *Streptococcus pneumonia*, use ComD/ComE to control competence development [81] and *Staphylococcus aureus* use AgrC/AgrA which regulate the pathogenesis process and is under QS regulation [29].

Signalling Molecules by Fungi

Farnesol, produced by *Candida albicans*, is the first signalling molecule reported in eukaryotes and its structure is 15 carbon isoprenoid alcohol. Oxylipins are oxygenated fatty acid produced by fungi for fungal-mammalian interactions. The signalling pathways or receptors in fungi are not a simple cell-cell communication like in bacterial species, it is complex and hence difficult to study. Hence the signalling cascades controlling the gene expression under QS regulation in fungal species is not well studied yet. Farnesols regulates filamentation in *C. albicans*, such as the Ras-cAMP-PKA pathway and the general repressor TUP1 [33, 42, 78]. Farnesols interferes with the activity of the Ras-cAMP-Efg1 signalling cascade and responsible for hyphal formation [11]. Induction of oxidative stress depends on Ras1-adenylate cyclase signalling pathway [18]. It effects cAMP signaling and isoprenoid pathway to inhibit adenylyl cyclase activity in *C. albicans* [32]. Gene expression in presence of farnesols leads to upregulation of genes which are exclusively regulated by Mitogen-activated protein kinase (MAPK) pathway [78]. The farnesols cannot hinder filamentation or biofilm formation in *C. albicans* and mutants that lacked Chk1p (histidine kinase mutant) did not show QS signalling [45].

Mechanisms Controlled by QS

Quorum sensing regulates varied mechanisms such as motility, sporulation, virulence, antibiotic synthesis, cell differentiation, nutrient flux, biosurfactant and biofilm formation etc. Nutritional parameters and motility linked with biosurfactant and biofilm are explained below. However, quorum sensing mechanisms related to biosurfactant production and biofilm formation are discussed in next section.

Nutrient Flux

Nutrients are the essential elements required for growth, metabolism and regulatory networks in microorganisms. Bacteria exhibit different growth patterns and growth rates based on the availability of nutrients. For example, in *Pseudomonas aeruginosa*, the critical regulators of virulence, biosurfactant production and biofilm formation are governed by the availability of the nutrient components. Production of biosurfactants is governed by the nutrient availability i.e., C and N sources [69]. One of the most reported biosurfactant rhamnolipid by *P. aeruginosa* is produced at high C: N ratio, high cell density, N exhaustion and stress condition [67]. The operons involved here are *rmlBDAC* and *rhlAB* whose transcription and posttranscriptional modifications are regulated by factors associated with QS system.

The regulation of biofilm formation depends on certain metabolic pathways like acetate metabolism, magnesium transport glutamate production, arginine metabolism and the tricarboxylic acid cycle [102]. In *Burkholderia glumae,* it was hypothesized that the QS modulates glucose uptake through *ptsI* gene, and growth rate in *B. glumae* (*tofI* mutant BGS2) was rapid in contrast to the wild type [39]. Another study suggests that in *P. aeruginosa* nutrient inflow and waste outflow is regulated by QS. Similarly, in *Agrobacterium tumefaciens* diffusible QS molecule stimulates Ti plasmid conjugation along with TraR regulator and activates *tra* genes expression for opine synthesis [15]. Therefore, nutrient concentration and intracellular carbon flux plays a important role in altering the QS based expression of cellular properties that are vital for survival and metabolism of bacteria.

Motility

Bacterial motility is the ability of a bacterial cell to move from one place to other with the help of organelles like flagella and the different types of motility include swimming, gliding and swarming. QS system in particular, controls swarming motility which is movement of cells in an associated manner on solid surfaces demonstrated in *Azospirillum, Aeromonas, Burkholderia, Bacillus, Chromobacterium, Clostridium, Rhizobium, Vibrio, Yersinia, Sinorhizobium, Serratia, Salmonella* etc.

[75]. Swarming is hypothesized to rely on QS system and the signals are sensed and transmitted by two-component regulatory systems. Swarmer cells are noted to be hyper flagellated and allow cells to move in groups and increases cell-cell contact and available surfaces are colonized [37]. Studies also reveal that in nutrient limited conditions, AHLs modifies cellular motility and virulence of *Erwinia chrysanthemi* pv. [38] and swarming motility and biofilm formation in *P. aeruginosa* [72]. Phenotype regulated by AHLs in *Rhizobium* species includes motility, biofilm formation, production of antibiotic, exo-enzymes, auxins, indole acetic acid, root colonization and biological control [10, 93]. In soil bacterium, *Serratia liquefaciens* swarming motility is regulated by AHLs or other regulator genes such as *surR or synthase swrI* [49], QS system LuxS/AI-2 [1] in *E. coli* K-12, increases movement and stimulates biofilm production [30]. Likewise, very recent experimental evidences suggest the increased concentration of 3-oxo-C_8 -HSL molecules increases motility in *Rhizobium etli* RT1 [75]. In addition, the swimming motility in *Vibrio harveyi* (in wild and mutant types) involved QS regulator LuxR, which was demonstrated through motility assays and gene expression studies [97]. This explains that nutrient status (chemical signals), motility, virulence, biofilm formation are interdependent on cell communication.

Biosurfactant Production

Quorum sensing molecules regulate various physiological processes; one among them is biosurfactant production. Biosurfactants are amphiphillic compounds released out of the cell or membrane bound. They reduce interfacial and surface tension by aggregating or accumulating organisms between phases. Biosurfactants are categorized as: glycolipids, lipoprotiens, phospholipids, fatty acid salts and polymeric biosurfactants [9]. Three mechanisms i.e., induction, repression, nutrient element based regulation of biosurfactant production [17] of which induction is the general regulation mechanism which controls the onset synthesis of lipopeptide biosurfactant [8, 44, 84].

Quorum Sensing and Biosurfactant Synthesis

Biosurfactant production in *Pseudomonas putida* is regulated by *ppuI, rsaL and ppuR* genes. It was reported that *P. putida* PCL1445 produces, two lipopeptides, putisolvin I and II, consisting of a C 6 lipid moiety and a 12-amino-acid peptide, are synthesized by putisolvin synthetase gene designated as *psoA* [46]. Strains with mutations in ppuI and ppuR were generated which showed decreased expression of ppuI, which was complemented by synthetic AHLs. QS molecules include, C4-HSL for Rhamnolipids [22] and 5-amino peptide Glu-Arg-Glu-Met-Thr for lipopeptide biosurfactant production [64].

Quorum sensing based biosurfactant studies, include, *Bacillus subtilis comA* and *comP* that regulates surfactin production via 5-aminopeptide-Glu-Arg-Met-Thr (Acyl AHL quorum sensing molecules). Biosurfactant, Serrawettin W2 is synthesized by *swrI and swrR* genes via N-butanoyl and N-hexanoyl HSL in *Serratia liquefaciens*. In Pseudomonas aeruginosa rhlI and *rhlR* genes regulates quorum sensing molecules N-butanoyl HSL whereas genes *lasI, lasR-rsaL* regulate oxo-dodecanoyl HSL and result in the synthesis of biosurfactant, rhamnolipid. Rhamnolipid synthesized in *P. aeruginosa* has *rhlABR* gene cluster that synthesizes RhlR regulatory protein and a rhamnosyl transferase. Later Ochsner and Reiser identified *rhlI* regulatory gene, located down-stream of the *rhlABR* gene cluster. In RhlA, 32.5 kDa protein harbours a putative signal sequence, while RhlB, 47 kDa protein has at least two putative membrane-spanning domains. Active rhamnosyl transferase complex is located in cytoplasmic membrane with RhlA protein. Regulatory gene *rhlR* encodes transcriptional activator, RhlR protein which is 28 kDa and belong to LuxR family and gene *rhlI* encodes autoinducer synthase which synthesizes autoinducers. The regulatory protein is activated upon binding to autoinducer. The binding of activated regulatory protein to rhlA promoter initiates transcription of rhlAB operon encoding rhamnosyl transferase. Regulatory protein RhlR also controls *rhlI* gene expression [17].

Significance of Biosurfactants

Biosurfactants produced by one organism can be utilized by itself or other bacterial species or strains for various purposes like restoring deficient motility, changing viscosity of the surfaces, etc. with the aid of quorum sensing. For example, in vitro mutational studies in *P. aeruginosa* revealed that the mutant that do not produce biosurfactants, have decreased growth, and it was restored upon addition of rhamnolipid [64]. Biosurfactants are commercially used in food processing and preservative industries due to their properties like low toxicity, high surface or interfacial activity, excellent biodegradability, thermotolerance, chemical stability, product of renewable resources and ability to form microemulsions. Because of most of the above discussed properties of biosurfactants like rhamnolipids, USEPA approves its uses for human consumption and industries. Not only the above said properties biosurfactants also possess antimicrobial property, which can be used in increasing shelf life of food products with minimum concern for human health. Biodegradation of hydrocarbons by natural microbial population via quorum sensing is the primary mechanism by which bioremediation is carried out. Biosurfactants induce resistance in plants there by reducing infections and also play a role in non-specific immune responses. Examples include biosurfactant capable of stimulating defense genes in tobacco, protect monocotyledons against biotrophic fungi, inhibiting zoospore forming plant pathogen that are resistant to chemical fertilizers [73]. Amphisin, a cyclic lipopetide, is QS regulated and displays antifungal activities against *Pythium ultimum and Rhizoctonia solani* [2].

Biofilm Formation

Biofilms are tightly enclosed, highly organized polysaccharide matrix structures formed by the microbial communities. From the previous studies it is known that, the triggering of genes for biofilm production is directly related to nutrient availability and presence of QS molecules [26]. During this biofilm formation the microorganisms undergo profound change from free swimming form to a complex surface attached community. Certain reports also suggest, this bacteria and fungi have certain machinery for cell to cell-communication, biofilm formation and maturation and back to their original forms [60]. Bacteria synthesize organic soluble microbial products (SMP) and insoluble concrete exopolysaccharides (EPS). The EPS can reinforce the structure of biofilm which is interconnected with polymers within microbial communities [6]. In biofilms, the bacterial attachment in the beginning is reversible due to weak vander Waals forces in the colonies which can be removed by detergents, and upon continued adherence the anchorage becomes permanent by adhesins, proteins etc. Variables effecting biofilm formation includes type of cell membrane, hydrophobicity, appendages, EPS-substratum, texture, physicochemical traits etc- flow velocity, pH, temperature, presence of ions and antimicrobial agents in bulk fluid [20]. Many bacterial genera are involved in biofilm formation viz., *Escherichia coli, P. aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, Enterobacter cloacae, Klebsiella pneumoniae, Actinomyces israelii, Haemophilus influenza, Burkholderia cepacia* [56]. Autoinducer-2 molecules are globally reported for biofilm formation [40] which is structurally determined by NMR as 2-hydroxy-4-[(methyl amino) (phenyl) methyl] cyclopentanone (HMCP) [47].

Quorum Sensing and Biofilm Formation

Pseudomonas sp, is a well researched bacterium, considered for studying various biological processes, like biofilm synthesis via different pathways, one of which is QS [14] involving AHL's [92]. The studies on QS mutant strains of *P.aeruginosa* i.e., lasll/rhll and lasR/rhlR and pilA and fliM revealed that biofilm formation is dependent on carbon source and swarming motility respectively. In *P. aeruginosa* there are three QS systems i.e., las, rhl and quinolone signal system (PQS system). In the las and rhl QS system the autoinducers signaling molecules N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) are produced through LasI and RhlI and perception through transcription factors LasR and RhlR, whereas in PQS system, quinolone signals interact with AHLs intricately [83]. Biofilms are observed in different walks of life, by default these are formed by many microorganisms which are both beneficial and harmful to biotic and abiotic entities on earth.

Significance of Biofilms

Microorganisms forms an exopolymeric coat on abiotic surfaces in hospital environment and responds poorly to chemotherapy and significantly contributes to emergence of drug resistant strains. The commonly found pathogens causing nosocomial infections on devices like catheters, tubes, implants, valves etc. are, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Proteus mirabilis*, *Diptheroids*, *Streptococcus* spp., *E. coli*, *Clostridium perferingens*, *Neisseria meningitides*, *Corynebacterium*, Enteric Gram negative bacilli, *Peptostreptococci*, *Enterococcus faecalis*, *Candida* spp. [51]. These pathogens cause urinary tract infections [3], middle ear infections, chronic otitis [34], cystic fibrosis, chronic rhinosinusitis [55], dental plaques and native valve endocarditis [51].

Significance of biofilms in ecological studies is quite vast and appreciative. Role and activities of bacterial biofilms in association with plants and in soil is profound. In plant microbial association, biofilms are formed both on the root and shoot systems. On the aerial plant parts, the biofilm consists of less cell density than compared to biofilms formed on root system. These biofilms help in over all growth of plants, in nutrient uptake, water adsorption and protection from pathogens. Rhizosphere and phyllosphere microflora like *Pseudomonas* spp., *Bacillus* spp., *Aspergillus* spp., *Streptomycetes* spp. can be considered as examples. For example, *Pseudomonas fluorescens* is reported to form coat on plant roots and protect against phytopathogens [61]. In contrast to this, the microorganisms forming biofilms can also become opportunistic and cause infections to the crops effecting the yield and plant growth leading to its death. Example PA01 and PA14 strains of *P. aeruginosa* which when inoculated on *Arabidopsis* and Sweet basil plants in vitro and in soil, lead to extensive damage of tissues in root and shoot systems and caused plant death [90]. Biofilms also helps in maintaining the soil structure by holding the soil particles and retention of soil nutrients, moisture and microflora.

Bacteria – Plants: QS and Biofilm Formation

Microorganisms or the plant viable microbiome is equally dynamic and employ various mechanisms to cope with biotic and abiotic stress as the plant system. The plant root exudates include many small molecular weight compounds [7] and studies by group of researchers detected QS mimicking root exudates in rhizosphere region [43, 53, 80]. Furthermore, the methanol extracts of pea root exudates are found to be structurally similar to Gram negative AHLs [80], similarly tobacco and potato rhizosphere region is found to have AHLs [71, 82]. Apart from AHLs the root exudates are found to influence biofilm formation by inhibiting the bacterial QS mechanism [65]. The chemical and molecular evidence for QS inhibition is reported

in a marine red alga [98], which produces structural analogs of AHLs and halogenated furanones, which competitively bind to specific receptors leading to proteolytic degradation and inhibition of associated QS signals [79].

Similarly the production of AHL lactonases by the soil bacterium *Bacillus thuringiensis* degrades AHLs [19]. Previous studies explains AHL responding bacterial cells and AHL producing host root cells need not be in close vicinity [28, 63]. As of now except for AHLs/AHL like molecules secretion, there are no evidences suggesting that plants secrete or utilise AHL-degrading enzymes which hinders bacterial association. However there are possibilities of engineered plants which produces QS degrading enzymes considerably changing the scenario of root-pathogen interactions [19].

Microbes: Biosurfactants and Biofilms

In some microorganisms, biosurfactant production is essential for the biofilm development, examples include synthesis of rhamnolipid and biofilm formation in *Pseudomonas aeruginosa*, surfactin and biofilm formation in *Bacillus* spp. [64]. In contrast, for strain *Pseudomonas putida* that produces biosurfactants putisolvin I & II inhibits development of biofilm and breaks the formed biofilms. Similarly, an rsaL mutant overproduced AHLs, and putisolvins production was induced early. The determination of biofilm development as a carrier inferred that ppuI and ppuR mutants were capable to form thicker biofilm compared to the wild type PCL1445, constituting to low putisolvins production, and early putisolvin production is attributed to delay in biofilm development by rsaL mutant [21]. This explains the relation of QS signals for biofilm and biosurfactant production. The Table 1 below mentions recent examples of microorganisms and the QS systems.

Conclusions

Microorganisms have their own social network interactions called as QS, wherein bacteria extracellularly produces and identifies the chemical signals, gauging cell number and regulation of gene. The bacteria cross talk is used to modulate vital characters as biosurfactant and biofilm development, horizontal and catabolic gene expression, synthesis of exopolysaccharide, chemotaxis and movement. Hence, there is a need to understand and decode the bacterial language in terms of biofilms and biosurfactants looking at the enormous practical applications in agriculture, bioremediation and health.

Table 1 Microorganisms and QS systems responsible for production of biofilm and biosurfactant

Sl. No	Bacteria	Quorum sensing molecule/ mechanism	Impact	References
1.	*Serratia* sp.	C6-HSL and *N*-(3-oxohexanoyl) homoserine lactone	Prodigiosin pigment production, swarming motility and biofilm	[54, 70, 91]
	Serratia marcescens SS-1	Lux R	Biosurfactant	
2.	*B. subtilis*	*Spo0A, 0B, 0H, 0F; yveQ, R; kinC, D, E; yhxB; sipW; yqxM; ecsB; yqeG* to *M; ylbF; ymcA; tasA; com k;*	Synthesis of antibiotic, degradative enzymes; plant growth promotion, resistance against various pathogens	[5, 74, 87]
3.	*P. aeruginosa*	*AHLS-GacS/A* and *RetS/ LadS; las; rhl; pelA* to *G*	Therapeutic application for developing drugs/antibiotics/ rhamnolipid	[22, 83, 100]
		N6P6 strain-AHLs	Bioremediation	[58]
4.	*Streptococcus mutans*	Signalling peptides- *sgp; dgk; ccpA; brpA; comACDE*	Bacteriocins production	[99, 101]
5.	*Salmonella enterica*	LuxS	Food and water borne infections	[77]
6.	*C. albicans*	Farnesol	Future antimicrobial therapies to control drug resistance infections	[16]
7.	*A. tumefaciens*	AHLs-*traR&I*	Crown gall-tumor inducing plasmid	[103]
8.	*P. fluorescens*	mass A, B, C; nrps	Antibiotic production	[12, 13]
9.	*P. putida*	*bscA to H, Q, Z*; *galE*, *peaA* to *I*	Help plant cope with water and solute stress	[59]
10.	*Lactobacillus* sp.	Agr (accessory gene regulator)	Probiotics	[52]
11.	*Yersinia* sp.	AHLs- Lux	Bioluminescence, antibiotic biosynthesis	[4]
12.	*Staphylococcus* sp.	Extracellular matrix synthesized by *ica* genes	Biofilm formation on catheters and medical devices causing nosocomial infection	[85]
13.	*Rhizobium* sp.	*cinI*	Effects growth, nitrogen fixation	[50]
14.	*Burkholderia* sp.	*AHLs*	Siderophore production, reduces ROS synthesis	[86]

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Quorum Sensing in Microbes and their Function in Modulating Antibiotic Synthesis

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Abstract The intercellular communication, commonly called as Quorum Sensing (QS) or auto induction, has been attributed for various coordinative and community phenomena in Gram positive and Gram negative bacteria. The signalling is facilitated by diffusible signal, auto inducers, in response to population of neighbouring bacteria. Consequently, QS influences bacterial phenotype such as the production of antibiotics. Current understanding of how bacteria mediate antibiotic synthesis in the natural environment is limited to classical quorum sensing receptors and 'orphan' quorum sensing receptors. The genetic studies and biochemical investigation of carbapenem synthesis in *Serratia* and *Erwinia carotovora* have acknowledged a group of nine genes complex in the assembly namely carRABCDEFGH which are responsible for antibiotic assembly. N-(3oxohexanoyl)-L-homoserine lactone (OHHL) is produced as a product of the independent carI gene activates CarR transcription factor. This OHHL reliant transcriptional activation permits the cells to synchronise expression of carbapenem with cell density. An orphan quorum-sensing receptor, discovered as the soil bacterium *Burkholderia thailandensis,* differs from classical quorum sensing as this receptor does not respond to characteristic quorum sensing signalling partners. The orphan receptor however responds to antibiotics, such as trimethoprim and sulfamethoxazole. Consequently, eliciting the expression of the genes malA-M which is involved in synthesis of the cytotoxic antibiotic malleilactone. This controlling pathway might be vital to sense and compete in mixed communities. The synthesis of antibiotic is very expensive for the bacteria therefore the induction and modus of induction is tightly regulated. This work is an understanding of the current view of quorum sensing and their function in modulating antibiotics synthesis.

Keywords Quorum sensing · Auto induction · Antibiotic synthesis · Orphan receptors

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Introduction

The intercellular communication, commonly called as Quorum Sensing (QS) or auto induction, has been attributed to various coordinative and community phenomena in Gram positive and Gram negative bacteria [1]. The signalling is carried out by diffusible signal, auto inducers, in response to population of neighbouring bacteria. Bacteria guard the expression of specialized gene sets in response to their cell density [2]. The bacteria sense various environmental factors such as the magnitude of their population by sensing the amount of tiny, diffusible, signal molecules termed as auto-inducers as they are produced by self synthesis. These molecules mediate signalling by secretion and reabsorption into the cells by facile diffusion among other processes. These signals thereby accumulate in the extra cellular environment as a function of the growth of the cells. At certain critical concentration which corresponds with a threshold population density, these signals serve as co-inducers to regulate transcription of target genes. The output of which presumably are of asset to the bacteria in the particular habitat. Consequently, QS influences bacterial phenotype such as the production of antibiotics.

The phenomenon of quorum sensing was explained by studying the bioluminescent bacteria *Vibrio fischeri*. The symbiotic bacteria is commonly found in marine hosts such as fish and cephalopods and is bioluminescent when their number reach threshold population [3]. The study of the bacteria helped decipher the molecular mechanism. The bacteria release its auto inducer, *V.fischeri* auto-inducer (VAI) during their growth in the marine host's light organ. Upon reaching the threshold population, *Vibrio fischeri* auto-inducer activates the transcription of the lux operon using a transcription activator, LuxR [3]. At the necessary concentration of VAI, VAI and LuxR interact and consequently convert LuxR into a function transcriptional activation factor [4]. The functional active LuxR activates the lux operon. LuxI is the first gene on the operon, the protein product of the gene catalyzes the synthesis of VAI [4]. This auto inducer mediated amplification of the auto induction allows increased amount and amplification of the transcription of the gene in the target operon. Thus, the auto inducer increases its production in accordance with the population and bacterial dependent production of desired target gene on the operon.

Quorum sensing has been exhibited to regulate different genes in many bacteria of different genera due to continuous and sustained study. The extensive study has allowed characterization of several genes such as *Agrobacterium tumefaciens* which regulates conjugal transfer of plasmid by quorum sensing. The conjugal transfer is controlled by using the transcriptional activator TraR and the diffusible molecule AAI [5]. Pseudomonas aeruginosa regulates several gene systems by systems control genes, those include genes needed for the production of rhamnolipids, and extracellular enzymes and toxins [6]. The genes are regulated by two quorum-sensing systems; every system has has its own cognate transcriptional regulator and diffusible signal molecules. Another pseudomonad, *P. aureofaciens*, monitors production of phenazine antibiotics by quorum-sensing [7]. These antibiotics have been implicated in the disease of wheat suppressive disease. *Erwinia carotovora* are

plant pathogens which guarded the expression of pathogenic traits by quorum sensing systems. [8] *Serratia* is also mediates quorum sensing which regulates the synthesis of antibiotic carbapenem and pigment prodigiosin [9]. Literature and studies indicate the similar regulatory strategies are employed by the bacteria albeit different target genes are controlled by the auto induction differ in accordance with the bacterial requirements.

Current understanding of how bacteria mediate antibiotic synthesis in the natural environment is limited to classical quorum sensing receptors and 'orphan' quorum sensing receptors [6]. The molecular level investigation of carbapenem synthesis in both *Serratia* and *Erwinia carotovora* have reported a group of nine genes engaged in the synthesis of the antibiotic (carRABCDEFGH) [9]. N-(3oxohexanoyl)-L-homoserine lactone (OHHL) synthesized by the product of carI gene which activates the transcription factor named as CarR. This transcription factor permits the cells to work in density depended manner in order to express carbapenem. Another quorum sensing receptor also known as orphan receptor has been discovered in the *Burkholderia thailandensis which is a soil bacterium* [10], differs from classical quorum sensing as this receptor does not respond to common quorum sensing signalling partners. Infact, these receptors respond to selective antibiotics, such as trimethoprim and sulfamethoxazole [10]. Consequently, expression of the genes mal, involved in synthesis of the cytotoxic antibiotic malleilactone is triggered. This regulatory pathway may be important to sense and compete in mixed communities. The synthesis of antibiotic is very expensive for the bacteria therefore the induction and modus of induction is tightly regulated.

The present work explains the classical quorum sensing mechanism as described in various bacteria. The auto inducer and their homologues control the target gene on the operon. The work will focus on the antibiotic synthesis as a result of the quorum sensing by the bacteria. The antibiotic synthesizing gene attributes survival of the population in a mixed bacterial community and also pathogenicity in certain strains of bacteria.

Auto Induction in *V. fischeri*

The *V. fischeri* auto inducer (VAI) is 3-oxo-N-(tetrahydro-2- oxo-3-furanyl) hexanamide also known as N-3-(oxohexanoyl) homoserine lactone. The auto induction phenomena of this species help them to distinguish between the plantonic and colonized bacteria within the host and thus induces the luminescences system in the density depended manner only when the bacteria is interacted with the host. At intracellular critical concentration on the order of 10 nM the transcription of the luminescent genes are activated. The bacteria passively diffuse the auto inducer, VAI at low cell densities. On reaching the threshold population VAI accumulates in the bacteria equivalent to the extracellular concentration. In presence of VAI Lux operon transcription activator LuxR is activated. LuxR is encoded by one unit of the lux operon. It is a 250 amino acid protein which in the presence VAI transcribes

luminescent genes encoded in the other unit of the cluster. The cluster is divided into two units whose start sites separated by about 150 bp. One unit encodes luxR and the other unit is the operon, luxICDABEG [11]. The LuxR binding site or the lux box has a dyad symmetry is about −40 bp upstream from the start of luxICDABEG transcription. The sequence is required for luxICDABEG activation by LuxR and for positively or negatively auto regulate luxR depending on the VAI and LuxR levels in the cell. As a consequence of the cellular accumulation of VAI and the activation of LuxR luxI gene is transcribed. The luxI gene coded for a 193-amino-acid protein is considered to be an auto inducer synthase as the LuxI catalyses synthesis of VAI leading to its amplification [12]. The other genes in the luxIC- DABEG operon carried out the synthesis of light. lux4 and luxB code for the a and b, subunits of luciferase. luxC, luxD, and luxE encode for components of the fatty acid reductase complex required for synthesis of the aldehyde substrate for luciferase. luxG might code for a flavin mononucleotide reductase that produces reduced flavin mononucleotide as a substrate for luciferase [3]. This genomic cluster shares homologues in other bacteria engaging in quorum sensing and encodes gene required by the bacteria for desired traits.

Auto Inducer

Autoinducer are the small molecules releases in respond to population depended manner. Majority of them are N-acylated derivatives of L-homoserine lactone (acyl-HSLs).The synthesis of these molecules is carried by different organism which varies in the length, the positioning of 3-carbon and the availability of unsaturated bonds within the acyl side chain.The illustration can be well explained by the autoinducers of VAI and Agrobacterium species. The autoinducer of VAI is N (3 oxo hexanoyl) L HSL whereas the N(3 oxo octanoyl) L HSL belongs to the Agrobacterium species. The another example of different nature of AI can be well understood by closely observing the difference in *Chromobacterium violaceum* which manufactured the N hexanoyl L HSL, while the AI produced by isolates of Rhizobium spp. is N-(3-hydroxy-7-cis-tetradecanoyl)-L-HSL.

Orphan Receptor

LuxR proteins are transcription factors that are activated by acyl-homoserine lactone (AHL) signals. Recent studies into the signalling molecules and their receptors have identified conserved LuxR family protein, MalR in *B.thailandensis* which induces genes independently of AHLs. MalR is needed for expression of genes coding for synthesis of the cytotoxic malleilactone [10]. The mal genes are induced to synthesize MalR which consequently synthesize malleilactone needed few antibiotics, such as trimethoprim. The mechanism needs to be elucidated of the pathways

that the antibiotic trimethoprim employs to increase malR expression. At sufficient amounts of malR expression, MalR functions solely of any external signal. It has been reported that the muted biosynthetic genes of malleilactone get activated by MalR which works in a AHLs independent manner.The absence of cognate ligand lends the name orphan receptor to the receptors [6].

LuxR, Transciption Activator

LuxR is an important transcription activator although no homologues have been identified in vitro. It has two domain polypeptide containing GroEL and GroES which folds into an active conformation [1]. Mutational studies and analysis of the LuxR polypeptide identified regions which were responsible for its function. Amino acids at the N terminus mainly residues 10–20 have an inhibitory role which is reversed upon auto inducer accumulation [3]. The amino terminus is responsible for downregulating of the luxR gene but isn't responsible for the binding to the lux box or activation of luxICDABEG. The mutational studies further showed that removing residues 2–156 results in an increase of an auto inducer-independent luxICDABEG operon transcription [12]. Thus indicates its regulatory function. In addition, the study shows that the C-terminal region, residues 190–210, has a DNA binding function through a helix-turn-helix (HTH) motif and is responsible for transcriptional activation [4]. While the amino-terminal half of the protein has an inhibitory role that is overcome by auto inducer. Single-amino-acid mutations between residues 79 and 127 affected the interactions with VAI. VAI binds to LuxR by a multimer formation by the residues in the region of 116–161 and subsequent binding to the lux boxes [13]. The studies further showed that the residues 230–250 may not be required for DNA binding but they may be needed for activation by making contacts with RNA polymerase.

LuxI

The luxI gene encodes the protein LuxI which amplifies the accumulation and synthesis of VAI in both *V. fischeri* and *E. coli* [3]. The function makes a strong case for the gene to encode VAI synthase. It has been shown that The LuxI protein catalyze the production of VAI from S-adenosylmethionine and 3-oxohexanoyl coenzyme A but the amount of 3-oxohexanoyl coenzyme A needed to saturate the enzyme activity are high, suggesting that 3-oxo- hexanoyl acyl carrier protein to be the obligatory substrate for VAI synthase [14]. Production of *V. harveyi* autoinducer, HAI uses the D isomer of its fatty acid precursor indicating that the acyl moieties of AI are derivative of intermediates lipid biosynthetic pathways [13].

Homologous Systems

Several bacteria contain homologous system of quorum sensing which act through LuxI and LuxR homologues for desired traits. Literature indicates systems for antibiotic synthesis in several bacteria such as *Erwinia carotovora*.*It*is a plant pathogen which colonizes vascular tissues of host plants. The bacteria produces cell wall-degrading exo-enzymes required for virulence [15]. The study of bacteria led to the identification of LuxI homologue ExpI. Quorum sensing by the bacteria *E. carotovora* is mediated by an auto inducer identical to VAI. VAI induces synthesis of the antibiotic carbapenem in *E. carotovora* [8]. While no LuxR homolog have been identified and shares sequence similarity with LuxR open reading frame downstream from expI. This indicates it encodes VAI dependent regulator.

The synthesis of antibiotics in Streptomyces spp. by quorum sensingis depend upon the molecule called butyrolactones which is similiar to VAI. A factor from *Streptomyces griseus* regulates a number of secondary metabolic processes including streptomycin synthesis thus the butyrolactone-mediated regulation seems similar to auto inducer-dependent [16]. It is showed that the LuxR-type proteins activate transcription but the A-factor receptor is a repressor thus is not homologous to LuxI.

Apart the canonical quorum sensing cascade to synthesize the secondary metabolite, the antibiotic has several functions. In certain systems they act as signals while in certain systems their induction deviates from the classical LuxR-LuxI system. Such systems are important indicators of different modalities of quorum sensing and the role of secondary metabolites.

Erwinia carotova

Erwinia carotova is a plant pathogen which releases exo-enzymes to degrade plant cell wall, it also releases beta- lactam antibiotic to compete against other bacterial population in a mixed community [17]. The release is cell number is dependent, which enables the pathogen to mount a sustained and successful attack on the host. The synthesis of the antibiotic is regulated by quorum sensing wherein the antibiotic synthesis follows the LuxR system [18]. The auto induction mediates beta-lactam (carbapenem) synthesis on intracellular accumulation of auto inducer *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) [9]. The accumulation of HSL intracellularly mediates the amplification by LuxI homologue CarI. CarI mediates synthesis of 3-oxo-C6-HSL consequently the HSL binds to LuxR homologue CarR. Studies show unequivocal evidence of the LuxR superfamily protein being receptor of HSL ligands [8]. LuxR homologue, CarR attains its active form on HSL binding. This binding activates the CarR transcription activator mediated activation of car genes [17]. The binding of HSL activates the CarR to its active protein form due to its intrinsic tendency to multimerize. The activation induces the transcriptional activation of genes [8].

Serratia also produces beta-lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid [18]. Extensive study into the genetics of the antibiotic synthesis has identified a cluster of nine genes (barABCDEFGH) [9]. Functions have been assigned to all the genes in the cluster except carH. The gene cluster encodes the enzymes involved in the beta lactam biosynthesis, a novel beta-lactam resistance machinery and also a positive regulator of the carbapenem gene. carR in the gene cluster encodes CarR which is LuxR family homologue [18]. Thus lactone auto inducer N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) synthesized by the unlinked gene product CarI activates CarR which in turn activates the transcription of the remaining genes in the cluster. Thus the auto inducer dependent activation coordinates the expression of carbapenem with cell density and therefore called quorum sensing. The production of antibiotic is regulated by protein Rap in *Serratia* and Hor in *Erwinia* [9]. The regulators directly bind to the DNA and activate the genes in the stimulon. Despite, the key similarities the bacterial systems are not identical. The bacteria induce the antibiotic production depending on the growth phase in *Erwinia* while it parallel to the growth in *Serratia*. Further unlike *Erwinia, Serratia* can also induce carbapenem synthesis in response to certain pheromones.

Pseudomonas aeruginosa System, Antibiotic as Terminal QS Signal

Pseudomonas aeruginosa is a gram negative bacteria which regulates gene expression by quorum sensing. Extensive studies have identified two systems controlled by their transcription activators LasR and RhIR. These systems respond to auto inducer AHLs 3-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL) and butanoyl-homoserine lactone (C4-HSL) respectively [6]. The two systems induce gene expression by accumulating the auto inducers in the cells. The accumulation and binding of respective ligands to transcription activators activates a molecular switch to active protein. The transcription activator mediates transcription of lasI and rhII genes and other genes in the operon. The proteins LasI and RhII amplify AHLs 3-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL) and butanoyl-homoserine lactone (C4-HSL) synthesis respectively [11]. These systems are LuxR system homologues but recent studies into the bacteria revealed a third system responsible for antibiotic synthesis [2]. The signal is found to be a part of the *P.aeruginosa* quorum sensing through a quinolone 2-heptyl-3-hydroxy-4-quinolone (PQS). PQS production is regulated by quorum sensing and is similar to Pyo compounds [11]. These compounds are identified as antibiotics belonging to the 4-quinolones family. The quorum sensing mechanism of PQS is different from classical systems or LuxR systems because it is not an AHL. Further the genes responsible for the synthesis of PQS are not LuxI homologues. None-the-less, they are qualified as a quorum sensing signal as the signals are small diffusible compounds which are cell density dependent and accumulates in the cell. [7] The accumulation triggers transcriptional

response of specific genes thus PQS are defined in QS systems. The PQS system does not have canonical quorum sensing homologues but the signal and response system is in temporal cascade. AHLs are released in the early phase while PQS is released in the late exponential phase as the terminal signal in the temporal cascade [11]. *P. aeruginosa* positively regulates several virulent factors, monooxygenase and the proton driven efflux pumps of resistance nodulation cell division transporter mexGHI-opmD operon through MvfR [19]. These responses are mediated by PQS through PqsE. In addition to the virulent factors, PQS signalling produces phenazines in its response. Phenazines are heterocyclic, redox active compounds which are toxic to other prokaryotes and eukaryotes [19]. Phenazines are also released from cells at late exponential phase. Thus in the three signal system phenazine is the terminal product [7]. The quorum sensing cascade comprises of three signal which are interdependent and function in a growth stage manner. LasI and LasR synthesize AHLs 3O-C12-HSL and butanoylhomoserine lactone (C4-HSL) respectively. These signals bind to their transcription activators LasR and RhIR and in turn induce the gene expression [7]. This results in the synthesis and secretion of PQS, required for the production of phenazines. The terminal phenazine signal activates the transcription factor SoxR and induces the expression of mexGHI-opmD operon and virulent factor PA2274 [19]. The mechanism of activation of SoxR remains to be elucidated but it is found to be active in strictly anaerobic condition. Thus, the phenazine is a necessary signalling molecule to upregulate the expression of a set of gene. The genes are involved in the efflux and redox action; in addition to that they are also important in iron acquisition. *MexGHI-opmD* and PA2274 are controlled by the phenazine signal as well.

PQS on the other hand, controls the expression of the *phz* operon which synthesize phenazine. It has been shown that the production of phenazine connects expression of *mexGHI*-opmD and PA2274 to PQS. This explains the phenazines position as a terminal signal molecule in QS circuitry. SoxR controls the six genes which includes monooxygenase (PA2274) and two transporters, i.e. the RND efflux pump MexGHI-OpmD and the MFS transporter PA3718. PA2774 which is defined as monooxygenase act similar ActVA Orf6 of *Streptomyces coelicolor*, thus oxygenates actinorhodin. Actinorhodin is an aromatic polypeptide and similar to structure of phenazines [19]. The conversion of PCA to PYA is carried out by PhzS This enzyme is also involve in monooxygenase as well. Thus it clears that PA2274 might recognize phenazines, permits to work against the monooxygenase in either function as an enzymatic or act as a competitor. The PhzS might also works as a chaperone which enables the protection of the cellular environment from the toxic phenazines, or behave as receptors which sense phenazines, thereby affecting gene expression. MexGHI-OpmD is one of 10 potential RND pumps in *P. aeruginosa* [11]. It has indicated that the efflux pump is needed for the excretion of a PQS precursor. Further, it is also indicated that MexGHI-OpmD is implicit in the secretion of a toxic PQS precursor and also in the efficient to and fro of phenazines [11]

Phenazine and quinolones have been classically considered only as antibiotics due to their antimicrobial activities [7]. Recent studies add an additional dimension wherein quinolone and the phenazine PYO can function as intercellular indicators

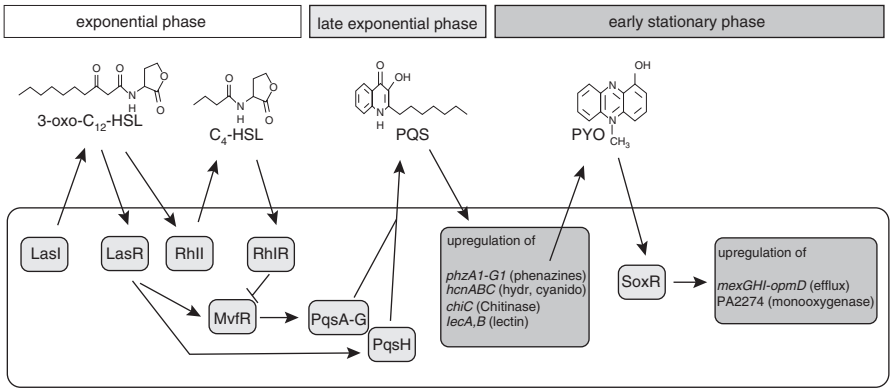

Fig. 1 Model of the network in *P. aeruginosa*. The quorum sensing system is a growth stage dependent flow of three signalling molecules wherein the LasI and LasR manufacture the AHLs 3O-C_{12}-HSL and butanoylhomoserine lactone (C_4-HSL) respectively, during the exponential phase. Consequently, interacting with transcriptional activators, LasR and RhlR respectively induce the gene expression. Which in turn potentiates the synthesis and release of PQS? PQS is mandatory for the production of phenazines and the phenazine PYO triggers the transcription factor SoxR. Thus increasing the manifestation of *mexGHI-opmD* and PA2274. (Source: Molecular Microbiology Volume 61, Issue 5, pages 1308–1321, 25 JUL 2006 DOI: https://doi.org/10.1111/j.1365-2958.2006.05306.x)

thus as a 'secondary' metabolites which maybe of significant importance adapting the cellular response to a particular physiological state [7]. The quorum sensing network is intricately designed to optimize the desired effect for a population. One sees such intricacy in phenazine production initiated under limited oxygen conditions. In such conditions when the phenazines are synthesized, they respond with oxidized species such as ferric oxide and consequently facilitate the acquisition of iron. Hence phenazine reins the countenance of genes involved in iron attainment and redox homeostasis, and genes that modulate the self-processing (*mexGHI-opmD)* [6] Fig. 1.

Thus, this bacterial system proves that the antibiotic synthesis is not just a secondary metabolite which is an important for the population survival in a mixed community or for successful host invasion, can also function as signalling during the quorum sensing cascade. Further, it can coordinate multiple responses which transcend their antimicrobial activities.

Burkholderia thailandensis, Burkholderia pseudomallei and Burkholderia mallei, Orphan Receptors

Burkholderia thailandensis, Burkholderia pseudomallei and Burkholderia mallei are associated bacteria referred in beta-proteo-bacteria category [20]. They are related with sequence identity which spans several genomic regions. Despite their

genomic identity *B. mallei* is a mammalian pathogen which obligatory chooses solipeds as host but the bacteria can infect human. *B. pseudomallei* are prevalent human infecting bacteria which are commonly found in soil and water. *B.thailandensis* is also a mammalian infecting bacteria endemic to central America. These bacteria are pathogenic in nature and quorum sensing has been known to contribute to its virulence. These bacteria have been studied for multiple quorum sensing systems. These systems are classical activated by self-produced extracellular signal that are population density dependent.

These bacteria produce acyl-HSL as their auto inducers. The signals are produced by acyl- synthases belonging to the LuxI superfamily. The acyl-HSL binds to LuxR superfamily proteins and active the transcription factor the LuxR homolog reacts effectively with the HSL synthesized by the equivalent LuxI homolog as their nature varies depending on different LuxI homologs. Therefore the gene for the homologs are present often on adjacent locus of the chromosome and are said to be cognate pair. The homologs in these bacteria are different genes in each and each bacterium has multiple lux homologs for multiple quorum sensing systems. The homolog genes are known as bma in *B. mallei, bps* in *B.pseudomallei* and *bta* in *B.thailandensis* [6]. These three have highly preserved R1-I1 genes, R3-I3 genes and possess two homolog of orphan luxR. The luxR homolog is R4 and R5. *B.pseudomallei and B. thailandensis* also have added quorum sensing genes called R2-I2 is absent in *B.mallei.* Despite this the bacteria have sequence similarity in the amino acid of non-orphan receptors R1-I1, with octanoyl-HSL as their cognate signal. The system has been implicated in siderophore synthesis, phospholipase C manufacture and oxidative stress reaction. The BmaR3-BmaI3 in *B.mallei* responds to N-3-hydroxy-octanoyl-HSL and BtaI3 of *B. thailandensis* in addition, responds to the same HSL [21]. 120 kb DNA region of BtaR2-BtaI3 in *B.thailandensis* is preserved in *B.*pseudomallei but lacking in *B.*mallei due to genomic size reduction by deletion during evolution. Similar phenomenon is seen with quorum sensing system associated with R2-I2 containing DNA element. Literature indicates that Bta system generates HSL and responds to the signal by production of antibiotic which would be active against various gram positive bacteria. Bta system produces 3OHC8-HSL and 3OHC10-HSL but BtaI2 is a LuxI homologs in B.thailandensis, further BtaR2 responds to both signals. Thus the HSLs are synthesized by cognate HSL synthase BtaI2 and BtaR2 responds to both the signal [20]. The BtaR2-BtaI2 system of quorum-sensing modulates two of the gene clusters that are present on the 120-kb element. The element is additionally present in *B. pseudomallei* but is lacking from *B. mallei*. This120-kb element is considered an island of quorum-sensing [21].

The *B. thailandensis mal* gene cluster expression can be triggered by certain antibiotics. Studies show that sufficient levels of malR expression are enough to stimulate mal gene manifestation. Further, studies indicate that MalR is an orphan receptor with no cognate HSL [20]. Other than the absence of ligand the MalR is similar to LuxR. It activates lux like sequence for antibiotic mallei-lactone synthesis. The MalR expression for malR gene is activated by some antibiotics in the environment. Antibiotics that triggered mal gene expression retard the development of B.*thailandensis*. Thus, development and mal activation are inversely correlated

[21]. Nonetheless, certain antibiotics slowed development but did not distress mal gene stimulation. Thus, manifestation of some antibiotics stimulates a precise cellular reaction pathway, consequently triggers expression of malR.

The *mal* genes encode for production of mallei-lactone. The molecule is a cytotoxin that additionally has antibiotic function against many Gram-positive bacteria. The mallei-lactone-biosynthetic gene clusters are conserved and MalR are identical across these three species. Trimethoprim and sulfamethoxazole are two antibiotics which activate mallei-lactone production in B. thailandensis by MalR mediated expression [20]. Thus the BtaR2-BtaI2 reins the synthesis of the ancillary metabolite along with antibiotic function against several of the gram-positive bacteria. The antibiotic production gives microbes a competitive edge in their environment over added antibiotic sensitive microbes in similar environment. Apart from the classical LuxR-LuxI system, orphan receptor activation by antibiotic also produces antibiotics which are important for population survival.

Summary

Quorum-sensing systems are widespread among the bacterial populations. Quorum sensing is facilitated by diffusible minor molecules released by the bacteria till it reaches a threshold population density. On reaching the critical density the molecules accumulate intracellularly to match the external levels. These molecules are recognized by self- receptors and elicit a response by activating a cascade of events which help in survival of population [17]. The system involves the auto inducers which are produced by participants of the LuxI family of synthases. The LuxR protein family members act as related auto inducer reliant transcription factors. The LuxI synthesizes the auto inducer which consequently binds to LuxR [16]. The binding activates the transcription factor and mediates the expression of desired trait. The genes beleaguered by the members of LuxR family have an 18- to 20-base inverted repeat in the promoter regions which is conserved through species [22]. These essentials are known as lux box-like sequences and function to serve as obligatory sites for the transcription factors. Such classical system is exists in several bacteria and has been studied to control the synthesis of antibiotics. Such system is present in *Serrati* and *Erwinia* which release Beta- lactam [23].

An emergent view is that antibiotics actually assist intercellular signalling roles as opposed to munitions against competitors. Such a system is seen in phenazine antibiotic released by *Pseudomonas aeruginosa.* The system uses the antibiotic not only as an important secondary metabolite but also as signalling molecule in the cascade of quorum sensing.

Burkholderia thailandensis has three sets of luxI-luxR-type genes namely, btaI1-btaR1, btaI2-btaR2, and btaI3-btaR3 [23]. The luxR homologs is related to its equivalent luxI homolog, similarly luxI-luxR-type gene sets in several other bacteria. This species have two added luxR homologs, btaR4 (now called malR or btaR5). Members of LuxR family (like MalR and BtaR5) are called orphans due the absence

of cognate ligand associated with LuxI [7]. These systems synthesize mallei-lactone as the antibiotic.

The synthesis of antibiotics is important for the bacteria to mount cell population dependent invasion into host, compete in mixed communities of bacteria and activate cascade needed for population survival [22]. These bacterial models of antibiotic synthesis cover the classical models and the present view of structural and functional significance in the pathways. The present work describes the quorum sensing system present in bacteria and the contributing element of the cascade. The homologues of the cascade and their function are elaborated. Further the secondary metabolite i.e., antibiotic is synthesized by several bacteria. This chapter also covered the three models of antibiotic induction using model bacteria. Wherein, *Erwinia* and *Serratia* mediated quorum sensing by classical LuxR LuxI system. Pseudomonad displayed the quorum sensing cascade where the phenazine was the desired antibiotic but its synthesis was also a terminal signal and was an integral part in the system. Finally the proteobacteria genera mediate quorum sensing by classical system as well as novel orphan receptor, which regulates the synthesis of antibiotic in response to the environmental antibiotic such as trimethoprim. Hereby, explaining the biosynthetic pathways and their cognate contributing factors.

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Part III
Quorum Sensing Regulated Behaviors in Fungi

Perspective of Quorum Sensing Mechanism in *Candida albicans*

Prerna Pathak and Parul Sahu

Abstract *Candida albicans* a polymorphic fungus can cause wide range of disease in humans, its potential to transform between yeast to hypha form and biofilm formation is associated with its virulence. The regulated expression of virulence genes plays a critical role during pathogenesis and immune evasion. The synchronized expression of genes in accordance with the population density is define as Quorum sensing (QS).There are enormous information about prokaryotic QS but the eukaryotic QS was concealed till the discovery of farnesol in *Candida albicans*. Farnesol is a sesquiterpine alcohol produce endogenously in plants, animals and fungi. It inhibits the initiation of hyphal formation. Apart from farnesol other quorum sensing molecule (QSM) in fungal kingdom includes aromatic amino acid derived alcohols like tyrosol, tryptophol and phenylethanol. This chapter emphasizes more on the various types of mechanisms involved in the fungal Quorum sensing, elaborating their physiological effects and quorum sensing involved during the host-pathogen interactions. Understanding of Quorum sensing mechanisms in *Candida albicans* may open the door of various therapeutic possibilities.

Keywords *Candida albicans* · Quorum sensing mechanisms · Farnesol

Introduction

Kingdom Fungi comprise of 7% of all the eukaryotic known species [22], out of them 600 species are pathogenic in nature [6]. These pathogenic fungi causes dimension of diseases from moderate skin infections to lethal systemic infections, the fungi having the ability to cause Invasive fatal infections (IFIs) are *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Cryptococcus neoformans* and *Candida albicans*. Infections due to *Candida* species are frequent of the fungal infections. Candidiasis

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is one of the most common hospital acquired infections in the United States, with almost 50% death rate [25, 36].

C. albicans is a commensal, dimorphic and opportunistic fungus; it is present as a commensal in the oral cavity, skin, genitourinary tract and gastrointestinal tract of healthy individuals. It has been defined as clinically important pathogen as it is more prevail in immunocompromised patient suffering from AIDS. Since, *C. albicans* is an opportunistic pathogen, individual undergone for organ transplantation and chemotherapy treatment are most targeted host. For survival under the harsh hostile environment and to meet up there metabolic requirements, *C. albicans,* switch from yeast to hyphae form and also form biofilm. It has been reported, that the dynamic morphological changes of this class of fungi is associated with the virulence.

The hyphae and pseudo hyphae form of *C. albicans* is pathogenic in nature; however it is present as a commensal in its yeast form. The major factor which causes this morphological switching is contact sensing i.e. upon contact with the host tissue yeast form convert to hyphae form. Hyphae are invasive in nature, moreover hyphae secretes several virulence factors such as adhesions, cytolytic peptide toxin, tissue degrading enzymes etc., which helps hyphal form of growth to invade the host tissue and cause damage. This chapter will focuses on the types of Quorum sensing molecule and mechanism involved along with their physiological effects. However the research in this direction is still in its juvenile stage, understanding the mechanism of action and pathways involved may help in the development of new antifungals.

Pathogenicity Mechanisms of *C. albicans*

The ability of this dimorphic fungus to infect such a wide range of host is due to its multiple virulence factors and adaptive trait. Following are some of its traits which help this pathogen to evade host immune system, being dimorphic in nature it undergoes morphological switching between yeast and hypha form. There is expression of various hyphal specific gene during this transition, hyphal form of growth is more invasive in nature than that of yeast form.

C. albicans expresses a set of adhesins gene which intercede cell to cell adherence, and adhesion to the host cell surface, agglutinin-like sequence (ALS) proteins consist of eight members *ALS 1–7* and *ALS 9*, are the know adhesion protein in this fungus, of all the Als protein Als 3 plays an vital role during adhesion [23, 26, 36].

Apart from phenotypic switching and adhesion the other factors which are responsible for the virulence of this pathogenic fungus are biofilm formation, secretion of hydrolytic enzymes, metabolic adaptation to extreme conditions like pH, nutrients availability etc.

Drug Resistance in *Candida albicans*

There are six different class of antifungals used- azoles, polyenes, echinocandins, allylamines, nikkomycins and sodarins. The mode of action each antifungal is different, azoles inhibits the ergosterol synthesis pathway, polyenes target the ergosterol, echinochadins inhibit glucansynthases enzyme and target the cell wall, allylamines inhibits squalene epoxidase a enzyme required for ergosterol biosynthesis, nikkomycins are the chitin synthesis inhibitors, sodarins inhibits fungal protein synthesis.

In spite of the availability of so many different antifungals clinical resistance has been observed for all these classes, no single class of antifungal is effective against all type of fungal infections. Most pathogenic fungus has developed strategies to avoid or minimize the toxic effects of these antifungals. There are diverse mechanisms of antifungal drug resistance like modification and degradation of drugs, reduction in permeability of drug due to change in membrane composition, overexpression of drug efflux pumps like *CDR1*, *CDR2* and *MDR1* and target alteration by point mutation. Each and every class of drug has its own pros and cons, new generation of drug which specifically target biofilm development are need to be explored since biofilm formation is a major cause of azole resistance.

From the past many decades, Biofilm study has been under lime-light. It has been reported that biofilm is responsible for over 80% of all microbial infections [9]. Biofilms are consist of aggregate of micro-organism, enclosed in a self prepared polysaccharides and attached to the substratum. To convert into aggregates from the planktonic stage of microorganism, the phenomena involved termed as Quorum sensing. There is a synchronized expression of genes in accordance with the population density. The mechanism of bacterial communication was observed first time in late 1960s, during the study of bioluminescence in marine bacteria *Vibrio fischeri* [4]. After this the phenomenon of QS was contemplate in many species of bacteria regulating the group behaviours, like secretion of virulence factors, sporulation, formation of biofilm, motility, competence and antibiotic production [21]. In pathogenic organisms, the coordinated expression of virulence factor enhances the chances of host infection [11, 35].

Quorum Sensing in *Candida albicans*

There is enormous information about prokaryotic QS but the eukaryotic QS were concealed till the discovery of farnesol as a quorum sensing molecule (QSM) in *Candida albicans* [18]. Farnesol is a sesquiterpine alcohol produce endogenously in plants, animals and fungi. It inhibits the initiation of hyphal formation. Apart from farnesol other molecules which mediate QS in fungal kingdom includes aromatic amino acid derived alcohols like tyrosol, tryptophol and phenylethanol. This chapter initially emphasizes on the various types of QSMs, followed by the mechanisms involved in the fungal Quorum sensing.

The First Quorum Sensing Molecule in Fungal Kingdom: Farnesol

In contrast to bacteria, where all QSMs belong to acyl homoserine lactones family [7], the *Candida* QSM is sesquiterpene farnesol. The discovery of farnesol in *C. albicans* brought insight in the fungal quorum sensing mechanism, a phenomenon once thought to be confined in bacterial kingdom. Farnesol is an acyclic sesquiterpene alcohol comprises of three isoprene units (Fig. 1). Hornby et al. reported for the very first time the effect of farnesol in regulating the *C. albicans* filamentaion, they showed that farnesol inhibit the yeast to hypha and pseudohyphal transition [15]. However, farnesol have no effect on cells which already started hyphal development [27]. Biofilm being the major cause of drug resistance (Ramage et al). check

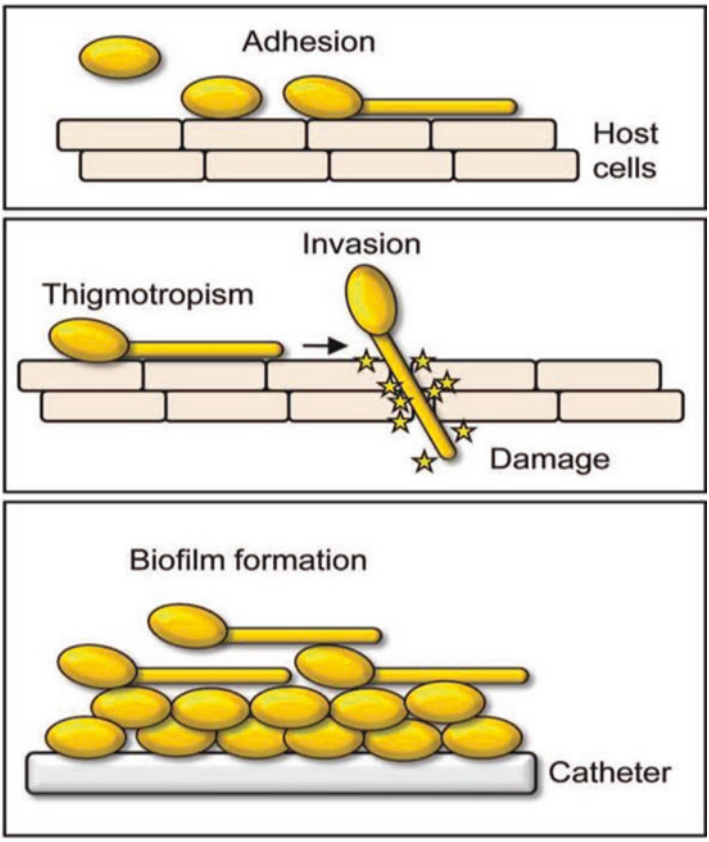

Fig. 1 An overview of *C. albicans* pathogenicity mechanism. (Source Mayer et al. [14])

Fig. 2 Structure of farnesol

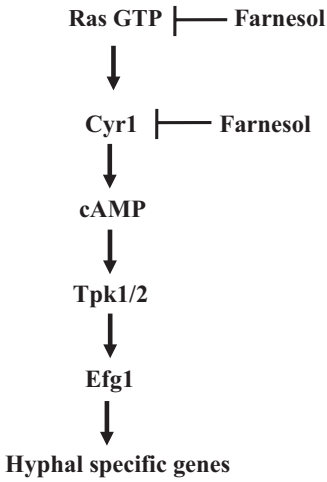

Fig. 3 The proposed model indicate the possible target of farnesol in Ras1 signaling pathway

the effect of farnesol on the development of biofilm, they observed that biofilm formation is inhibited by farnesol [7]. Microarray analysis of farnesol treated biofilms shows that genes related to cell wall, iron transport, cell surface hydrophilicity and drug resistance proteins were up-regulated with respect to hyphae formation genes [13] (Figs. 2 and 3).

Farnesol signaling in *Candida albicans*

Previous studies have reported that Farnesol affects this pathogenic fungus in multiple ways. Ras1 which is a monomeric G protein believed to be the target of farnesol. Ras1 activates adenylate cyclase Cyr1 which in turn leads to the increase cAMP levels [5, 20] which further stimulates Tpk1 and Tpk2. Tpk1/2 are essential during hyphal growth [3], the Ras1/Cyr1/PKA pathway plays an crucial role during yeast to hyphal switch in presence of various environmental stimulus [33]. The second mechanism by which farnesol affects is by disrupting the membrane environment, farnesol being the lipophilic molecule disrupt the membrane and interfere with the localization of the Ras1. The third possible way of farnesol inhibition is that it interferes with the Ras1 interaction with its signaling partners.

Farnesol Effect on Other Fungi

Apart from *C. albicans* farnesol have deleterious effects on many other fungi, such as *Saccharomyces cerevisiae, Staphylococcus aureus*, *Paracoccidioides brasiliensis, Mycobacterium smegmatis, Aspergillus* species and *Mycobacterium smegmatis* [1, 32]. The effect of farnesol in these fungi is described in brief.

***Saccharomyces cerevisiae*:** Farnesol inhibit the growth of *S.cerevisiae* by arresting the G1 stage of cell cycle, there is also a reduced DAG levels in its presence [19]. It is also shown that there is formation of reactive oxygen species (ROS) in the presence of farnesol [28].

***Aspergillus nidulans*:** Farnesol induces apoptosis in this fungus and lead to the formation of reactive oxygen species [31]. In addition it is also reported that this apoptosis is due to autophagy and protein kinase C function.

***Aspergillus fumigatus*:** In case of this fungus farnesol affect the signaling pathway which maintain the integrity of the cell wall and lead to the mislocalization of Rho protein which causes problem in hyphal morphology [12].

***Candida dubliniensis*:** Similar to that of *C. albicans* farnesol does not have inhibitory effect on this fungus, but it inhibits its hypha formation [17].

***Candida parapsilosis*:** Farnesol causes dose dependent killing of *C. parapsilosis*, this killing is due to the overexpression of genes associated with ageing. It also affect the genes involved in biofilm formation, sterol metabolism and oxidation reduction [29].

***Paracoccidioides brasiliensis*:** Farnesol inhibit the growth of *P. brasiliensis* at higher concentrations, while at minimum concentrations it suppress the yeast to hyphae transition [10].

Tyrosol is a derivative of phenethyl alcohol, it is an antioxidant present in various natural sources like argan oil, olive oil etc. Apart from farnesol tyrosol also act as QSM in *C. albicans* (Fig. 4).

Tyrosol affects the growth of this fungus by decreasing the length of its lag phase, In contrast to that of farnesol tyrosol activates the filamentation and biofilm formation [2, 8]. Tyrosol promote the germ tube formation opposite to that of farnesol.

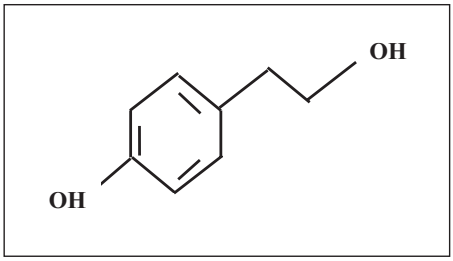

Fig. 4 Structure of Tyrosol

This antagonistic regulation of germ tube formation by both these alcohols displays a tight metabolic regulation in response to environmental factors. In contrast to farnesol signaling there is very little information about tyrosol signaling, there is need to explore more about the components involved and mechanism of tyrosol signaling.

Other Quorum Sensing Molecules in *Candida albicans*

In addition to farnesol and tyrosol *C.albicans* also secretes other aromatic alcohol like tryptophol and phenylethanol. Production of these aromatic alcohols depends on the condition of growth like availability of amino acids, pH, oxygen level [16, 24]. However there is no such information so far whether, these aromatic alcohol act as a QSMs or not. Future work in this field needs to be done in order to explore the role of these aromatic alcohols.

Quorum Quenching in *C. albicans*

The molecules which have the ability to effectively reduce the quorum sensing phenomenon are called as Quorum quenchers or Quorum sensing inhibitors (QSIs). Similar to that of QSMs, QSIs should be small, stable, and specific to the target QS system [28]. There are three possible ways of inhibiting QS signaling: blocking the interaction of QSM with its receptor, restrict the QSM production and degradation of QSM [30]. There are several molecules in fungal kingdom which act as a QSIs either to different fungal species or to some prokaryotic organism like patulin, penicillic acid etc. Farnesol is the only know QSIs in *C. albicans*, besides acting as a potent QSM it also work against non albicans species of *Candida* [34].

Conclusions

It is almost two decades since the discovery of density dependent mechanism of communication in the fungi. Hornby et al. were the first to describe the farnesol as a QSM in the dimorphic fungus *C.albicans*. However, only few aromatic alcohols like farnesol, farnesoic acid, tyrosol, tryptophol and phenylethanol are know so far as a QSMs. Except for the farnesol the mechanism of action of other QSMs is not yet know. Fungal quorum sensing system is need to be explore more, future work in this system will unravel different molecules and pathways involved.

The most explored QSM in fungal kingdom is farnesol. Its role in *C.albicans* morphology, biofilm inhibition, and interactions with host cells is already being

established. So far the farnesol signaling is well studied in fungal kingdom. Several studies on tyrosol depicts its role in QS but there is need to explore more, the pathway of tyrosol signaling and the molecules involved in this is not yet reported. There is need to investigate more about the other aromatic alcohols and their role as a QSM. Similar to that of bacterial QS, fungal QS is need to be explored to that extent, this could open a new door for the more efficient treatment of fungal infections.

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Conflict of Interest Authors have no conflict of interest.

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Part IV
Quorum Sensing Regulated Behaviours in Gram- Negative Bacteria

Vibrio fischeri Symbiotically Synchronizes Bioluminescence in Marine Animals via Quorum Sensing Mechanism

Pallaval Veera Bramhachari and G. Mohana Sheela

Abstract The process of intercellular communication called quorum sensing (QS) was first described in the marine bioluminescent bacterium *Vibrio fischeri* which lives in symbiotic associations with a number of marine animal hosts. A luciferase enzyme complex is found to be responsible for light production in *V. fischeri*. The bioluminescence emitted by these bacteria is a striking result of individual microbial cells coordinating a group behavior. In *V. fischeri*, QS controls bioluminescence, the ability of the bacteria to produce light, at high cell density. The mechanism of sensing involves an AI synthase, LuxI in *V. fischeri*, which makes small auto inducer molecules (AHLs). The autoinducer builds up in medium at high concentrations binds to a transcription regulator, LuxR in *V. fischeri*, which then alters the gene expression by coordinating bioluminescence among the local cell population. The genes responsible for light production are principally regulated by LuxR-LuxI QS system. This review primarily emphasizes the role of AHL signal molecules in QS network between the bacteria-animal symbiotic associations.

Keywords *Vibrio fischeri* · Quorum sensing · Autoinducer molecules (AHLs) · Bioluminescence

Introduction

Bioluminescent organisms are diverse and widely distributed in freshwater, marine and terrestrial ecosystems. The first exemplar of 'cell to cell communication' was explained in *Vibrio fischeri,* marine bacterium [51] controlling genes for bioluminescence. Understanding the regulation of luciferase genes revealed bacterial intercellular communication, which provided further insights on microbial pathogenesis

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and associations of microbes in the environment [5, 61, 63]. *V. fischeri* are most abundant and broadly distributed light emitting organisms [41] which form species specific symbioses with marine animals, together with squid and fish. *V. fischeri* are model systems for discerning bacterial colonization factors and host specificity determinants [38]. Interestingly association is extremely specific where; certain strains of *V. fischeri* colonize squid [54] and utilize bioluminescence released by *V. fischeri* for disguising itself. The squid disguises by interrupting its surrounded shadow by impeccable light [31]. The squid *E. scolopes* recruits *V. fischeri* populations to initiate and establish symbiosis within light organ and induce changes in light organ through bacteria-derived signals called microbe associated molecular patterns to use bioluminescence. For several decades, *V. fischeri* bioluminescence was observed as an exemplary example of microbial group behavior [55].

The inception of bacterial bioluminescence has led to the discovery of an intriguing phenomenon at this instance generally called quorum sensing (QS) [69]. QS is the means for a bacteria to sense the situations and activate the action that is beneficial to bacterial cells only when carried out in a group. In addition to bioluminescence, QS results in biofilm development, virulence and other traits [19]. There is plethora of information a propos to QS biochemistry [28]. It is extensively established that bacteria understand this process of synthesis, secretion and recognition of chemical signaling molecules known as auto inducers (AIs). Moreover, AI and its concentration plays an important role based on AI concentration profile throughout the colony. QS-responsive bacteria detect the AIs binding to their specific receptors and trigger cascade of intracellular signaling resulting in a phenotypic switch. As a consequence, cells in the colony become 'quorum-active' and behave in a synchronized manner.

Multiple QS systems in *V. fischeri* regulate the expression of operon lux *ICDABEG* to control luminescence of them LuxI-LuxR QS system is important one. Required amounts of AI (3-oxo-C6-HSL) is synthesized by LuxI [58], which binds and triggers LuxR [20]. The complex LuxR/3-oxo-C6-HSL binds upstream to operon luxICDABEG as a dimer recruiting RNA polymerase and initiate transcription [62]. In addition LuxS-LuxP/Q and AinS-AinR QS systems modulate transcription of luxR and control luminescence. AinS and LuxS synthesizes AI (C8-HSL) and AI-2 respectively, where histidine kinase AinR detects AI and periplasmic protein LuxP accepts AI-2 [3]. In this chapter, we emphasized the significance of Biochemistry and Molecular Biology of lux genes and its functional regulation of LuxIR in QS of *V. fisheri.*

Bioluminescence Phenomena

Bioluminescence signifies the activity of emitting visible light in living microbes facilitated by catalyst enzyme. The phenomenon of bioluminescence has been pragmatic in numerous organisms such as bacteria, fungi, fish, insects, algae, and squids. The enzymes luciferases catalyze bioluminescence reaction and luciferins are substrates for this reaction. The discoveries of biochemistry, genetic control,

molecular biology and physiology of bacterial bioluminescence have revolutionized the area of biotechnology, environmental microbiology, medicine significantly [14, 24, 41, 43, 45]. The understanding of regulation of luciferase genes regulation revealed intercellular communication among bacteria, which provided with insights on bacterial associations and their pathogenesis in the environment [5, 61, 63]).

Biodiversity of Bioluminescent Bacteria

Bioluminescent organisms are diverse and widely distributed, inhabiting freshwater, marine and terrestrial ecosystems occurring within proteobacteria [41]. Luminescent is predominate in marine bacteria *Vibrio* and *Photobacterium* species (e.g., *V. fischeri, V. harveyi,* and *P. phosphoreum*). There are non-luminous bacteria in the genus *Vibrio* and *P. angustum, P. damsela, P. histaminum, P. iliopscarum and P. profundum*). In eukaryotes the fireflies (*P. pyralis*), and click beetles (*P. plagiophtalamus*) exhibit luminescence. Noteworthy differences exist between bioluminescence mechanism of prokaryotic and eukaryotic luminescent organisms with respect to substrates and luciferase structure and properties. The only widespread features in both prokaryotic and eukaryotic luminescence are prerequisite for oxygen molecule and luciferase enzyme [7]. Each species of light emitting bacteria vary in a several properties, including growing conditions and reaction kinetics of enzyme luciferase [25]. Despite the diversity among species of luminous bacteria, light is produced using extremely homologous biochemical systems in luminescent microorganisms. A central signalling pathway tightly regulates the inception as well as the energy output of light-producing molecular machinery [46, 63].

Ecological Significance of *Vibrio fischeri*

Most mesmerizing quality of light emitting bacteria is their tendency to form a symbiotic association with twenty fish families that have light organs and *V. fischeri* is a symbiont of one family Monocentridea [25]. Bacterial bioluminescence is predominant in marine ecosystems, particularly among fish [61], *Euprymna scolopes* (Squid) – *V. fischeri* mutualism [6, 26]. The ecological advantage is established for both fish and squid living in a symbiosis with luminescent bacteria [49]. In a fish – *V. fischeri* mutualism both organisms are benefited; the fish makes use of nutrients that are drifted to the floor of ocean. Bacteria come across an environment which is nutrient-rich in the fish gut, where they can propagate, get excreted and maintain the cycle. Overall, bioluminescence has helped in understanding the intricacies of microbial ecology. It has also guided to noteworthy findings on how a bacteria may interact among themselves and with higher organisms. In a Squid – *V. fischeri* mutualism the host organism can use the light emitted by bacteria to attract prey, escape from predators or for communication. However, it is not understood what specific

benefits symbiotic bacteria derive from producing light. Albeit one could imagine some advantages for bacteria living in light organs of animals, it seems incredible that the establishment of such a symbiosis could be main evolutionary drive to develop very complex light-emitting systems [6]. The purpose of light production in higher organisms by and large falls under three categories: (i) to assist in predation (offense), (ii) to aid in avoiding predators (defense) and (iii) intraspecies communication such as courtship. The biological role of luminescence in free-living bacteria remains even more mysterious.

Quorum Sensing in *Vibrio fischeri*

Bioluminescence is regulated by auto induction or quorum sensing, which was first reported in *V. fischeri*, where 'cell-to-cell communication' links gene expression to bacterial cell density [12]. Quorum sensing includes self-production and accumulation of AI, which functions as signal moiety to bring about a characteristic effect with group of cells [50, 63]. Quorum sensing facilitates numerous roles which are essential for continued existence of *V. fischeri*. For example, symbiosis between *V. fischeri* and *E. scolopes* requires synchronous activity of cells of luciferase to emit sufficient light for its host to prevent its exposure [57]. The AI once reaches its threshold; it activates luciferase synthesis and other enzymes. Thereby, estimating the density of populations to assure that luminescent product is sufficiently high to affect environment [63]. AI and N-acyl homoserine lactone (AHL) were assumed to be species specific, but recent studies have proved that AHL is a signaling molecule for more than 16 genera of gram-negative bacteria. This provides insights on AI which assists in communication [68] allowing the bacteria to monitor self and other species. Quorum sensing is common in bacteria which influence their ecology and higher organisms [5, 63]. The light organ of squid *E. scolopes* is colonized specifically by *V. fischeri* in low abundance of surrounding seawater. *V. fischeri* uses its QS system to activate genes for luminescence in high-density light organ environment (Fig. 1). This model system has provided insight not only into the role of QS in an animal host–bacterial interaction, but also about how a microbiome, albeit a simple one, can persuade host development [39, 40].

Biochemistry of Bioluminescence in *Vibrio fischeri*

In bacterial, aldehydes are important for bioluminescence reaction, while substrate is long-chain aldehyde. Long chain aldehydes are derived by fatty acid reductase from fatty acid precursor [42, 45]. Light emission happens due to reaction of molecular oxygen with aldehyde and Flavin mononucleotide catalyzed by luciferase, to produce following long chain fatty acid and FMN.

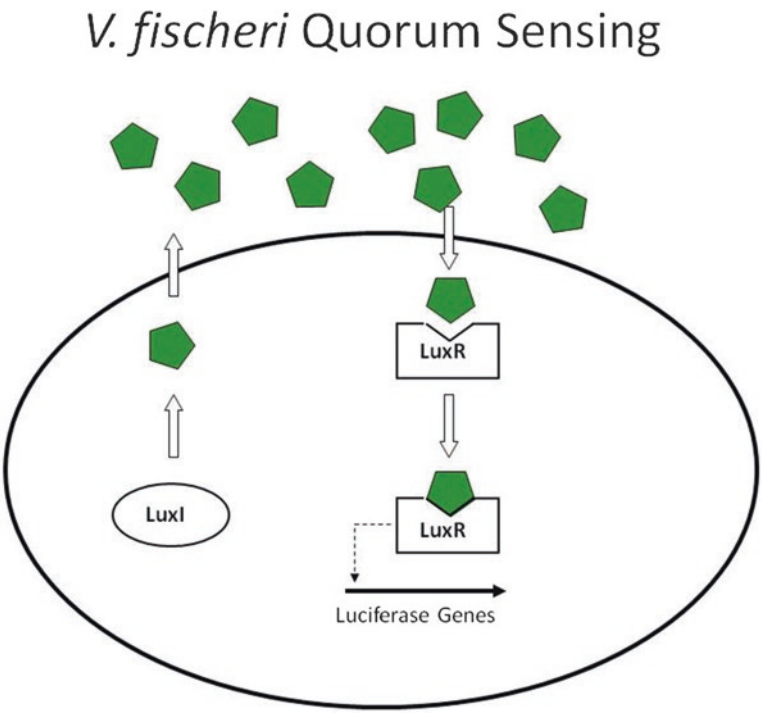

Fig. 1 QS regulatory mechanism (activate genes for luminescence) in *Vibrio fischeri*

$$\mathbf{FMNH_2 + RCHO + O_2 \xrightarrow{Luciferase} FMN + RCOOH + H_2O + Blue\ green\ light\ (\lambda\ max = 490\ nm)}$$

Bacterial luciferase catalyzes this bioluminescent reaction which linked to respiratory pathway. Luciferase enzyme is a heteropolypeptide protein having α and β subunits of nearly 40–44 kDa and 35–40 kDa, respectively, ascended by gene duplication [42]. The α-subunit of luciferase has active site, but β subunit is vital for light-emitting reaction. Neither α nor β subunit alone displays light emission, but when both subunits united possess activity signifying that individual subunits are not active. Long chain aldehyde binds to interface of α and β subunits [24]. In contrast, firefly luciferase is active in monomer form with a molecular weight of 62 kDa [13] (Fig. 2). In bacterial bioluminescent systems electrons are shunted from reduced substrates to O_2 via two flavin enzymes [22]. Luciferase may have ascended as efficient terminal oxidase substitute to cytochrome system [23], as progression of cytochrome deficient bacteria rely on luciferase induction and iron. Iron is important for cytochrome synthesis, but suppresses luciferase synthesis [24]. Coupling among respiration and bioluminescence was identified by using respiratory inhibitors cyanide and carbonyl cyanide-*m*-chlorophenyl hydrazone.

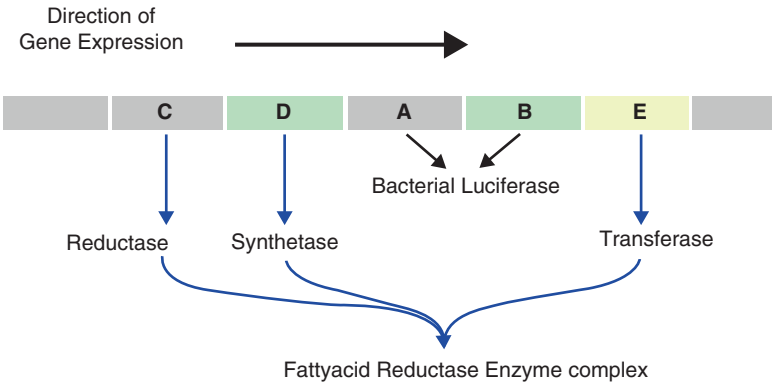

Fig. 2 Arrangement of *Lux*CDABE open reading frame (genes in *lux* operon of *Vibrio fischeri*

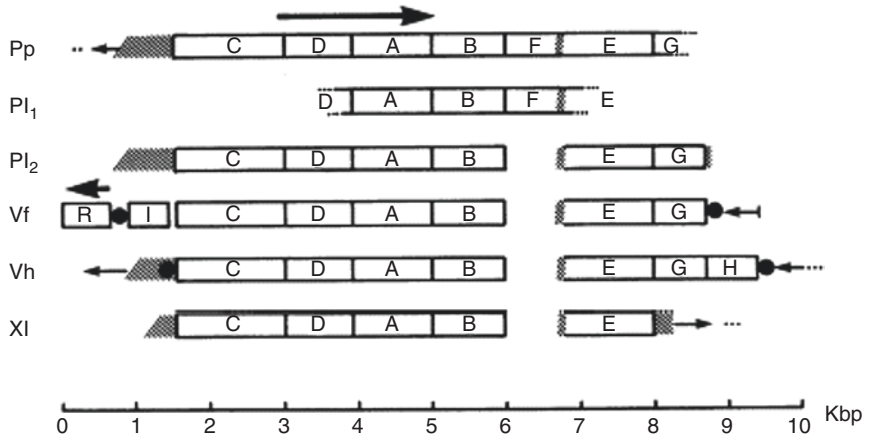

Fig. 3 *lux* gene organization for *P. phosphoreum* (Pp), *P. leiognathi* (Pl), *V. fischeri* (Vf), *V. harveyi* (Vh), *and X. luminescens* (Xl). The nucleotide sequences have been determined for all regions represented

Molecular Biology of Bioluminescence in *Vibrio fischeri*

Bacterial lux *Genes*

Bacterial bioluminescence was well described genetically and biochemically [21, 24, 41–44]. Engebrecht et al. [16] first reported about Luciferase enzyme, regulatory mechanisms essential for expression of phenotypes and established key facets of genetic organization [15]. Genes that encodes luciferase subunits (*luxAB*) and fatty acid reductase polypeptides (*luxCDE*) regulates aldehyde biosynthesis (substrate for luminescent reaction). *luxABCDE* genes sequenced from at least three genera: *Photobacterium*, *Vibrio* and *Photorhabdus* by cloning (Fig. 3). The *lux CDE* genes

flank *lux AB* genes in different luminescent bacterial species with transcription in the order *lux CDABE*, although an additional gene is located between *lux B* and *lux E* in *Photobacterium phosphoreum* [41, 43, 44]. Fatty acid reductase multienzyme complex was identified from *P.phosphoreum* [15]. The structural genes (*lux CDABE*) of *V. fischeri* and *V. harveyi* are much conserved, signifying that light emitting systems are analogous in both species. In *V. harveyi*, there is no open reading frame more than 40 codons in 600 bp start site of *luxC*, where *luxI* is traced in *V. fischeri* (Fig. 3). Regulation of light emission by *V. fischeri* strains is regulated transcriptionally through AI. The AI signals accumulates in culture medium when inducer concentration attain a threshold level (10^7 cells/ml), which induces transcription of structural genes [47].

Classical *LuxIR* QS Regulation in *Vibrio fisheri*

Bioluminescence in *V. fischeri* is produced by *luxICDABEG* operon, which codes for bacterial luciferase and enzymes for synthesis of luciferase substrate [41]. Luciferase is controlled by two genes (*luxR* and *luxI*), located in two divergent operons (Fig. 4). LuxI-like proteins are AI synthases of N-3-oxohexanoyl- L-homoserine lactone (3OC6HSL). AHL molecules produced, bind to its cognate receptor LuxR which then acts as transcriptional activator for *lux* operon. AI molecules freely diffuse through cell membrane and accumulate at threshold concentrations in (nM) to stimulate *V.fischeri* bioluminescence [52]. These regulators involve in a positive feedback loop, so that LuxR-AHL complex stimulates synthesis Lux enzymes, along with 3-oxo-C6-HSL, thus intensifying light production. LuxR is self-regulated at transcriptional level through a complex phosphorelay pathway which consist of two SKs (sensor kinases), LuxP/Q, and additional downstream regulators, with histidine phosphotransferase, LuxU, σ^{54}-dependent RR, LuxO [37, 52]. At low cell density (LCD), SKs act as kinases to autophosphorylate and to phosphorylate protein LuxU. Phosphorylated LuxU, donates a phosphoryl group to

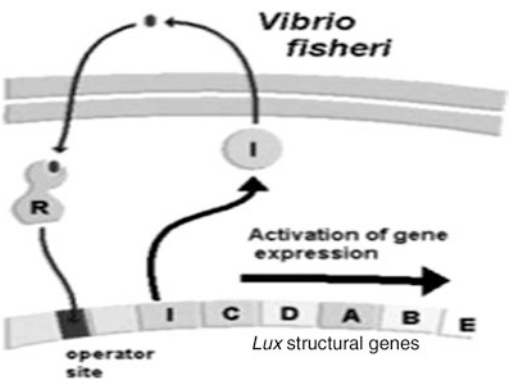

Fig. 4 Mechanism depicting the regulation and expression of lux genes in *V. fischeri*

LuxO. Phosphorylated LuxO (at low cell density), interrupts negative regulation of LitR, (activator of LuxR) and it stimulates expression of sRNA, *qrr1,* which hinders translation of *litR* mRNA [48]. *LitR* is a direct transcriptional activator of *luxR*; therefore, inhibition of *litR* guides inhibition of bioluminescence [48, 53]. At high cell density (HCD), phospho-transfer pathway is reversed, where SKs function as phosphatases for removal phosphoryl group from LuxU (and LuxO) [12, 18].When LuxO is dephosphorylated (at high cell density), *qrr1* levels decreases, *LitR* is translated, *luxR* is transcribed, and *lux* operon is expressed. In addition to regulating luminescence via LuxR, AinS/R also regulates a number of other behaviors, such as motility, acetate utilization and colonization [37].

Natural Bioluminescent Bacteria as Biosensors

Marine luminous bacteria created an attention among the ecologists because they are cost-effective, resourceful, employ numerous nutrients and inhabit diverse econiches in marine environment, their bioluminescence being awfully sensitive to toxicants that were employed in bioassays for detecting nano to picomolar concentrations of impurities in pharmaceuticals [23], food industry [4], and water quality testing [8] commercially available Microtox test is based on inhibition of bioluminescence in, *P.phosphoreum* when exposed to toxic substances, including solvents and toxic metals [8]. Changes in bioluminescence relative to a control used on same day indicate the presence of toxicants, where the exact nature of the toxicant cannot be identified, as this test indicates only the presence of some form of toxicants. Nevertheless, the dynamics of dose-related reductions in bioluminescence can signify the classes of toxins in marine environment [56]. Additionally, intact freeze-dried cells were used for testing toxicity in long-term assays with toxic substances in Mutatox test [2]. The Mutatox test employs a typical variant of *V. fischeri,* which produces bioluminescence after incubation at 27 °C for 16–24 h in presence of genotoxic agents.

Genetically Modified Luminous Bacteria as Biosensors

Hitherto to the advances in molecular biology, it has been feasible to design clone natural bioluminescent bacteria that, by insertion of *lux* genes [59, 60, 67]. Several bioluminescent bacterial sensors for uncovering of toxic metals along with organometals have been customized by genetic manipulation of *E. coli*. By using transcriptional fusion of Tn21 Hg resistance encoding (*mer*) operon from *V. fischeri* with *lux* CDABE; three biosensors for Hg (II) was constructed and tested [59, 60]. This *mer-lux* biosensor evidenced semi quantitative detection of inorganic Hg (II) in range of 0.1–200 ppb levels which was a an excellent system to identify bioavailable forms of mercury [60]. Recombinant luminescent bacteria were manipulated

and used for general toxicity testing including heavy metals [35]. Metal-specific recombinant bacterial sensors have been constructed and used for detection of inorganic mercury [59, 60, 66, 67], organomercurials [30], zinc, cadmium, cobalt and lead, cadmium and nickel [64–66]. In a metal-specific bacterial sensor the expression of a reporter gene is controlled by a genetic regulatory unit, i.e. reporter-receptor concept by [36] is used. Majority of the regulatory units used in construction of metal-specific sensor bacteria that hold natural specifically regulated resistance systems towards heavy metals. Heitzer et al. [27] designed a bioassay to evaluate the bioavailability of naphthalene and salicylate in contaminated soils, using genetically modified *P.fluorescens* carrying the *nah-lux* reporter plasmid competent of degrading together. Applegate et al. [1] have constructed a *tod-lux* fusion and cloned into *P. putida* F1, which was employed as a whole-cell reporter for toluene, benzene, xylene and ethylbenzene, (BTEX) sensing and bioavailability determination. A novel mutagenicity assay for detection of mutagenic pollutants in marine environment has recently been developed by using genetically modified *V. harveyi* strains [11]. Ever since, environmental stressors, such as organic solvents and heavy metals were inaccurately measured by using recombinant *E. coli*, fused with bacterial *lux* as reporter genes. Recently, Thouand and colleagues designed a bioluminescent (BL) biosensor device that employs recombinant BL bacteria [32–34]. In 2011, Charrier et al. developed a novel multichannel Lumisens biosensor III [9, 10]; which enhanced third generation of their 2007 Lumisens biosensor II [29]. They concocted a disposable card that was used in assemblage of a multi-strain biosensor. Jouanneau et al. studied a precise decision tree method to detect heavy metal in water samples [32, 33]. Additionally, Jouanneau and group also designed a marine microbial biosensor that possessed wild bioluminescent bacterial strain *Aliivibrio fischeri* [34].

Future Perspectives and Conclusions

One of the mysteries in bacterial QS understands how a microbe combines the clues obtained from many signal inputs. *V.fischeri* regulates its bioluminescence through QS mechanisms that receive input from three AI signals. The recent advancements in molecular biology discovered a puzzling fact that three parallel signal transduction circuits exists in *V. fischeri.* AI-1 is employed for intra-species communication, CAI-1 for Vibrio genus level communication and AI-2 for inter-species communication, in the sense to differentiate between self and others in order to counter to alteration of population densities in a timely approach main goal of exploring QS systems of *Vibrio* sps is to comprehend the molecular and cellular levels used by bacteria for cell-cell communication. Nonetheless it indecisive how crosstalk between C8HSL and 3OC6HSL affects the information that bacterium obtains through QS. Multiple QS systems control luminescence in *V. fischeri* by regulating expression of LuxI-LuxR QS system. QS Network employs both RNA-based and protein-based regulatory factors. Hitherto, the sRNA-arbitrated gene regulation was underappreciated in microbes. The complexity of these pathways raises numerous

questions about why sRNAs are ideal for QS regulation and what choice does multiple sRNAs provide the circuit? Whether the Qrr sRNAs have different affinities for their targets? if so, does this interrupt on flux of QS transitions. What molecular mechanisms emphasize the distinct expression of qrr genes?

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Conflict of Interest The author declares that there is no conflict of interest.

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Quorum Sensing System Regulates Virulence and Pathogenicity Genes in *Vibrio harveyi*

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Abstract Quorum sensing (QS) is intracellular communication among bacteria that perceive population density, regulates the formation of biofilm, virulence factors production and provides resistance to antibiotics through extracellular signal molecules. *Vibrio harveyi*, a marine pathogen, and major cause for loss of productivity in aquaculture hatcheries, farms and for the growth of industry. *V. harveyi* uses multi-channel quorum sensing system, each consisting of an autoinducer-sensor pair that controls the expression of genes required for bioluminescence, virulence, biofilm formation. The multi-channel system is mediated by the *V. harveyi* autoinducer 1 (HAI-1), autoinducer 2 (AI-2), *V. cholerae* autoinducer 1 (CAI-1) which activate or inactivate target gene expressions by a phosphorylation/dephosphorylation signal transduction cascade. The production of extracellular virulence factors are involved in regulation of virulence and pathogenesis of *V. harveyi*. This article focuses on chemical communication mechanism, its regulation of virulence factors and pathogenicity of *Vibrio harveyi*.

Keywords Quorum sensing · Virulence · *Vibrio harveyi* · Pathogenesis · Signal transduction cascade · Gene expression

Introduction

Vibrio harveyi, a gram negative, pathogenic, marine fluorescence emitting bacteria commonly present in gut microflora of aquatic invertebrates viz. crustacean, molluscs and vertebrates viz. fishes. *Vibrio harveyi,* an oligotropic pathogen reported to be associated with Bright red syndrome [33], luminous vibriosis [17], to aquatic invertebrates and skin ulcers, eye lesions, gastro-enteritis [7], vasculitis to vertebrates which impedes the commercial development of aquaculture around the world [3] (Table 1).

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Table 1 Examples of diseases caused by *V.harveyi*

Host	Disease	References
Peneause monodon (Tiger prawn)	Luminescent vibriosis	[17]
Litopenaeus vannamei (White shrimp)	Bacterial white tail disease (BWTD)	[44]
Epinephelus coioides (Estuary cod)	Gastroenteritis	[8]
Rachycentron canadum (Cobia fish)	Gastroenteritis	[21]

Quorum sensing is an intracellular communication among bacteria that enables them to change behaviour in response to variations in cell density. QS includes the species specific synthesis and release of signalling compounds extracellularly named as autoinducers [39]. Accumulation of autoinducer molecules increase as population density of bacteria increases. These changes of autoinducers concentration in surrounding environment was monitored by bacteria to track their cell density and to modify array of gene expression [19]. QS regulates the formation of biofilm, bioluminescence, virulence factor expression, motility, responsible for pathogenicity and virulence of *V.harveyi* [39].

Auto Inducers and Receptors

V. harveyi was first discovered bacterial species to drive communication using chemical signals (auto inducers) and remained the model organism to understand how bacteria process chemical blends. *V. harveyi* synthesizes and process three different auto inducers for communication between intra-genera, intra and inter-species. *V. harveyi* lives in diverse inhabitants probably combat complex mixtures of chemical molecules produced by their own species, their surrounding flora, which act as competitors. *N*-acylated HSL (homoserine lactones) are most common class of auto inducers detected and synthesized by *V. harveyi* for intra-species communication.

AI-1 Autoinducer-I molecules are acyl HSL synthesized by LuxM synthase benefitted for interspecies communication**.** HAI-1 *[N*-(3-hydroxybutyryl) homoserine lactone] acts as ligands and are produced by LuxM and sensed by LuxN receptor specific to *V.harveyi*. LuxN is a two-component protein comprise of two domains a kinase domain which acts as a sensor and response regulator domain. HAI-1 molecules were constrained to *V. harveyi* and closely related sps *V.parahaemolyticus*, signifying HAI-1 role in intraspecies signalling [5–7].

CAI-1 *V. harveyi* senses (*Z*)-3-aminoundec-2-en-4-one, closely related *V.cholerae* autoinduer molecule known as cholera autoinducer 1 (CAI-1). CAI-1 was first identified in *V.cholerae.* In *V.cholerae* CAI-1 molecule is synthesised by CqsA (CAI-1 autoinducer synthase). CqsA utilises SAM (S-adenosyl methionine) and decanoyl-CoA to synthesise amino-CAI-1. Amino CAI-1 undergo spontaneous hydrolysis and by dehydrogenase to form CAI-1. Interestingly, CAI-1 molecule prevails in

cell-free extracts while, both amino-CAI-1 and CAI-1 are biologically functional molecules. *Vibrio* spp. Synthesis many CAI-1 moieties with different acyl chain lengths and modifications. CAI-1 is detected by CqsS receptor has six transmembrane helices and utilises for intra-species communication. Derivatives of CAI-1with altered acyl sidechains fail to stimulate CqsS, however autoinducer with extended head group switched the molecule to an antagonist.

AI-2 Autoinducer-2 (AI-2), a furanosyl borate diester one of the few biomolecule having boron and was first identified in *V.harveyi*. AI-2 has a set of interconverting molecules which are derivatives of 4,5-dihydroxy-2,3-pentanedione (DPD). LuxS, (DPD synthase), exists in >500 bacterial species, synthesises AI-2 molecules [9]. AI-2 is the most common bacterial autoinducers known yet. DPD is very reactive and spontaneously cyclizes to form furanone moieties with varied structures. Different bacterial sps respond to diverse forms of DPD. Interestingly, AI-2 molecules has boron in *V. harveyi* sps, while, *E.coli* and *Salmonella* spp., contain non-borated cyclized DPD moiety as AI-2 [34]. As the different DPDs rapidly interconvert, AI-2 provides a means for inter-species communication.

AI-2 signal is sensed and transduced by periplasmic protein LuxP (binding protein). LuxP interacts with LuxQ (hybrid two-component sensor kinase protein) to enable signal transmission [6]. LuxQ transduces signal to its shared histidine phosphotransferase protein (LuxU), which transmits signal to LuxO. LuxO, along with σ54, modulates the expression of target genes [12]. In the absence of AI-2 signal, 2 proteins LuxP and LuxQ complexes to form a symmetric heterotetramer. Binding of AI-2 creates large conformational change that stimulate protomer rotation in periplasmic region and disrupt LuxPQ–LuxPQ tetramer symmetry which inhibits phosphorylation of cytoplasmic domains. Interestingly, binding of AI-2 promotes formation of clusters by LuxPQ–LuxPQ tetramers, which can effect sensitivity of AI-2 and its response dynamics [25].

Quorum Sensing in *Vibrio harveyi*

V. harveyi QS circuit system depends on three cognate transmembrane receptors. However pros and cons of cytoplasmic DNA-binding transcription factors against membrane-bound receptors is yet unidentified. Nonetheless both types of receptors avoid response to autoinducers produced endogenously before reaching ‘a quorum’. Initiation of QS in *Vibrio* sps is decoupled by differential localization of receptors (on membrane) and site of autoinducers synthesis (cytosol) and from recognition in periplasm [26] *V. harveyi* uses CqsS, LuxPQ and LuxN as QS receptors, which binds with CAI-1, AI-2 and HAI-1 signal molecules respectively [19]. At low density of chemical signals, LuxPQ, LuxN, and CqsS acts as kinases and autophosphorylates. Therefore, phosphorylated receptors phosphorylate phosphorelay protein, LuxU which phosphorylates downstream target LuxO (response regulator

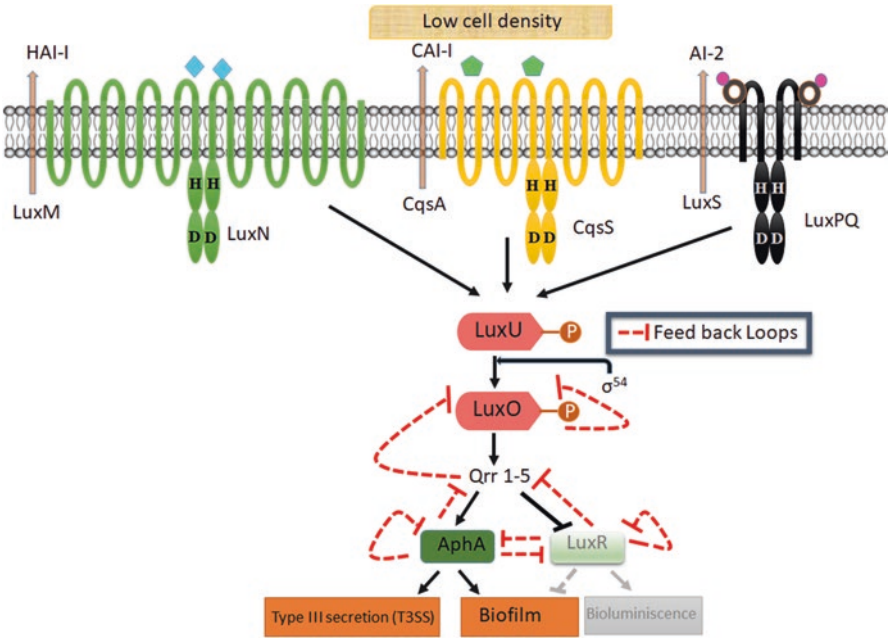

Fig. 1 QS cascade at Low Cell Density. LuxM, CsqA, LuxS synthesises 3 autoinducers that mediate QS in *V.harveyi*. At LCD the receptors acts as kinases and autophosphorylates LuxU and LuxO. Phosphorylated LuxO induces the expression of Qrr sRNAs and degrade/destabilise LuxR a master regulator for LCD. This promotes the expression of T3SS structural genes, biofilm formation, and motility. Feedback loops which plays an important role in Quorum sensing dynamics are represented in red color

protein) [12] LuxO interacts with σ^{54} and activate the transcription of target genes that encode for five homologous quorum regulatory sRNAs (Qrr 1–5) [20, 36]. Qrr sRNAs now modulates the expression of target mRNAs gene through base-pairing which activates/repress translation of 20 mRNAs. Qrr sRNAs stimulate translation of AphA, mRNA at LCD (low cell density master regulator) while limiting translation of LuxR mRNAs (high cell density master regulators) [32] (Fig. 1). At high cell density, binding of autoinducer hinders autophosphorylation, which enables the action of phosphatases. Dephosphorylated LuxO is less active and prevents *qrr* genes expression. In lack of Qrr sRNAs, gene expression of *luxR* is activated while *aphA* is repressed. LuxR is a master transcriptional regulator that activates >70 genes that promote collective QS behaviors [38].

Qrr sRNAs also repress translation of *luxMN* mRNA by coupled degradation. The Qrr sRNAs inhibit *luxR* through catalytic degradation of *luxR* mRNA, supress translation of *luxO* by sequestration [36], and stimulate *aphA* by revealing of ribosome binding site [10] (Fig. 2). However, Qrr sRNAs mediated catalytic degradation of *luxR* mRNA has no effect on Qrr pool, while sequestration (*luxO*) and coupled degradation (*luxMN*) reduce Qrr sRNAs from system [36]. These regula-

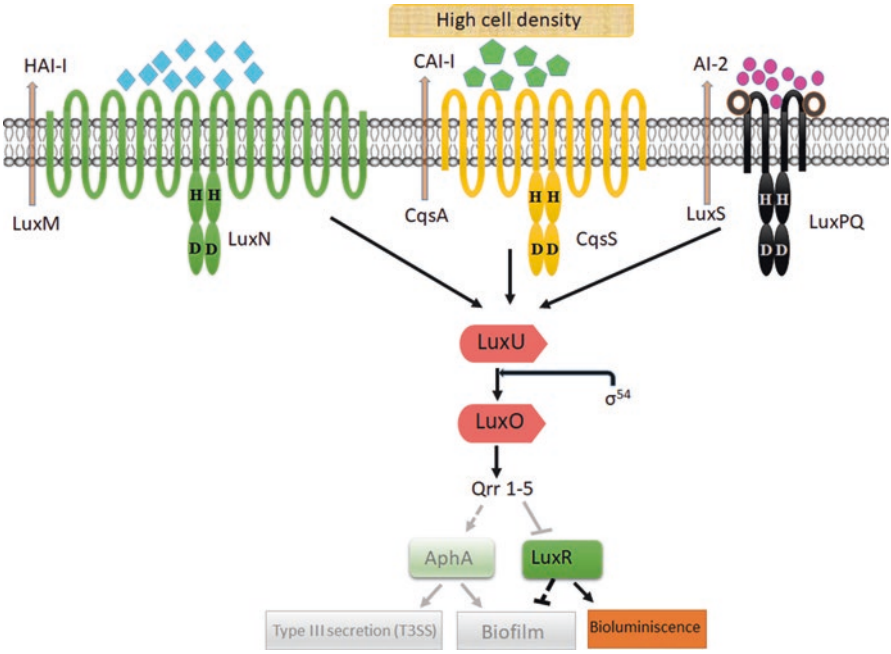

Fig. 2 QS cascade at High Cell Density. At HCD the receptors acts as phosphatses dephosphorylates LuxU and LuxO. LuxO reduces the expression of Qrr sRNAs and promotes synthesis of LuxR and represses synthesis of AphA. LuxR promotes the expression of bioluminescence, biofilm formation while repressing the expression of T3SS genes. H indicates His residues and D indicates Asp residue (phosphorylation targets)

tory pathways are important for the maintenance of defined Qrr pools of system and overall QS dynamics. HAI-1and AI-2 act synergistically, to mimic HCD conditions [23]. Under LCD conditions, LuxN acts as a kinase in the absence of HAI-1 (AI-2 is present). This function effects the net phosphorylation of protein LuxO which is essential to sense LCD environment. Accumulation of HAI-1, converts LuxN to acts as phosphatase. Now both sensors LuxN and LuxQ are phosphatases and trigger dephosphorylation of total LuxO. This transition senses low- to high-cell-density mode [12].

QS receptors in *V.harveyi* are two component receptors with both kinase and phosphatase activities which phosphorylate/dephosphorylate LuxU. QS system is completely turned on/off unless all the autoinducers are present or absent, respectively. Further QS in *V.harveyi* is controlled by feedback loops and regulatory feedbacks which may fine tune flow of information by chemical signals (Fig. 1).

(i) LuxO auto represses its own transcription [28, 36]
(ii) Qrr sRNAs sequester the *luxO* mRNA, which supresses translation of *luxO* gene. In LCD (low cell density) these two loops reduce synthesis of LuxO protein, this reduces protein level below which Qrr sRNAs cannot further represses QS [10, 36].

(iii) LuxR activates *qrr* genes expression, and the synthesized Qrr sRNAs destabilize *luxR* mRNA. This double loop drives LuxR- mediated QS transitions faster [20, 38].
(iv) LuxR limits its own transcription, which evades, limited synthesis of protein at HCD, therefore regulating QS output. LuxR family of proteins, the master global transcription factors targets expression of downstream genes in response to alterations in cell density [28].
(v) AphA and LuxR mutually supress each other's transcription, which allows maximal expression of AphA protein on LCD and optimal expression of LuxR HCD [22].
(vi) During LCD, Qrr sRNAs enable degradation of *luxMN* mRNA, results in reduced synthesis of HAI-1. This loop minimises HAI-1 signal at LCD and intensifies HAI-1 sensitivity at HCD [35]. Presumably, all these feedback loops promote fidelity, optimal dynamics between quorum sensing states.

Group Behavior and Co-ordination

Motility

Bacterial motility is one of the important virulence factors in most pathogens. Motility is essential during the early stages of infection for pathogenic bacteria in to weaken repulsive forces between host tissues and bacterial cell. This facilitates bacterial cells attachment to the host. However, regulation of chemotaxis and/or motility is common to *V.harveyi* regulons, LuxR stimulate motility gene expression. *V.harveyi* display maximal motility at HCD. In *V. harveyi*, LuxR positively controls expression of chemotaxis genes, while in *V. parahaemolyticus* and *V. cholerae*, OpaR/HapR negatively control homologous genes. QS positively controls motility by targeting flagellar biosynthesis which significantly affects virulence of *V. harveyi* [41].

Single Cell Heterogeneity

Heterogeneity is essential for bacterial group behaviour to share 'Public goods', viz., substances for ECM (extracellular matrix) or degradative enzymes among the population [1]. Quorum sensing does not frequently effect the homogeneous behaviours of cells instead exhibits phenotypic heterogeneity/diversification of behaviours in clonal populations [16]. QS system induced heterogeneity within the population was reported in *V. harveyi* [1, 2], *Aliivibrio fischeri* [27], *Listeria monocytogenes* [13], *Salmonella enterica* [11] using single cell analysis. Anetzberger et al. [2] reported that some AI-regulated genes (*luxC, vscP* and *vhp)* exhibit

functional heterogeneity in a *V. harveyi* in wild type cells. The two genes (*vscP*, *vhp)* exhibit wide intercellular variation in response to AIs at transcriptional levels. AIs regulate expression of *luxC, vscP* and *vhp* genes by binding to their promoter regions – that are essential for expression of bioluminescence, type III secretion proteins and exoproteolysis respectively. At HCD (high concentration of autoinducers) *lux* operon and exoprotease gene expressions were induced, while expression of *vscP* is repressed. However *luxS,* an AI-independent gene, is expresses largely in homogeneous manner. AI molecules plays a crucial role in the phenotypic diversification of clonal population (heterogeneity). Nonetheless, AIs not only serve as indicators for cell density but also coordinates cooperative behavior to share and synthesize 'public goods' and harmonizes QS-regulated processes [1, 2].

Biofilm Formation

Although a plethora of studies reported that LuxR-type proteins regulate biofilm genes expression, the correlation among QS and formation of biofilm in *V. harveyi* is not well established yet [1, 37]. Anetzberger et al. [1] reported that QS positively controls biofilm formation in *V.harveyi*. However, in *V. cholerae*, HapR limits the expression of *VpsR* and *VpsT* genes (activators for biofilm formation), which results in formation of biofilm at LCD.

Virulence and Pathogenicity in V. harveyi

The pathogenic mechanisms responsible for virulence in *V. harveyi* was not yet elucidated completely however, pathogenicity is thought to emerge via adhesion to host cell/surface, colonisation and production of lytic enzymes such as siderophores, hemolysin, proteases, lipases, gelatinase and caseinase [29, 42, 43]. In *vibrio* sps, virulence gene expression can be stimulated by several features of host environment, including low iron, oxygen, phosphate levels, mucin, catecholamines, bile salts and cholesterol [30]. Nonetheless, QS differently regulates different virulence factors viz., metalloprotease, gelatinase and caseinase activities are positively regulated QS, while phospholipase genes, T3SS genes are negatively regulated. In *V. harveyi* hemolysin and lipase activities are independent of QS system [24, 30, 40].

T3SS and T6SS proteins have complex needle like structures that penetrate cellular membranes to deliver effector proteins interfere with various cellular processes to cause cell death [15]. T3SS are usually rupture eukaryotic membranes, whereas T6SS can breach both eukaryotic and prokaryotic membranes [4]. *vscP* and *vhp,* are the two genes essential for pathogenesis of *V. harveyi* encode for a component of type III secretion system and an exoprotease, respectively. Some of the virulence factors generated by pathogenic bacteria translocate to cells exterior by type III

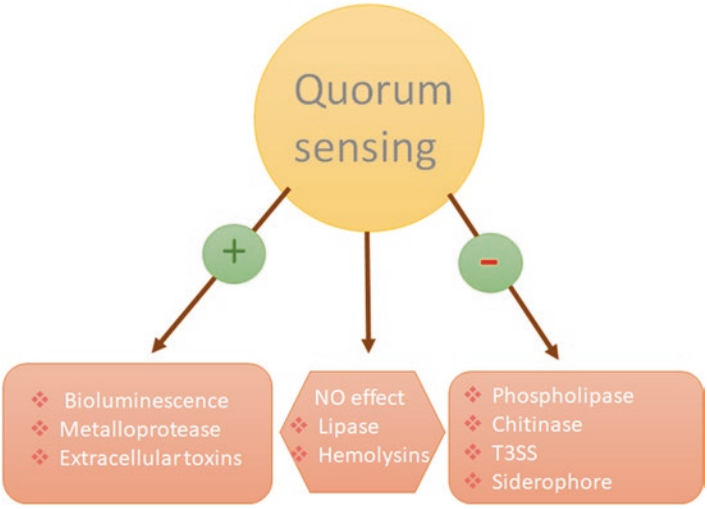

Fig. 3 Regulation of QS and virulence

secretion system (T3SS) [14]. T3SS locus consists of three adjacent operons on chromosome 1 (*vopD, vscP, vcrD* genes) and one operon located 15 kb apart [30].

In *V.harveyi* expression of T3SS structural genes is activated by ExsA a transcriptional modulators belongs to AraC/XylS family [18]. LuxR suppress expression of T3SS operons, together with genes that encode for structural, effector proteins and transcription factors of T3SS system. Expression of T3SS operon greatly varies between low and high cell density, but the expression is highly enhanced during infection in a QS dependent way. LuxR, a LCD modulator activates expression of two promoters of *exsBA* operon (*exsA, exsB*) and promotes production of ExsA. However, deregulated expression of the *exsBA* operon, critical for the QS-mediated control of T3SS genes at HCD [40]. At LCD, AphA represses the expression of >40T3SS genes. Nonetheless repression of T3SS genes during LCD and HCD by AphA and LuxR respectively, results in T3SS genes expression at mid-cell density [4]. Thus, expression of Type III and VI secretion systems (T3SS/T6SS genes) are regulated by QS in various *Vibrio* species [18, 31] (Fig. 3).

Conclusions and Future Perspectives

In this review, we have provided an overview of current knowledge on virulence genes and their regulation in *V. harveyi.* Quorum sensing among *V. harveyi* is central feature for many cellular processes, virulence, heterogeneity, inter and intra species communication, group behaviors. Several complexities of QS networks were disclosed and yielded an important understanding on the role of LuxR type proteins however several questions remain unknown. Does LuxR controls expression of

proteins at transcriptional/translational level? Mechanism through which QS positively regulates biofilm formation. How does environmental factors affect expression of virulence factors? How mellaoproteases promotes virulence in *V. harveyi.* Therefore a better understanding of regulatory mechanisms involved in virulence gene expression of *V. harveyi* (LuxR regulon) hosts several cues in the years to come.

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Quorum Sensing Complexity of the Gut Enterobacteria *Escherichia coli* and *Salmonella enterica*

Chandrajit Lahiri

Abstract The human alimentary canal is the reservoir of a diverse range of bacteria, of which the gram negative strains of *Escherichia coli* and *Salmonella enterica* mostly present themselves as beneficial and opportunistic pathogens, respectively. The complex environment of the human gut necessitates an adaptation by these bacterial species, which, primarily, is done through interspecies communication mediated by cell-density dependent gene regulation. This phenotype of sensing the quorum a.k.a. quorum sensing (QS), has been shown to play roles in bioluminescence, formation of biofilm, swarming motility and virulence for bacterial species over the years. For *E. coli* and *S. enterica*, quorum sensing (QS) a.k.a. intracellular signalling has been mediated by more than one mechanistic pathway involving the proteins and biomolecules such as the autoinducer-1 (AI-1) type LuxR homolog SdiA, AI-2 type LuxS, AI-3 type epinephrine/norepinephrine and/or indole. A usage of these proteins and/or biochemicals in combination is a hint towards their adaption to the influencing factors in the external environmental milieu of the host human gut. Notably, high osmolarity, low or neutral pH and preferred carbon sources affect such adaptation processes. While numerous bioactive compounds like Artemisin, Digoxin, Flavonoids, Ginkgo, Phenols, Punicalagin, Stilbene, Taxol, Vincristine and Vinblastine act as anti-QS products and have been explored, novel brominated N-heterocycles have started gaining importance as new measure for the antimicrobial resistance threats posed by such *Enterobacteriaciae*.

Keywords *Escherichia* · *Salmonella* · Quorum sensing · LuxS · AI-1 · AI-2

The association of the host with the microbe and their interactions has been subject of studies for quite some time now. The essentiality lies in the roles the microbes play in affecting the host's immunity and their maintenance of the metabolic homeostasis [1]. The niche to initiate, mediate and complicate such human body

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processes is the gastrointestinal (GI) tract, harbouring a dynamic and diverse population of bacteria ranging from beneficial to opportunistic and pathogenic types. Till date, the comprehensive human-associated microbial repertoire compilation from MetaHit and the Human Microbiome Project have identified 2172 species from 12 different phyla, having 386 strict anaerobes [2, 3]. The phylogenetic classification has 93.5% microbes from Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes [3]. Among these, the gamma-Proteobacteria, *Escherichia coli*, mostly present themselves as facultatively anaerobic opportunists and pathogens while the phylogenetically closely related *Salmonella enterica* are strictly pathogens. Several of their interactions with the gut microbiome are similar, including but not limited to, the utilisation of fucose and sialic acid from the gut microbiota mucins [4, 5]. However, with huge diverse bacterial population inhabiting the gastrointestinal environment, there lies a variety of similarity and differences in their mode of intra- and inter-species communication within the gut microbiome, of which cell-density dependent gene regulation is a part. Essentially, this is referred to as quorum sensing (QS) wherein upon the attainment of quorum i.e. a particular threshold corresponding to a high cell density of the bacteria, a myriad of coordinated expression of specific genes are exhibited by the entire population.

The Diversity

The phenomenon of QS entails the cellular communications within and between the community of bacterial populations encompassing regulatory processes namely biofilm formation, bioluminescence and virulence. Reported for the first time in *Pneumococcus*, for controlling the cell's competent state [6], sensing of a quorum is generally conceived through small biomolecules called autoinducers (AI). These were first identified as intracellular chemicals controlling the activity and synthesis of the luminescent system of the marine bacterium *Vibrio harveyi* [7]. Detected from diverse range of Gram-negatives and Gram-positives, the AI-1 type LuxR homolog SdiA, AI-2 type LuxS, AI-3 type epinephrine/norepinephrine and/or indole are the several quorum-sensing systems used by *Escherichia coli* and *Salmonella enterica* to achieve intercellular signaling and involve them for interspecies communication [8]. Of these, interkingdom communication is being mediated by the QS system AI-3/epinephrine/norepinephrine [8] (Fig. 1). Interestingly, both these species have virulence as the phenotypic effect of all these systems [9–11]. Evolution of these systems by these bacteria, residing primarily in the human gut, probably reflects their adaptation to the gut environment [8].

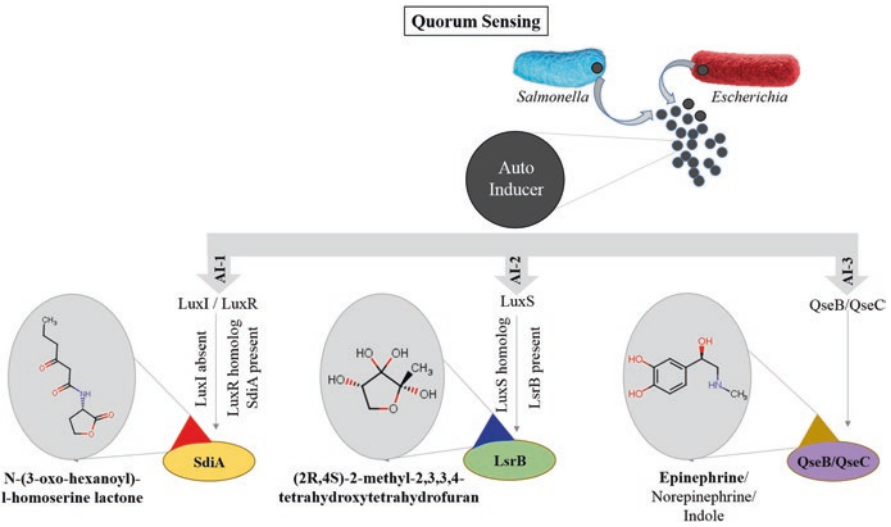

Fig. 1 Quorum sensing phenomenon involving the three autoinducers (AI) of pathogenic *Escherichia* and *Salmonella* species. The gene products involved in binding/recognizing each type of AI are represented in light yellow, light green and medium purple coloured ellipses. The AI molecules are denoted by red, blue and olive colours, respectively for the types 1, 2 and 3. The representative chemical structure of each AI molecule types are shown in bubbles with their names mentioned underneath

The Detection

QS in *S. enterica* and *E. coli* were observed through secretory soluble organic molecule, when strains like LT2, and AB1157, respectively, were grown in Luria-Bertani medium containing glucose [12]. The molecule, being heat labile and glucose inducible, ceased with the depletion of glucose and around the onset of stationary phase thereby getting affected by factors like high osmolarity, low or neutral pH, logarithmic growth and preferred carbon sources [12, 13]. These factors relate to the complex conditions in the intestinal lumen and the phenomenon was related to QS. This communicates the bacterial cell density along with the metabolic potential of the lumen environment thereby playing a crucial role in the behavioural regulation of bacteria in the pre-stationary growth phase [12]. This soluble molecule was produced by several clinical and laboratory strains of *S. enterica* serovar Typhimurium and *E. coli*. However, DH5α strains of the latter did not produce any such molecule which indicates the loss of the necessary biosynthetic gene(s) and/or machinery probably due to high domestication [12]. Grossly, this was attributed to the *luxS* gene product which mediates the production of aforementioned soluble molecule and is referred to as AI-2 [13]. This is also supplemented by the fact that the domesticated *E. coli* DH5α strain produces AI-2 upon *luxS* gene introduction from the pathogenic O157:H7 strain [14].

The linkage of *luxS* gene to AI-2 production was reported for the first time upon MudJ transposon mutagenesis of *S*. Typhimurium LT2 genome which, out of 10,000 transconjugants, gave rise to one insertion mutant [15, 16], lacking detectable AI-2 in mid-exponential phase culture fluids [16]. Sequencing the PCR amplicon helped in determining the site to be mapped on to *ygaG*, an open reading frame (ORF) having unknown function in *E. coli* MG1655 genome [16]. Moreover, a complementation assay with the *E. coli* O157:H7 *ygaG* gene and *Vibrio harveyi luxS* gene revealed a 1.5 times more AI-2 activity than *V. harveyi* for both the AI-2 lacking strains *S. typhimurium* CS132 and *E. coli* DH5α [16]. Furthermore, a high sequence homology with 77% identity was observed when *E. coli* YgaG protein was compared with the LuxS from *V. harveyi*. Similar results were obtained for a protein sequence comparison of the *S.* Typhimurium *ygaG* ORF [16]. Later, such AI-2 production was observed for *S. enterica* serovar Typhimurium and *E. coli* O157:H7 in chicken broth and milk under different conditions, using *V. harveyi* luminescence assay [17].

Despite the aforementioned similarity in their genes, the chemical molecule of *Salmonella* AI-2, however, differs from the classical molecule for signalling, viz. AI-2 of *V. harveyi*, though both are derivatives of the same 4,5- dihydroxy-2,3-pentanedione (DPD). Thus, the structure of *V. harveyi* AI-2 complexed with the receptor protein LuxP, revealed a (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (S-*THMF* borate) [18]. Such borate moiety in *S*-THMF-borate are found in high-borate environments encountered by marine vibrios [19]. On the contrary, a boron lacking chemically different AI-2 signal, (2R,4S)- 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF) is bound to a distinct AI-2 signal binding protein, LsrB, of S. Typhimurium, [19]. LsrB and its homologous periplasmic sugar binding proteins play role in AI-2 signal binding, internalization, and metabolism [20, 21].

Nevertheless, it is to be noted that *Escherichia* and *Salmonella* are unable to synthesize the N-acyl-L-homoserine lactone (AHL) quorum-sensing signalling molecules by themselves despite having the ability to detect AHLs produced by other bacterial species [22]. For instance, Enterohemorrhagic *E. coli* (EHEC) responds to AHL from bovine rumen and activates the acid fitness pathogenicity island while repressing the flagellar genes [22]. Similarly, *Salmonella* could detect *Yersinia enterocolitica* AHLs produced in the Peyer's patches of mouse and activate the gene *srgE,* encoding a putative Type III secreted effector as well as the *rck* operon [22].

The Sensing

AI-1

In general, the phenomenon of QS leads to a differential gene expression aided by a mechanism of population density dependent cellular communication [23]. This is mediated by the synthesis of the AI-1 type AHLs by a pair of proteins encoding acyl

homoserine lactone synthase (LuxI) and a transcription activator (LuxR) or their homologs [23–26]. While this is the case in most Proteobacteria, Enterobacteriaceae members *E. coli* and *S. enterica*, lack LuxI homologues and thus, cannot synthesize AHL [27] despite having a LuxR family transcription factor [28]. This transcription factor namely, SdiA in the *E. coli* genome, suppresses the cell division inhibition [29]. SdiA of *S. enterica* and *E. coli*, however, responds to AHLs of other bacterial species and synthetic AHLs [30–32, 43], thereby resembling the mammalian paracrine signalling systems [34]. For example, *Salmonella* AHLs, regulate phenotypes such as biofilm formation on polystyrene, adhesion to HeLa cells and invasion of HEp-2 epithelial cells besides aiding in the survival in rabbit and guinea pig serum [35–37]. Similarly, for *E. coli*, such AI-1 regulate the resistance to acidic pH, biofilm formation on polystyrene and adhesion to HEp-2 epithelial cells [38–40]. Again, the growth, motility and adhesion leading to biofilm formation under anaerobic conditions on a polystyrene surface, were observed to be influenced by the AI-1N-dodecanoyl-DL-homoserine lactone (C12HSL) of *S. enterica* serovar Enteritidis PT4 578 [42]. Moreover, the stationary phase associated organic acids levels and the protein abundance were also impacted by such AHL of *S.* Enteritidis [41].

Contrary to such normal cases, the effect on SdiA regulation of other phenotypes in the absence of AHLs in *E. coli* and *Salmonella* have also been reported [31, 33, 43–45]. For instance, constitutive activation of the SdiA Enterohemorrhagic *E. coli* O157:H7 (EHEC) is induced by the binding of a molecule 1-octanoyl-*rac*-glycerol (OCL) produced by prokaryotes and eukaryotes [44] as a monoglycerol signaling molecule to be used as membrane synthesis substrate and an energy source [46, 47]. Additionally, besides OCL in the absence of AHLs, different ligands such as *N*-(3-oxo-hexanoyl)-l-homoserine lactone (3-oxo-C6-HSL) and *N*-(3-oxo-octanoyl)-l-homoserine lactone (3-oxo-C8-HSL) complexed with EHEC SdiA protein, affected its conformational changes [44].

Moreover, the QS regulator SdiA directly regulates the *pefI-srgC* operon which encodes the Rck invasion by *S. typhimurium* [48]. In fact, host cells invasion by *Salmonella* occurs through. the Trigger and the Zipper mechanisms [49]. Of these, the *Salmonella* pathogenicity island-1 (SPI-1), encoding the type III secretion system-1 (T3SS-1), triggers the process of eukaryotic cell invasion [50]. The zipper mechanism is mediated by the T3SS-1-independent entry system involving the outer membrane protein Rck [51], named so for its property of ‘resistance to complementary killing’ [52]. Moreover, the SdiA- and AHL-dependent activity was displayed only by the predicted distal promoter upstream of *pefI*, PefIP2 compared to the SdiA independent very low activity of the predicted proximal PefIP1 promoter as determined using plasmid-based transcriptional fusions [48]. Furthermore, surface plasmon resonance studies and electrophoretic mobility shift assays identified a direct and specific interaction of SdiA with the PefIP2 region [48].

AI-2

The regulatory mechanism of QS, sensing the density of cell population through the signalling molecule, achieved a new dimension with the evidence of *luxS* gene necessity for *Salmonella* virulence phenotypes [9]. This was reported through transcription assays on *S. enterica* serovar Typhimurium recombinants with deleted luxS gene, which reflected the abolishment of cell-density-dependent *invF* gene induction and related *invF*-regulated genes expression of the *Salmonella* pathogenicity island 1 (SPI-1) [9]. The restoration of expression happened with the synthetic signal molecule supplementation or plasmid copy of the *luxS* gene introduction [9]. This reduced necessary-SPI-genes expression lead to the attenuated virulence phenotypes, both *in vivo* and *in vitro* [9]. The impact of such *luxS* gene deletion from *S. enterica* serovar Typhimurium was also on the flagellar phase variation which polarized it towards the more immunogenic phase 1 flagellin [53]. Though this was QS-independent phenomenon [53], the importance of the two different types of flagellar subunits, viz. FliC (phase 1) or FljB (phase 2) have been reported [54, 55]. These include the swimming motility of Salmonellae helping to invade the host cells [56, 57], host immune response stimulation through Toll-like receptor 5 binding [58] and macrophage-induced bacterial killing [59, 60]. Despite the QS-independent phenotypic effect of *luxS* having importance in the motility, invasion and interaction within the host gut milieu, the heat and acid adaptation/resistance of *Salmonella* were not affected by such AI-2-based quorum sensing [61], though different microorganisms exhibited the usage of QS to resist various stresses e.g. *Pseudomonas aeruginosa* to resist oxidative stress [62], *Vibrio vulnificus* and *Vibrio angustum* for adaptation to starvation and resistance to stress [63, 64] *Streptococcus mutans* for combating hydrogen peroxide [65] and *Staphylococcus epidermidis* for the regulation of general and oxidative stress-response factors [66].

Despite the necessity of the LuxS/AI-2 QS system for normal SPI-1 expression, its mechanism was largely unknown until more than a decade of discovery of this phenomenon in *S.* Typhimurium. It was found that LsrR protein, a transcriptional regulator, negatively controls flagellar and SPI-1 gene expressions thereby regulating the SPI-1-mediated *Salmonella* virulence [67]. Reportedly, the binding of the phosphorylated AI-2 inactivates LsrR while the active LsrR decreases the flagellar and SPI-1 gene expressions in the luxS mutant thereby impairing the invasion of *Salmonella* into epithelial cells [67]. Moreover, a plasmid based overexpression of the LsrR regulator decreased similar gene expression [67]. This was relieved by exogenous AI-2, which binds and thus, inactivates LsrR [67].

AI-3

Catering to the need of timing the virulence determinants with the human gastrointestinal tracts, it has been proposed that the pathogenic bacteria are aided by secreted AIs, either by themselves or by other intestinal bacteria, for interspecies

communication [68]. These AIs, along with the neurotransmitters like norepinephrine (NE), released by the human sympathetic nervous system controlling motility, secretion and vasoregulation in the highly innervated gastrointestinal tract of the human host [69] have reported to be serving as quorum sensing signals to *Salmonella* and *E. coli* [70]. The research by [70] has demonstrated that the NE, produced by the host (often during stress) along with a medium pre-conditioned with 10% AI-3, enhanced the motility of the wild-type *S. enterica* serovar Typhimurium strain, besides transcriptionally inducing the motility genes, as evidenced by DNA microarray and qRT-PCR analyses. The enhancement is facilitated by the *qseC* gene, encoding the QseC sensor kinase of the bacterial two-component quorum sensing system, QseBC [70]. Their research demonstrated the importance of the *S.* Typhimurium QseBC system for colonizing the swine gastrointestinal tract along with porcine tissues such as cecum, tonsil, ileocecal lymph nodes and ileal Peyer's Patches [70]. Again, besides NE, host epinephrine has also been shown to be activating specific bacterial genes pertaining to growth and motility [10]. In an attempt to investigate the role of epinephrine and conditioned medium (containing AI), a significant increase was observed in the expression of F4 fimbriae, motility and heat-labile toxin (LT) of enterotoxigenic Escherichia coli (ETEC, E68) culture [10].

Coordinated

Notwithstanding the fact that *Salmonella* and *Escherichia* do not synthesize AHLs by themselves though can detect them, the combined effects of AHLs and QS signalling compounds like and AI-2 has been tested out. For instance, research showed that the growth of *S.* Typhimurium and *S.* Enteritidis are affected by the presence of AHLs and AI-2 signalling compounds in the cell-free culture supernatants (CFS) of *Pseudomonas aeruginosa*, *Serratia proteamaculans* 00612, *Yersinia enterocolitica*-like GTE 112, *Y. enterocolitica* CITY650 and CITY844 [71]. The results recorded for area, detection times (Tdet) and slope of conductance curves indicated the complexity of inter-species bacterial communication [71]. Again, the coordinated QS and cellulose production gene expression levels were analysed under three different conditions of growth *viz.* aerobiosis, anaerobiosis and micro-aerobiosis for eleven (11) *S. enterica* strains to evaluate the relationship between the expression of genes involved in biofilm formation and quorum sensing-related phenomenon [72]. Results reported that the gene expression of cellulose synthesis (csgD and adrA) and quorum sensing (sdiA and luxS) were lessened in micro-aerobiosis and anaerobiosis in all strains of *S. enterica* compared to the tested serotypes of *S. enteritidis* and *S. typhimurium* where they are less reduced indicating the influence of atmospheric conditions to be considered during food processing under modified atmospheres or vacuum [72].

Furthermore, other interesting observations have been reported for coordinated behaviour pertaining to QS across *S.* enterica serovar Typhimurium and *E.* coli. For instance, a conservation of such coordinated behaviour were made by [73]. Using

an in-house grown algorithm on the COnserved MODules across Organisms (COMODO) to identify the conserved expression modules between two species, the co-expression conservation across three organisms namely, *Bacillus subtilis*, *E. coli* and *S. enterica* were detected [73]. Reportedly, regulatory interaction networks were conserved between *S. enterica* and *E. coli*, albeit, in some parts of the regulon of local regulators, stress sensing and signalling pathways which was not the case between these bacteria and *B. subtilis* [73]. This was directly in contrast without any conservation for pathogenic genes of these species thereby reflecting their varying lifestyles [73]. Another interesting application of the coordinated effect of QS was made for *Salmonella* with the hypothesis that upon integrating a density-dependent switch, the proteins in tightly packed colonies within tumors would only be expressed [74]. The critical density for initiation of protein expression was determined by measuring the fluorescence and bacterial density in culture and in a tumor-on-a-chip device from a GFP reporter into non-pathogenic *Salmonella* along with a clone of the AI-2 QS system [74]. A report for the calculations of autoinducer concentrations, indicating sigmoidally density dependent and inversely average radial distance dependent expression, led to the conclusion of using QS *Salmonella* that will target drugs to tumors while preventing damage to healthy tissue and thus, be perceived as a promising tool for the treatment of cancer [74].

The Quenching

The phenomenon of QS now has gained attraction due to its potential role in virulence. Reducing the effect of QS through an inhibitory effect, better known as quorum quenching (QQ), thus, has formed a major focus of research. Such QQ phenomenon probably can be achieved through the inhibitory reversal effect of the coordinated behavior of other organisms or by inhibiting the crucial role-playing protein in the organism of interest (Fig. 2).

Interspecies Association

A brilliant example of such interspecies association is between the human intestine infecting enterohemorrhagic *E. coli* O157:H7 (EHEC) and *Lactobacillus sakei* NR28, with the latter serving as a new candidate strain for AI-2 related quorum quenching [75]. The pathogenicity of EHEC ‘wild-type’ strain *E. coli* ATCC 43894, controlled by the LuxS/AI-2 signalling system and mediated by the mechanisms such as motility, attachment and biofilm formation was significantly reduced by *L. sakei* NR28 [75]. To determine the relationship between the virulence reducing effect of *L. sakei* NR28 and its AI-2 inhibiting ability, the purified AI-2 molecule and a luxS deficient mutant of EHEC strain ATCC 43894 were used along with an AI-2 independent EHEC mimicking strain of *Citrobacter rodentium* [75]. In another

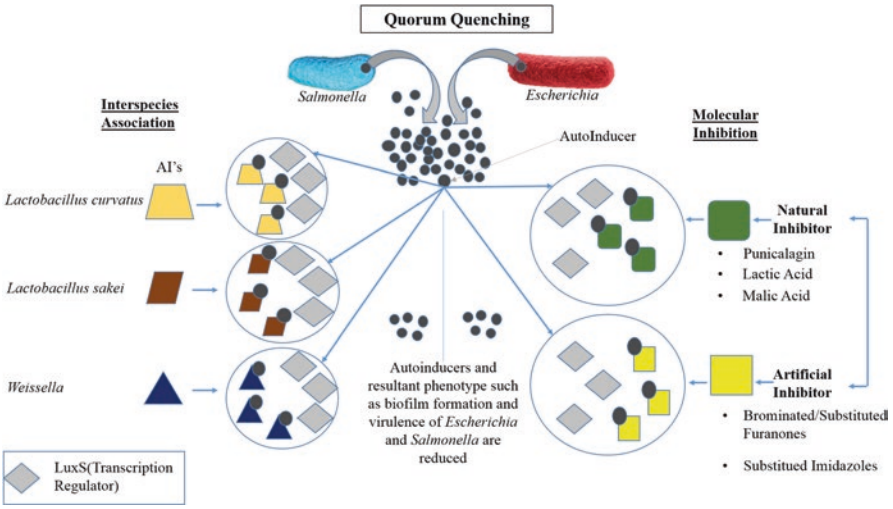

Fig. 2 An overview of the quorum quenching phenomenon for the AI of pathogenic *Escherichia* and *Salmonella* species. The LuxS type transcriptional regulator releasing AI-2 molecules by pathogenic strains are reduced or inhibited through quenching by AI molecules released from species in close association in the human gastrointestinal tract. The pathogenic AI-2 can also be quenched through synthetic products like brominated furanones and its side chain substituted derivatives. Else, natural products like punicalagin, malic and lactic acid can play the role of natural quenchers

instance, the AI-2 activity of kimchi, the lactic fermented food, was measured to find different AI-2 signalling intensities [76]. Reportedly, the AI-2 properties of the 229 lactic acid bacterial isolates obtained from the kimchi samples, was detected using a modified AI-2 bioluminescence assay [76]. AI-2 signal was either reduced or inhibited by the isolates of dominant species of the genera *Lactobacillus*, *Weissella* and *Leuconostoc*. On the contrary, no AI-2 activity was seen from isolates of the dominant species *L. sakei* (75 isolates) and *L. curvatus* (28 isolates) while no AI-2 inhibition could be detected from *L. plantarum* (31 isolates) [76]. All these results suggest the AI-2 activity of kimchi to result during fermentation of associated microbial food cultures (MFCs) having a coordinated interaction [76].

Molecular Inhibition

Earlier reports on the QS inhibitory molecules' effect focussed on the competitive inhibition with structural analogues of AHL like brominated furanone from the secondary metabolites of *Delisea pulchra*, an Australian red alga [77]. Such *Delisea* furanone molecules showed inhibition of bacterial swarming and affected the process of surface colonization of the opportunistic human pathogenic bacteria *Proteus*

mirabilis [78]. Later, in an attempt to efficiently synthesize better AHL analogues, cyclodehydration of brominated levulinic acids have been deployed, under mild reaction conditions, to yield brominated 4-alkyl-2(5H)-furanones with a single chromatographic separation compared to a six-steps procedure [79].The antagonistic effect of such furanone class of compounds against the biofilm formation by *S. typhimurium* was tested by a synthesized library comprising 25 10-unsubstituted and 10-bromo or 10-acetoxy 3-alkyl-5-methylene-2(5H)-furanones and two 3-alkyl maleic anhydrides [80]. With the quorum sensing regulated bioluminescence of *Vibrio harveyi* serving as control, the activity of the furanones in both the biological test systems were observed to be drastically enhanced upon the introduction of a bromine atom on the position 10 of the 3-alkyl chain [80]. Moreover, the potential of the (bromo) alkyl maleic anhydrides was demonstrated as a new and chemically easily accessible class of biofilm and quorum sensing inhibitors [80]. Furthermore, for an enhanced biofilm inhibition, another library of 80 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazo [1,2-a] pyrimidinium salts and 54 2 N-substituted 4(5)-aryl-2-amino-1H-imidazoles was synthesized and tested for the antagonistic effect against biofilm formation by *Pseudomonas aeruginosa* and *S. typhimurium* [81]. The inhibitory potential of the compounds with certain aromatic substituents at the 1-position, such as piperonyl or 3-methoxyphenetyl along with the 2N-substituent. Compounds with a n-butyl, iso-butyl, n-pentyl, cyclopentyl or n-hexyl chain at the 2N-position exhibited an improved activity as compared to their unsubstituted counterparts [81].

Additional attempts to focus on the structurally viable AI-2 quorum-sensing signaling molecules from *S. typhimurium* yielded in the development of a dendrimer-based multivalent probe for specifically recognizing Lsr-type AI-2 receptors [82]. This was based on the alkyl-DPD (4,5-dihydroxy-2,3-pentanedione) analogues reported to have worked uniquely as the quorum-sensing antagonist in both *S. typhimurium* and *V. harveyi* [83]. A list of next generation antimicrobials having antagonistic effects against the *lsr* gene expression in *S. typhimurium* has been reported [84]. Newer dimensions in such QS-antagonist research have been added with brominated heterocycles using aminobromopyrrolone, bromopyridazinone and hydrazinyl furanone arising from the reactions of brominated furanones with bi-nucleophiles, such as substituted or unsubstituted hydrazines [85]. Unfortunately, these have been found to be effective QS inhibitors against *Pseudomonas aeruginosa* only [85]. However, molecular docking studies on the SdiA protein of *S. enterica* serovar Enteritidis PT4 578 based on the three crystallized SdiA structures from Enterohemorrhagic *E. coli* (EHEC) were performed with different ligands like AHLs with 12 carbons, brominated furanones and 1-octanoyl-rac-glycerol in a recent study [27]. The results of this study indicated that the use of brominated furanones to inhibit phenotypes controlled by QS in *Salmonella* and EHEC may present a good strategy as these inhibitors are specific competitors of AHLs for binding to SdiA in both pathogens [27].

Other classes of attempts to hunt for compounds and molecules having anti-QS potential, led to the usage of bioactive compounds like Punicalagin, an essential component of pomegranate rind [86]. Other natural products like Artemisin,

Digoxin, Flavonoids, Ginkgo, Phenols, Punicalagin, Stilbene, Taxol, Vincristine and Vinblastine and non-protein amino acids have been shown to have some QS activities against *S. typhimurium* and other bacteria [87–89]. Of these, Punicalagin significantly decreased bacterial swimming and swarming motility, which corresponded to downregulation of the motility-related genes (*fliA*, *fliY*, *fljB*, *flhC*, and *fimD*) [86]. Moreover, it significantly reduced the invasion of colonic cells ($P < 0.01$) by *Salmonella* without any impact on adhesion [86]. Further attempts to search for anti-QS molecules include organic acid based antimicrobials like malic and lactic acids having autoinducer activity against the selected strains of *E. coli* O157:H7 and *S. typhimurium* [90]. These strains were screened for AI-2 like activity on cantaloupe and spinach homogenates using autoinducer sensing biosensor strains of *V. harveyi* [90].

Future Perspective

The present review aimed at bringing out the diverse complexities of the phenomenon of QS in two of the Enterobacteriaceae *S. enterica* and *E. coli* and the intra- and inter-species signalling molecules involved in the same. The genes and proteins involved have been discussed with an ultimate aim to simplify the goal of targeting these pathogens effectively for treating the infections caused by them. It is probably imperative for the QS system of pathogenic *S. typhimurium* and *E. coli* that, without specific AI-1 type AHLs being produced and AI-3 type molecules interfering with the host signalling system, the safer way to target the proteins for a reduced or inhibitory effect could be the AI-2 type molecules.

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Quorum-Sensing Mechanism in *Rhizobium* sp.: Revealing Complexity in a Molecular Dialogue

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Abstract Nitrogen fixation by a biological process is an important phenomenon for improving agricultural soil fertility by fixing atmospheric nitrogen in the form of ammonia, which is mediated by the symbiotic association between *Rhizobium species* and leguminous plants. During symbiosis bacteria aggregate to form biofilms and coordinate their behavior in response to environmental conditions by a process called Quorum sensing (QS). The mechanism of quorum sensing depends on the interaction between signal molecule and a sensor that helps bacteria to communicate and regulate gene expression related to nodulation, biofilm formation and symbiosis and nitrogen fixation. *Rhizobium* utilizes N- acyl homoserine lactones (AHLs) as signalling molecules to coordinate and regulates gene expression. In addition to this, host response to bacteria is important to combat pathogenic bacteria and attract beneficial ones. For this leguminous plants sense the presence of bacteria precisely and release chemical compounds like flavonoids to make appropriate responses to symbiosis. The review clearly emphasizes interkingdom chemical signaling governing molecular interactions between leguminous plants and *Rhizobium species* in the establishment of symbiosis and nitrogen fixation.

Keywords Quorum sensing · Rhizobium · Leguminous plants · Symbiosis · Nitrogen fixation

Introduction

Members of the nitrogen fixing soil bacteria collectively called rhizobia, are notable for their ability to establish a symbiotic association with leguminous plants by fixing atmospheric nitrogen. During the process of symbiosis, rhizobia reduce atmospheric nitrogen to ammonia inside the root nodules of plants and obtain carbon

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source from the plant [1, 2]. The rhizosphere, the narrow region of soil having direct contact with plant roots plays a pivotal role in driving communication between bacteria and host plants. The exchange of chemical signals for interaction during symbiosis between leguminous plants and rhizobia has been termed as molecular dialogue. In rhizosphere flavonoids secreted by leguminous plants, initiate molecular dialogue by inducing *nod* gene expression in bacteria which in turn synthesize Nod factors required for nodulation. After invading the roots bacteria colonizes and form biofilms that are crucial for their survival and establishment of symbiosis [3]. To undergo a shift from free-living state to endosymbiotic state, bacteria employ complex environmental sensing mechanism *i.e.* Quorum sensing(QS) that sense ecological niche, density and distribution of their own population and helps them to adapt to the given environmental conditions by regulating their gene expression [4]. The process is regulated by the presence of a signaling molecule in the environment which is referred as autoinducer. In gram-negative bacteria like *Rhizobium*, a variety of N-acyl homoserine lactones (AHL) that work as autoinducers have been identified. These help bacterium in regulating growth, nodulation and symbiosis [5]. While many details of these interactions remain unknown, efforts were made to interpret the governing mechanism of host-bacterial specificity that leads to symbiosis. In this chapter, we described molecular factors leading to symbiosis between rhizobia and leguminous plants and the role of quorum sensing in regulating symbiosis and nitrogen fixation.

Molecular Players in Determining Legume-Rhizobial Specificity for Symbiosis

Legume – rhizobial symbiosis is highly specific where specific rhizobial strains interact only to a specific leguminous plant and *vice versa* [6]. Due to this symbiotic specificity nitrogen fixation efficiency varies between different host-rhizobial combinations. This symbiotic specificity requires the exchange of signal molecules between the partners and can be observed at different stages such as bacterial infection, nodulation and nitrogen fixation.

Plant – Microbe Interaction: Early Interactions Between Host and Bacteria

During the early interaction, host-bacteria interact at the growing tip of root hair and legumes initiate the molecular dialogue by secreting phenolic compounds rich in flavonoids. In rhizosphere, flavonoids from the root system attract only specific bacteria and enter the bacterial cell by passive diffusion to activate NodD proteins [7, 8]. The mechanism where a spectrum of flavonoids produced from different legumes

shows specificity towards NodDs secreted by different rhizobia can be considered as early-checkpoint for legume-rhizobial symbiosis. NodDs activates *nod* genes *nodABC* that synthesize Nod factor that is responsible for nodulation and play an essential role in symbiotic association in most of the legumes. Whereas *nod* genes like *nodL*, *nodEF* are required for structural modifications of Nod factors important for the initiation and maintanance of infection thread during symbiosis [9–13]. Nod factors have a basic structure with oligosaccharides containing N-acetyl glucosamine in the backbone and fatty acyl chain at the non- reducing ends. Nod factors from different rhizobia show variation in length of back bone, size and structure of fatty acyl side chain. The amount of Nod factors and modifications towards reducing ends of side chain such as glycosylation and acylation determines the specificity of the host to which they interact [14]. It was stated that elevated level of Nod factor production inhibited nodulation process in soybean varieties [15] Mutation in any of the *nod* genes alters the structure of fatty acyl chain which in turn affects the specificity towards the host. For example, flavonoid inducers from different legumes activates expression of mutant *nodD* from *Rhizobium leguminosarum* bv. *trifolii* which are normally inactive [16]. The secreted Nod factors are recognized by Nod factor receptors (NFRs) present on the root surface (Fig. 1). These NFRs contain LysM motifs suggesting their involvement in binding Nod factors [17]. The NFRs induce changes in the root hair to trap bacteria. After successful interaction, the root hair bends back to grow inversely trapping few bacteria in the narrow passage to form infection thread. In the infection thread bacteria divide continuously by synthesizing Nod factors to initiate infection structure inside the root and finally reach root cortex where nodulation takes place [1, 18]. In addition to activate *nod* genes, Nod factors appears to induce expression of plant genes such as early nodulin (ENOD) genes that are involved in cell growth, cytoskeletal remodeling and suppress the expression of genes involved in defense responses [19].

Biofilm Formation and Establishment of Symbiosis

Rhizosphere, which is the soil zone surrounding the plant root forms suitable environment for the formation of biofilms that helps bacteria to survive under unfavorable environmental conditions such as altered pH, temperature, nutrient limitation, desiccation etc. Biofilm formation starts with the reversible attachment of bacteria to the inert or biotic surface followed by their multiplication in order to form three dimensional structures permeated with channels that help bacteria to exchange signals and nutrients [20]. Major cell wall components of bacteria, such as exopolysaccharides (EPS), flagella, and lipopolysaccharides plays a significant role in the formation of biofilm and symbiosis [21]. In biofilm matrix, exopolysaccharides (EPS) acts as physical barriers by preventing entry of toxic compounds and protect cells. Primary adherence of bacteria to the surface of the root is facilitated by interaction of bacterial glucomannan to the host protein lectin [22, 23]. In addition to this, proteins like PlyA glycanase and PlyB glycans produced by PrsD-PrsE type I

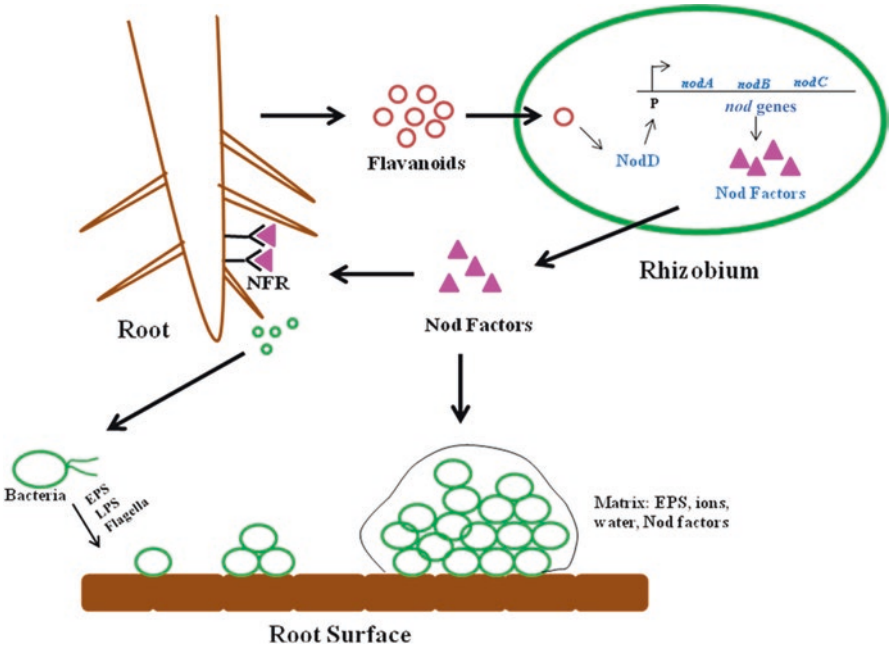

Fig. 1 Early interactions between host and rhizobia. During symbiosis initially, the plant produces flavonoid compounds that diffuse inside the bacteria and activate bacterial NodD proteins. This NodD protein in turn activates the expression of *nod* genes that codes for Nod factors. These secreted Nod factors are recognized by Nod factor receptors (NFRs) present on the root surface. These NFRs induce changes in the root hair to trap bacteria. Downstream to these Nod factors rhizobia uses cell wall components of bacteria like Flagella, Exopolysaccharides and Lipopolysaccharides for colonization, stabilization of biofilm and nodule development

secretion system are also involved in attachment and maturation of biofilm [24]. However, mutations in *plyA* and *plyB* genes form biofilm with an atypical structure whereas mutations in *pssA*, a key gene responsible for EPS synthesis, abolishes biofilm formation [25]. Rhizobial adhesion proteins, RapA1, RapA2, RapC are shown to be involved in early adhesion and colonization of rhizobia by promoting autoaggregation [26].

Further attachment of bacteria to host is mediated by other polysaccharides such cellulose, gel-forming exopolysaccharide, and glucomannan synthesized by *celA, gelA*, and *gmsA* genes. Among these glucomannan interacts with host plant lectin and helps in adherence of bacteria to root surface [23]. The flagellar protein responsible for swarming motility helps in expansion of biofilms by moving bacteria in a coordinated fashion on a solid surface for colonization and subsequent expansion to form communities [27]. It was reported that the O- antigen lipopolysaccharide is required for colonization [21].

Quorum-Sensing Genes in Rhizobia and Their Regulation

Four different N- acyl homoserine lactones (AHLs) based quorum sensing (QS) systems have been identified in *R. leguminosarum* bv. *viciae*, that regulates various stages such as adhesion to the host, biofilm formation, plasmid transfer and nodulation [5, 28]. One of the important characteristic of QS in rhizobia is its diversity towards the host as no two strains analyzed have the same QS system. The proteins that catalyze the synthesis of AHLs belong to LuxR family and are termed as AHL synthase. These AHL synthase genes are present in same locus in operonic arrangement with AHL regulators. *Rhizobium leguminosarum* bv. *viciae* produces four LuxI-type AHL synthases (RhiI, CinI, RaiI, and TraI) whose expression is regulated by five LuxR-type regulators (RhiR, CinR, RaiR, TraR, and BisR). All these AHL based control systems along with small bacteriocin regulate the growth of bacteria, plasmid transfer and nodulation (Fig. 2). Among these *cinI*/*cinR* system is located at the top of regulatory network and controls the expression of all other AHL dependent QS systems [29–32]. However, *rhiA*/*rhiR* are linked to *nod* genes and assumed to have role in process of nodulation [33, 34]. Genes of *traI*/*traiR* together with *bisR* are located on the symbiotic plasmid and induce expression of plasmid transfer genes for symbiosis [28, 29, 35], whereas the function of *raiI*/*raiR* present on non-symbiotic plasmid has yet to be identified (Fig. 2).

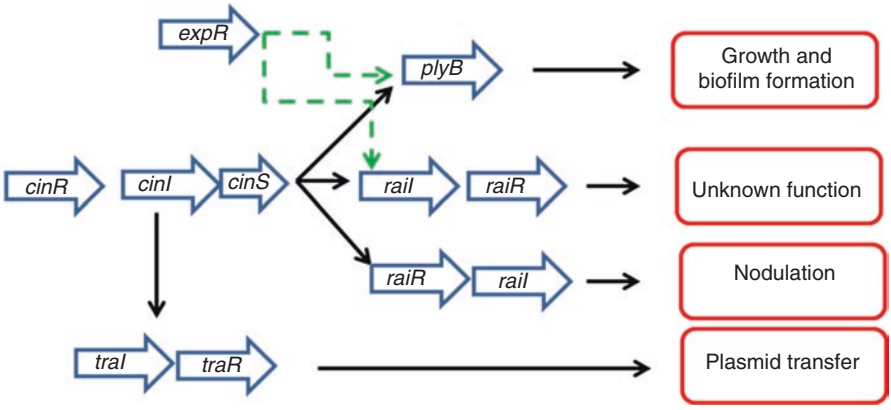

Fig. 2 Quorum sensing genes in rhizobia and their regulation. *cinI*/*cinR* QS system is present at the top of regulatory networks and induces the expression of other quorum sensing systems in rhizobia. *cinI* induces expression of *traI* gene and regulates plasmid transfer. *cinS* which is cotranscribed with *cinI* controls expression of *rhiI*, *raiI* and *plyB* genes.The *rhiI* system is involved in the nodulation process whereas the function of *raiI* system is yet to be identified. *expR* another regulatory gene controls the expression of *raiI* and *plyB* where PlyB is shown to be involved in growth and biofilm formation

Regulation of Growth (EPS)

CinS of *cin* quorum sensing system is reported to be involved in growth and formation of biofilm in *Rhizobium*. The small protein CinS is cotranscribed with autoinducer synthase CinI and regulates the expression of regulatory genes *rhiR, raiR* and also *plyB* [36]. PlyB is a glycanase required to cleave acidic exopolysaccharide required for biofilm formation in *R. leguminosarum* [37]. In addition to CinS, another protein ExpR whose function is independent of CinI also induces both *raiR* and *plyB*. Recently, it was shown that disruption of CinIR enhances biofilm formation in *R. leguminosarum* [36].

Regulation of Plasmid Transfer

The best plasmid transfer system understood in *R. leguminosarum* is pRL1JI symbiotic plasmid system. In this plasmid transfer is regulated mainly by TraR whose expression is under the control of *bisR* gene present upstream to *traR*. The AHL made by *traI* activates *traR* expression [28, 29, 35]. The AHL synthesized by *cinI* [30] activates the expression of regulatory genes, *cinR* which is present on the chromosome and *bisR* present on the plasmid. BisR has a dual function where it acts as an activator to induce expression of regulator gene *traR* and repress the expression of synthase gene *cinI*. Rhizobial strains with pRL1JI plasmid, produce little or no CinI as BisR represses *cinI* expression. Consequently, expression of *traR* is reduced due to the low level of BisR. Low-level of *traR* expression is inhibited by the *traM* gene that produces TraM [29]. During quorum sensing when bacteria carrying pRL1JI plasmid come in to contact with a strain that doesn't have the plasmid, AHL produced by *cinI* from bacteria without plasmid is detected by BisR which in turn induces *traR* gene expression. More levels of TraR titer out TraM and strongly induces plasmid transfer genes that are under the control of TraI–TraR quorum sensing system.

Regulation of Nodulation

RhiR was the first identified QS regulator in bacteria that positively controls the *rhiABC* operon present between *nod* genes required for nodulation and *nif* genes that encodes nitrogenase complex involved in nitrogen fixation [33, 34]. RhiR controls the expression of *rhiABC* and *rhiI* in response to AHLs synthesized by *rhiI* [31]. Mutations in any of these don't have any observable biochemical effect but in absence of *nodFEL* mutations in *rhiA* or *rhiR* further decrease nodulation [33]. Recently it was shown that nodulation efficiency was not affected in *rhiR/rhiI* mutants but the formed nodules were unable to fix atmospheric nitrogen [38].

Host Response to Bacteria

In response to bacterial quorum sensing signals, the host system also has evolved mechanisms such as regulatory, metabolic and defence responses by synthesizing signals that mimic the bacterial signals they encounter. It appears that in the rhizosphere, when bacteria adhere to the root surface, plant root secretes effectors that elicit changes in QS regulated gene expression [39]. Inside the root system plant secretes a set of AHL compounds that mimic bacterial signals [40]. The plant compounds reported so far acts as agonists to AHL signaling rather than antagonists [39–41]. The plant effectors activate gene expression in bacteria in such a way to bring changes that are favorable to the host. It cannot be ignored that there is limited opportunity for the host when bacteria are not yet quorate.

Symbiotic and pathogenic bacteria produce effectors or microbe-associated molecular patterns (MAMPs) that facilitate host invasion [42, 43]. Plants have evolved with different mechanisms to discriminate between friends and foes [44, 45]. In legume- rhizobial symbiosis Nod factors and surface polysaccharides are presumed to inhibit host defense responses [46–49].

Conclusions and Future Prospective

Legume–rhizobial symbiosis depends on the molecular interaction between AHLs, exopolysaccharides, lipopolysaccharides, and Nod factors where modifications in any of these structures affect host – pathogen specificity. There are multiple checkpoints observed between host and pathogen during symbiosis starting from root infection to survival and fixing nitrogen in root nodules. It was hypothesized that initial talk between legumes and rhizobia begins with the pathogenic association and gradually develops into a symbiotic form by suppressing plant innate immunity. Another important aspect to understand is the interconnection of rhizobial biofilm formation with effective symbiosis as there is no direct evidence indicating its role in promoting successful symbiosis with the host. Identifying host targets of symbiotic bacterial effectors will help in understanding beneficial pathways involved in promoting infection and nodule development. This also helps in understanding tolerance of a leguminous plant to the substantial colonization of *Rhizobium* and is useful to transfer nitrogen fixation symbiosis mechanism to non-leguminous plants. Comparative genomics and transcriptomics of varied symbionts will help to understand molecular mechanisms governing symbiosis specificity. However, elucidating the mechanism of host perception to the molecule signal produced by bacteria would help in the understanding of interkingdom signaling.

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Quorum Sensing and Its Role in Agrobacterium Mediated Gene Transfer

Nageswara Rao Reddy Neelapu, Titash Dutta, and Surekha Challa

Abstract Quorum sensing (QS) is a bacterial communication mechanism where individual cells produce and respond to small chemical signals. Literature reports activities such as conjugal transfer of the Ti plasmid; production of specific enzymes, exopolysaccharide, antibiotics, cyanide (HCN), hemolysin, neuraminidase, pyocyanin and rhamnolipid; cell division, bioluminescence, expression of rhizosphere genes and swarming motility are modulated by QS system in different bacteria. Role of QS system, chemical signals, and regulators of QS in replication as well as horizontal transfer of tumor inducing (Ti) plasmid is well established. In this chapter, we review the importance of different types of QS systems, chemical signaling molecules and regulators in *Agrobacterium tumefaciens* to gain more insights in understanding the conjugal transfer of Ti plasmid.

Keywords Quorum sensing system · Chemical signals · Quorum sensors · *Agrobacterium tumefaciens*

Introduction

Quorum sensing (QS) in bacteria is a unique cell to cell communication process that regulates bacterial gene expression triggered due to changes in density of cell-population [1, 2]. The bacteria in QS are involved in production and release of signal molecules which are extracellular in nature and are termed as autoinducers [3]. These autoinducers accumulate as bacterial population density increases. QS detects minimal change in concentration of autoinducer thereby leading to alteration in gene expression [4, 5].

QS regulates a wide array of biological events that comprises virulence, competence, antibiotic production, biofilm formation, conjugation, symbiosis, motility and sporulation [5, 6]. QS was initially identified and documented in two marine bacteria *Vibrio fischeri* and *Vibrio harveyi* that exhibited luminescence some

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25 years ago [7]. Both these species produced and released autoinducers in the form of acetylated-homoserine lactone (HSL) signaling molecules in response to cell growth. As the concentration of autoinducers cross the threshold barrier, luciferase production begins which facilitates bioluminescence at high cell density [7]. QS communication system is widely utilized in both Gram-positive and Gram-negative bacteria where they regulate various biological activities. Both bacterial species use acylated homoserine lactones and processed oligopeptides as autoinducer molecules respectively [8].

In Gram-positive bacteria, cellular communication is mediated by structurally modified oligopeptides (signal molecules) and bi component membrane-bound sensor histidine kinases (receptor). Unlike Gram negative bacteria, in this case the oligopeptides serve as autoinducers. The receptors recognize these oligopeptides and initiate the signal transduction via a series of phosphorylation reactions. These reactions are involved in regulation of a regulatory protein associated with DNA binding at the transcriptional level as well as gene expression. QS has been widely studied in Gram positive bacterial species such as *Bacillus subtilis*, *Streptococcus pneumonia* [9] (with respect to DNA uptake), *Staphylococcus aureus* [10] (in response to virulence) and *Enterococcus faecalis*, (it aids bacterial conjugation) [11].

Quorum Sensing System (QSS) in gram negative bacteria contains two proteins namely LuxI and LuxR with regulatory function. The LuxI-like proteins are involved in the synthesis and regulation of specific acylated homoserine lactone molecule (HSL) termed as autoinducer. QS has so far been elucidated and studied in many Gram negative bacteria. Till date studies revealed presence of LuxI/LuxR-type QSS in 25 bacterial species and have been well documented in the *V. fischerii* [7, 12], *Pseudomonas aeruginosa* [13], *Agrobacterium tumefaciens* [14, 15] and *Erwinia carotovora* [16].

In this chapter, we review the importance and types of QS systems, molecular mechanisms of the QS systems, chemical signaling molecules and regulators in *A. tumefaciens* to gain more insights in understanding the pathway of conjugal transfer of Ti plasmid as well as highlighting the diverse roles of QS signal and response systems in generating a cumulative response in *A. tumefaciens*.

Quorum Sensing Systems

Bacterial QSS is distinguished based on the type of auto inducer signaling molecules. According to literature, there are three QSS: peptide based QS, N-acyl homocysteine lactones (AHL) and AI-2 QSS. In Gram negative bacteria, AHLs serve as the autoinducing signal molecule that triggers QS responses in the bacterial species as well as in the transformed plant. These AHLs are fatty acid derivatives. In Gram positive bacteria, these AHLs are replaced by small peptide molecules that are processed post transcriptionally and are termed as peptide based or Auto Inducing Peptides (AIP). Al-2 is another class of signal molecules that are prevalent in case of many Gram positive bacteria's. The different types of autoinducers (Fig. 1) and their respective QSS has been discussed in the upcoming section.

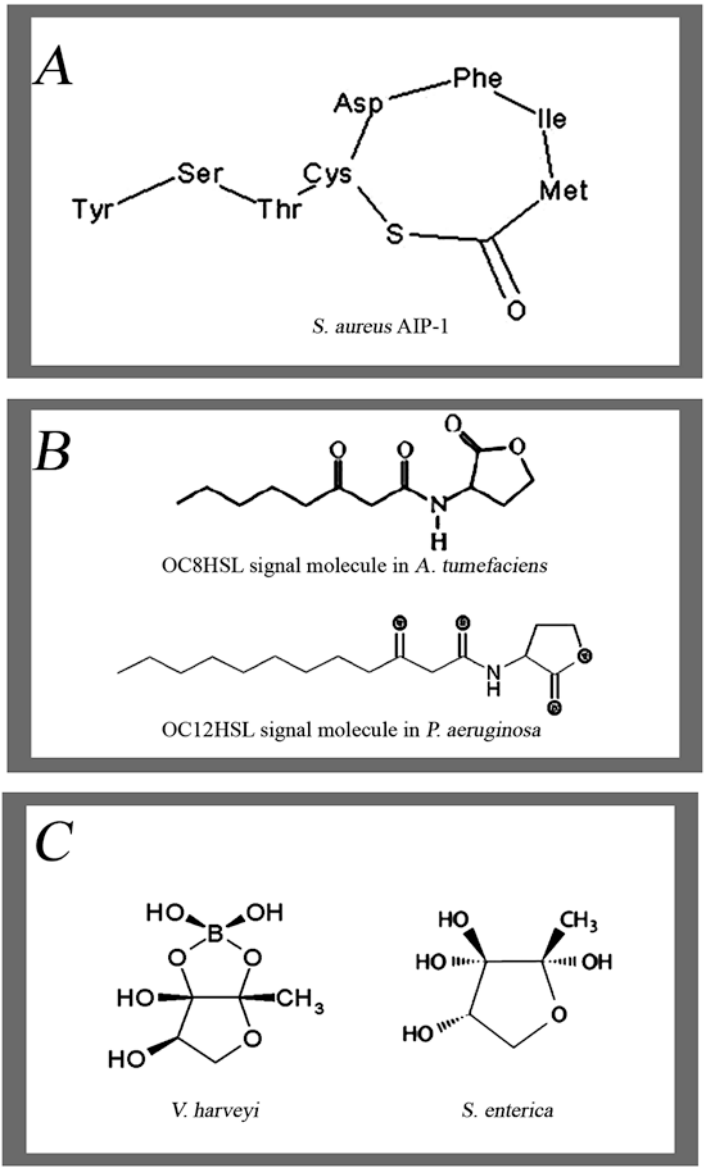

Fig. 1 Structures of different types of QS signal molecules identified in bacteria. (**a**) Peptide based Quorum sensing (AIP). (**b**) AHL based Quorum sensing. (**c**) Autoinducer-2 (AL-2) based Quorum sensing. (Source: LaSarre B, Federle MJ. Exploiting quorum sensing to confuse bacterial pathogens. Microbiology and Molecular Biology Reviews. 2013 Mar 1;77(1):73–111)

Peptide Based/Auto Inducing Peptides Quorum Sensing

Gram-positive bacteria do not possess the LuxI or LuxR homologues, instead oligopeptides serves as the autoinducer. These oligopeptides are products of specific genes and are synthesized intracellular. Once synthesized, the oligopeptides are transported out of the cell by specialized transporters since they are impermeable to the cell membrane. The process requires energy which is provided in the form of ATP molecules. The entire process of oligopeptide synthesis from its translation to export is subjected to various modification, processing or cyclization. The peptide signals are detected either within the cell or at the cellular surface. The most common detector responsible for detecting peptide autoinducers is a kinase enzyme sensor which is membrane bound. The kinase on interaction with an oligopeptide molecule gets activated thereby altering the phosphorylation state of the regulating molecule which subsequently leads to upregulation or downregulation of target QS genes. Extracellular oligopeptide signal detection is seen in the *agr* system of *S. aureus* and the *fsr* system of *E. faecalis*. Both these QSS are associated with production of control virulence factor. In case of the *agr* system of *S. aureus*, cyclic autoinducing peptides (AIPs) interacts with membrane bound cognate AgrC sensor kinases and gets activated due to increased kinase/phosphatase activity. This in turn facilitates the regulation of dispersal of biofilm as well as production of exotoxin in *S. aureus* [17]. However, the *fsr* system, GBAP (gelatinase biosynthesis activating pheromone) serves as the autoinducing oligopeptide. GBAP detection is achieved by membrane bound sensor kinase and is involved in gelatinase production [18]. In some bacteria such as *Streptococcus pneumoniae*, linear peptides also serve as autoinducers. The list of peptide based signaling molecules observed in major bacteria are tabulated in Table 1.

Table 1 List of QSS, peptide based signal molecules and their functions in bacteria

S. No	Organism	Type of QSS	Signal	Receptor	Function	References
1	*Staphylococcus aureus*	*Agr*	AIP	AgrC	Biofilm dispersal, virulence and exotoxin production	Thoendel et al. [19]
2	*Enterococcus faecalis*	*Fsr*	GBAP	FsrC	Production of gelatinase and protease	Nakayama et al. [11]
3	*Streptococcus pneumoniae*	ComC/ ComD	CSP	ComD	Competence, autolysis and virulence	Guiral et al. [20]
4	*Bacillus thuringiensis*	papR/ plcR	PapR	plcR	Exoenzyme production and plant colonization	Rocha-Estrada et al. [21]
5	*Bacillus cereus*	PapR/ PlcR	AIP	PlcR	Enterotoxins, proteases and hemolysins production	Rutherford and Bassler [4]
6	*Clostridium botulinum*	*Agr*	AIP	AgrC	Production of botulinum toxin., sporulation	Cooksley et al. [22]

AHL Based Quorum Sensing

N-acyl homoserine lactones (AHLs) are primarily used as autoinducer molecules in case of Gram-negative bacteria. AHLs are made up of a homoserine lactone (HSL) ring which is joined to an acyl chain (4-8 carbon atoms longs). AHLs can be differentiated on the basis of length and degree of saturation of their acyl chain. The LuxI family comprises of AHL synthases that synthesize AHLs from the substrate molecule *S*-adenosylmethionine (SAM) and are transported using an acylated acyl carrier protein (acyl-ACP) [23]. Once the AHLs are transported across the cellular membrane, they increase their accumulation until they reach the critical threshold concentration. As the AHL concentration reaches the required threshold level, its interaction with the receptor protein (LuxR) takes place in the cytoplasm. The LuxR family is comprised of transcriptional regulators in which ligand interaction alters their DNA-binding activities. The altered DNA-binding activity modifies regulation of target genes such as autoinducer molecules (AHLs) begin to accumulate. LuxI/R signaling is observed in *Chromobacterium violaceum*, where the autoinducer molecule AHL-C6HSL is used to regulate violacein production [24]. In some studies, it was observed that membrane bound sensor kinases are also involved in detection of some AHLs, such as LuxN receptor kinases of *V. harveyi*. These receptor kinases initially associate with the AHL by ligand binding and then initiate a signaling cascade that requires phosphorylation [23, 25]. Similarly, the AHL based QS has been identified and validated in case of the Gram negative soil bacterium, *A. tumefaciens* [26]. Table 2 contains the AHL based signaling molecules in major bacteria.

The AHL receptor proteins in major QS systems exhibited specific binding pattern with specific AHL. Moreover, it was found that each bacterium contains a synthase/receptor pair which is capable of responding only to specific AHL. This response is a result of specificity in the acyl chains lengths as well as the saturation degree of specific AHLs. The binding of these AHL autoinducer molecules depend on various factors namely acyl chain length, degree of its saturation as well as its oxidation state.

AI-2 Based Quorum Sensing System

AI-2 stands for autoinducer-2 and is generated from its precursor *S*-adenosylhomocysteine (SAH) in two sequentially enzyme catalyzed steps enzymes 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) and LuxS (metalloenzyme) [35]. The product of the two enzyme catalyzed steps is an unstable intermediate 4,5-dihydroxy-2,3-pentanedione (DPD), which simultaneously rearranges into interconvertible compounds that structurally represents cyclic furanones. These compounds are collectively termed as AI-2. These AI-2 autoinducers don't require the assistance of carrier proteins for their transport as they freely diffuse out of the cell membranes and accumulate extracellular. Many

Table 2 List of QSS, AHL based signal molecules and their functions in bacteria

S. No	Organism	Type of QSS	Signal	Receptor	Function	References
1	*Pseudomonas aeruginosa*	LasI/ LasR	3OC12HSL	LasR	production of virulence factors, rhamnolipids and biofilm formation	Hentzer et al. [27]
2	*Pseudomonas syringae*	AhlI/ AhlR	3OC6HSL	AhlR	Biofilm formation and plant colonization	von Bodman et al. [26]
3	*Burkholderia glumae*	TofI/ TofR	C8HSL	TofR	Adhesion, exopolysaccharide production and plant colonization	Goo et al. [28]
4	A. *Tumefaciens*	TraI/ TraR	3OC8HSL	TraR	Ti plasmid conjugation, virulence	von Bodman et al. [26]
5	*Chromobacterium violaceum*	CviI/ CviR	C6HSL	CviR	Production of exoenzymes, antibiotics and violacein	McClean et al. [29]
6	*Serratia liquefaciens*	SwrI/ SwrR	C4HSL	SwrR	Biofilm formation and swarming motility	Labbate et al. [30]
7	*V. harveyi*	LuxM/ LuxN	3OHC4HSL	LuxN	exopolysaccharide and protease production, bioluminescence and virulence	Anetzberger et al. [31]
8	*V. fischeri*	LuxI/ LuxR	3OC6HSL	LuxR	Bioluminescence, plant colonization and motility	Lupp et al. [32]
9	*Acidithiobacillus ferrooxidans*	AfeI/ AfeR	C12HSL, OHC14HSL	AfeR	Biofilm formation Involved in Cu^{2+} resistance	Wenbin et al. [33]
10	*Pseudomonas chlororaphis*	PhzI/ PhzR	OHC6HSL	PhzR	Phenazine antibiotic production and virulence factors	Khan et al. [34]

Gram-positive and Gram-negative species have been identified that use AI-2 based quorum sensing system such as *V. harveyi* and *S. enterica serovar typhimurium*. However, different bacterial species detect and interact with specific different forms of AI-2 molecules. For example, *V. harveyi* interacts with a boric acid form of AI-2 using the LuxP/LuxQ receptor/sensor kinase complex [36] whereas in case of *s. typhimurium*, there is interaction of the transporter LsrB with a non boric acid form of AI-2 [37]. Table 3 contains the AI-2 based signaling molecules.

Table 3 List of QSS, AI-2 based signal molecules and their functions in bacteria

S. No	Organism	Type of QSS	Signal	Receptor	Function	References
1	*Escherichia coli*	LuxS	AI-2	LuxP	Biofilm formation, Virulence, chemotaxis, motility	Barrios et al. [38]
2	*V. cholera*	LuxS/ LuxP	Al-2	LuxP	Virulence, biofilm formation and exopolysaccharide production	Rutherford and Bassler [4]
3	*Salmonella enterica*	LuxS/ LuxPQ	AI-2	LuxPQ	Biofilm formation	Choi et al. [39]
4	*Clostridium perfringens*	LuxS	AI-2	LuxS	Virulence and endotoxin production	Ohtani et al. [40]
5	*Streptococcus pyrogenes*	LuxS	AI-2	LuxS	Production of hemolysins and cysteine protease	Lyon and Novick [41]
6	*Clostridium difficile*	LuxS	AI-2	LuxS	Production of virulence factors	Carter et al. [42]

Overview of *Agrobacterium*-Mediated Gene Transfer

Agrobacterium-mediated gene transfer involves two basic components: transformation and tumor formation. Transformation involves the delivery and incorporation of T-DNA segment into plant DNA while tumor formation includes alterations in metabolic process of the host plant cell which is a direct result of transformation of host plant genome. It leads to cell proliferation and synthesis of specific compounds that aid in nutrition thereby providing a favorable niche or selective advantage for the *Agrobacterium* which is subsequently benefitted by the transformed plant cell.

The soil growing *Agrobacterium* is attracted towards the plant due to certain specific chemoattractants which oozes out of the exudates of wounded plant cells such as phenolic compounds (acetosyringone), amino acids and sugars [43, 44]. The *Agrobacterium* initially attaches very weakly to the plant cell via formation of acetylated polysaccharides which is gradually strengthened by extrusion of the cellulose fibers of the plant cell. As the *Agrobacterium* attaches to the plant wound site, activation of the vir regulon is simultaneously carried out by the VirA/VirG regulatory system. The vir regulon consists of a cluster of operons which are essential for generating and transferring the virulent segment of the DNA (T-complex). The chemoattractants present at the plant wound site, for example acetosyringone is responsible for autophosphorylation of VirA, which is a transmembrane receptor kinase [45]. Phosphorylation activates the VirA receptor kinase which in turn transfers its phosphate molecule to the VirG protein present in the cytoplasm and activates it. The activated VirB protein attaches to the *vir* box enhancer elements and upregulates the transcription of virA, virB, virC, virD, virE and virG operons [46]. The T-DNA strand can be synthesized utilizing any segment of the tumor-inducing (Ti) plasmid which lies between the 25 bp imperfect direct repeats (namely the left and

the right borders) flanking the entire T-DNA region. The VirD1 and VirD2 proteins co-regulate the synthesis of the T-strand [47]. The VirD2 protein remains bound to the 5′ end of the T-DNA strand and is coated by the VirE2 (a single stranded DNA binding protein). This T-DNA strand, VirD2 and VirE2 protein complex are collectively termed as the T-DNA complex which is transported from the *Agrobacterium* to cytoplasm of the plant via a bacterial type IV secretion system whose synthesis is regulated by the virB and virD4 operons [48]. The nuclear localization sequences of the VirD2 and VirE2 proteins favors the interaction of the T-complex with plant intracellular proteins (such as importin and cyclophilins) associated with targeting the T-complex to the nucleus of the plant cell. This is followed by successful integration of *Agrobacterium* T-DNA strand into the plant genome through non homologous recombination [49].

QSS in *A. tumefaciens*: TraI/TraR Virulence System

A. tumefaciens is a Gram negative soil bacteria known to cause crown gall tumors in susceptible plant species. The tumors are formed as a result of successful transformation of the host plant cell with the bacterium Ti plasmid [50]. The Ti plasmid contains genes associated with production and regulation of opines in the host plant. Moreover, they also contain genes that code for specific phytohormones involved in cell proliferation thereby resulting in tumor formation in the host plant cells [51, 52]. The opines synthesized are a vital source of nutrition for the *A. tumefaciens* in the plant [53]. Goodner et al. [54] provide detailed list of candidate genes of *A.tumefaciens* along with their function in relation to QS (Table 4).The conjugal transfer of the Ti plasmid between the *A. tumefaciens* and the host plant is regulated by the QSS prevalent in the bacterium [55]. The *A. tumefaciens* QSS contains two regulatory complexes named as TraI and TraR which are located on the transferable region of the Ti plasmid [56]. The conjugal transfer of the Ti plasmid is dependent on two sensory signals, host plant cell opine signal and the autoinducer (HSL) signal. *A. tumefaciens* uses *N*-(3-oxoctanoyl)- homoserine lactone as autoinducer molecule synthesized by *A. tumefaciens TraI* enzyme [57].

Opines accumulate at the infection site and initiate the QS pathway along with serving as source of nutrition for the bacteria. These opines initiate expression of the TraR regulatory complex. The conjugal transfer of the T_i plasmid can be regulated by two types of opines namely: octopine and nopaline [58]. The expression of the TraR complex is driven by the activator OccR in octapine mediated conjugal transfer of the T_i plasmid whereas in case of nopaline mediated conjugal transfer of the Ti plasmid, expression of the TraR is driven by inactivation of the repressor molecule AccR by the agrocinopine A and B [59]. The *A. tumefaciens* QSS is functionally opposite to the *V. fischeri* QSS. The level of production of the autoinducer is directly proportional to the expression levels of the *traI* gene. Opines secreted are responsible for activation of TraR which subsequently favors the binding of the regulatory complex TraR with the autoinducer. The TraR-autoinducer complex upregulates the

Table 4 List of candidate genes involved in QS regulation in octopine and nopaline type Ti plasmids

S. No	Gene name	atu code	Amino acid length	NCBI accession id	Function
1.	*traC*	*atu6126*	98	NP_396649.1	Conjugal transfer protein
2.	*traD*	*atu6125*	81	NP_396648.1	Conjugal transfer protein
3.	*traG*	*atu6124*	658	NP_396647.1	Conjugal transfer protein
4.	*Yci*	*atu6122*	104	NP_396645.2	Nuclease
5.	traA	atu6127	1100	NP_396650.2	Conjugal transfer protein
6.	*traF*	*atu6128*	176	NP_396651.1	Conjugal transfer protein
7.	*traB*	atu6129	398	NP_396652.4	Conjugal transfer protein
8.	*traH*	*atu6130*	209	NP_396653.1	Conjugal transfer protein
9.	*traI*	*atu6042*	211	NP_396559.1	Acyl-homoserine-lactone synthase
10.	*trbB*	*atu6041*	323	NP_396558.2	Conjugal transfer protein
11.	*trbC*	*atu6040*	133	NP_396557.2	Conjugal transfer protein
12.	*trbD*	*atu6039*	99	NP_396556.1	Conjugal transfer protein
13.	*trbE*	*atu6038*	822	NP_396555.1	Conjugal transfer protein
14.	*trbJ*	*atu6037*	267	NP_396554.1	Conjugal transfer protein
15.	*trbK*	*atu6036*	72	NP_396553.1	Entry-exclusion protein
16.	*trbL*	*atu6035*	398	NP_396552.1	Conjugal transfer protein
17.	*trbF*	*atu6034*	220	NP_396551.1	Conjugal transfer protein
18.	*trbG*	*atu6033*	284	NP_396550.2	Conjugal transfer protein
19.	*trbH*	*atu6032*	158	NP_396549.2	Conjugal transfer protein
20.	*trbI*	*atu6031*	438	P_396548.1	Conjugal transfer protein
21.	*traM*	*atu6131*	102	NP_396654.1	Transcriptional anti-activator
22.	*repA*	*atu6043*	405	NP_396560.2	Plasmid-partitioning protein
23.	*repB*	*atu6044*	370	NP_396561.1	Plasmid-partitioning protein
24.	*repC*	*atu6045*	439	NP_396562.1	Replication initiation protein

traI gene expression level thereby establishing a positive autoinduction loop. The TraR-autoinducer complex is involved in the regulation of the *tra* and *trb* operons, and the *traM* gene [59–61]. The *tra* operon encodes for genes associated with the transfer of the Ti plasmid, while the *trb* operon encodes candidate genes required for generating the mating pores. The *traM* gene is involved in the down regulation of QS as it binds to TraR regulatory complex thus blocking the binding site of TraR for DNA and subsequently inactivating target gene expression [62].

Scientists worldwide have contributed significantly in gaining insights of the molecular as well as structural role of QS in regulation of Ti plasmid conjugal transfer. It has been observed that TraR-autoinducer (AHL) complex is essential for correct protein folding and it serves as a scaffold. This is further validated by the location of the AHL ligand which has been found embedded inside the N-terminal domain of TraR regulatory complex [63]. The AHL autoinducer prevents proteolytic degradation of the TraR complex.

In case of the nopaline strain C58, the TraR is initially bound to the plamsa membrane but dissociates rapidly as it binds to the AHL and forms the TraR-autoinducer (AHL) complex. The AHL-TraR complex formed represents a dimer which facilitates the binding between the C-terminal helix-turn-helix domain and the major groove of the 18-bp tra-box recognition site. The tra-boxes are located upstream of the operons (traAFB, traCDG and traI-trb) [64, 65]. The regulatory complex, TraR triggers activation of the traI-trb operon and positively regulates the expression of *traI* [57]. Moreover, the activated TraR complex also upregulates the expression levels of the *traM*. The *traM* gene produces an anti-activator protein which has strong affinity towards C-terminal domain of TraR compex [60, 64]. AHL lactonase is an autoinducer synthesized by the *attM* gene and its concentration is directly proportional to the rate of conjugal transfer of Ti plasmid. It carries out an important role in *A. tumefaciens* QS regulation by governing signal-turnover events and bacterial attachment to the host plant cells [66].

Thus, we observe that opines as well as QS are used by *A. tumefaciens* to regulate the conjugation and replication frequency of Ti plasmid thus providing a favorable niche to the bacterium as well as the transformed plant. The opines serve as important nutrient source to the growing tumors and the *Agrobacterium* growing in the host plant. However, when the nutrient source starts depleting due to rapid increase in bacterial population, activation of Ti plasmid conjugation is facilitated by QS dependent regulation, which in turn presents the bacteria with an opportunity to regain the Ti plasmid. This gradually evolved mechanism is advantageous for all the bacterial species as it allows the bacterial cells to cause and spread new infections.

Activation and Regulation of *A. tumefaciens* QS System

Agrobacterium infection leads to transfer of opine synthesis genes in the host plant. The opines that are produced by the transformed plants are involved in activation of the *Agrobacterium* QS. Apart from being the candidate genes for opine synthesis, the OccR activator molecule activates the TraR regulatory complex. As the QS signal molecule N-(3-oxooctanoyl)- DL-homo serine lactone (3OC8-HSL) accumulates above the threshold level, it facilitates the binding of TraR to it forming the TraR–3OC8-HSL complex. This complex subsequently activates the traI regulatory complex and the tra/trb genes. The tra/trb genes are necessary for transfer of Ti plasmids and they modulate the expression of the *traM* gene.

Opines are involved in activation of the *Agrobacterium* TraR/TraI QS system, thereby playing an integral role in Ti plasmid conjugation and increases the copy number of Ti plasmid in the transformed plant cell upto eight-folds thereby facilitating maximum infection, pathogenesis and opine production [67].

Regulation of *A. tumefaciens* is targeted towards both QS signal production as well as QS signal degradation (quorum quenching). γ- amino butyric acid (GABA) and proline concentrations significantly regulate the *A. tumefaciens* QSS [68, 69]. High levels of proline have been associated with the well developed tumors and they

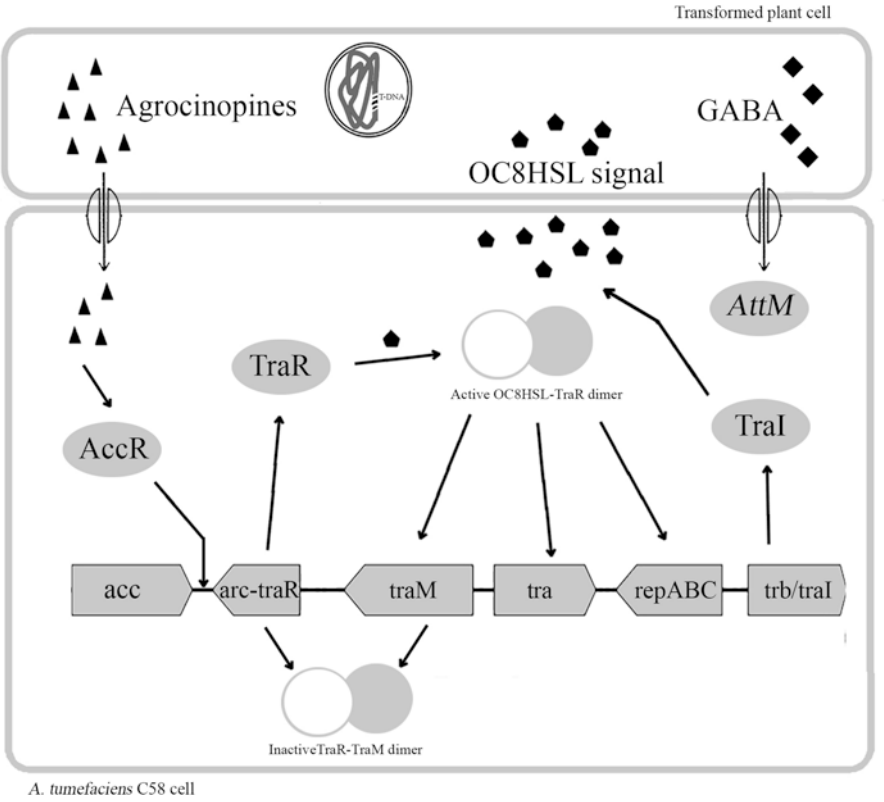

Fig. 2 Regulation of QS in A. tumefaciens C58 cell and transformed plant cell

positively regulate QS whereas high GABA levels have been observed in plants that are relatively less infected and produce smaller tumors. Thus high GABA levels are associated with low *Agrobacterium* pathogenesis and negatively regulate the conjugal transfer of the Ti plasmid (Fig. 2). Similarly salicylic acid (SA) has been found to downregulate the *A.tumefaciens* QSS.

Role of *Agrobacterium* Mediated Gene Transfer in Conferring Biotic and Abiotic Stress Tolerance

The role of QS in relation to conjugal transfer of Ti plasmid in *A. tumefaciens* has been well studied and documented. This finding has paved way for transfer of novel genes into plants of medicinal and economic importance targeting stress tolerance. Exposure to abiotic stresses predominantly salt, drought and extreme temperatures remains the major obstacle in the path of sustainable global crop productivity. The scenario is further aggravated by diseases caused by interaction of plants with biotic

factors (such as bacteria, fungi, insects etc.). Moreover perennial shifts in general climate pattern and uncertainty in rainfall has also worsened the situation and there is tremendous pressure in striking a balance between food supplies with the ever rising demands of the growing population. Biotic as well as abiotic stress factors interferes with the plant physiology affecting morphological characteristics, plant growth, crop yield and subsequently leads to plant death.

To minimize these drastic aftermath of the stress conditions, scientists worldwide focused on *A. tumefaciens* to counter this situation by exploiting the role of *A. tumefaciens* in the transfer of foreign gene. Moreover, in this chapter we highlighted the role of QS in the conjugal transfer of Ti plasmid from *A. tumefaciens* to plants. QS has enhanced the transfer of Ti plasmid and factors (such as *Agrobacterium* strain, cocultivation period, genotype, explants used etc.) [70, 71] improved the transformation efficiency which potentially revolutionized the field of genetic engineering. As transfer of T-DNA in *A. tumefaciens* is promoted by QS, this property has been used further for the transfer of specific genes in plants targeting biotic and abiotic stress tolerance. *A. tumefaciens* is used for genetic transformation of medicinal and economically important plants such as wheat, rice, pigeon pea, maize, pulses etc. [72, 73] subjected to abiotic and biotic stress conditions [74, 75]. In general genes encoding for transcriptional factors, osmolytes [76], aquaporins, LEA proteins etc. has been successfully transferred into respective host plants thereby conferring tolerance to stress conditions [77, 78]. Therefore it is of primary importance to study any possible cross-talks between QS and the transformed plant cell in relation to expression of the foreign gene and its products.

Conclusion

Bacteria have gradually evolved novel QSS and have adapted them significantly to facilitate the regulation of various activities ranging from conjugal transfer of the Ti plasmid, to production of specific enzymes, exopolysaccharide, antibiotics, cyanide (HCN), hemolysin, neuraminidase, pyocyanin and rhamnolipid; cell division, bioluminescence, and swarming motility of bacterial species. QS in bacterial communication is essential for their survival. QS facilitates coordination of behavior among bacteria and provides knowledgeable insights on building community networks and interspecies cell-cell communication among bacteria [79]. However, further research is necessary to infer the evolutionary, ecological as well as pathological facets of QS in improving the survival rate as well as optimum growth of bacteria.

In *A. tumefaciens*, the TraI/TraR regulatory system and the candidate genes involved in QS are conserved in both octapine and nopaline strains. The genes regulating QS in *A. tumefaciens* perform several functions such as conjugation and replication of Ti plasmid, positive feedback control using the OC8HSL-TraI enzyme complex as well as negative feedback control using the TraM antiactivator [80, 81]. Moreover, the *A. tumefaciens* QS system throws light on the biosynthesis and degradation aspects of QS signals thereby providing valuable knowledge that can be

incorporated to design latest antimicrobial strategies to counter bacterial pathogenesis. Though we have made sufficient progress to gain valuable insights on QS in *A. tumefaciens*, there is limited information regarding the role of QS during interaction of *A. tumefaciens* with the host plant, identifying chemical molecules that regulate *Agrobacterium* pathogenesis and elucidating the signaling pathway as well as the regulatory checkpoints of *A. tumefaciens* thereby facilitating the development of new antimicrobial drugs targeting *A. tumefaciens* pathogenesis.

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Quorum Sensing in *Helicobacter pylori*: Role of Biofilm and Its Implications for Antibiotic Resistance and Immune Evasion

Surekha Challa and Nageswara Rao Reddy Neelapu

Abstract *Helicobacter pylori* colonizes the host inducing gastritis, gastric ulcer and cancer. Successful infection in harsh environment requires a special mechanism to colonize the epithelial lining of the host stomach. Antibiotic resistance reports on *H. pylori* have driven the research to identify the factors contributing to antibiotic resistance. Many reports were there on the communities of microorganisms growing on different surfaces. Such communities of microorganisms growing on the surfaces like gastric mucosa are known as biofilms. The formation of biofilms by *H. pylori* on the surfaces of gastric mucosa can be one of the reasons for antibiotic resistance and successful infection in the harsh acidic environment. Several reports were also there on formation of biofilm and quorum sensing in *H. pylori*. The present chapter reviews formation of biofilm, quorum sensing and the advantages of *H. pylori* biofilm which are responsible for successful colonization.

Keywords *Helicobacter pylori* · Biofilm · Immune evasion · Antibiotic resistance

Introduction

Helicobacter pylori was discovered in human stomach, dental plaque, oral lesions, saliva, tonsil and adenoid tissue. *H. pylori* was known for causing gastrointestinal disorders like gastritis, ulcers and gastric cancer [1, 2]. Sometimes *H. pylori* may trigger some other diseases like otitis, sinusitis, phyrangitis, laryngitis and glossitis [3]. Most of the bacteria including pathogenic species like *H. pylori* form biofilms rather than existing in planktonic (cells) mode of growth, to survive in adverse environmental conditions. The adverse environmental conditions for bacteria can be presence of antibiotics and disinfectants; host's immune defenses; changes in nutritional support for bacteria; high temperatures or changes in temperature; changing pH etc. Literature reports that *H. pylori* often exist in biofilm mode usually in environment [4–9] and gastric mucosa of human [10–12]. According to Donlan and

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Costerton [13] microorganisms in a group showing another phenotype when compared with their corresponding planktonic cells is known as biofilm. Based on the composition, biofilms are two types: a) monospecies biofilm, and b) polymicrobial biofilms. Stoodley et al. [14] proposed a model on biofilm with various stages to understand the formation and maturation of biofilms. Stages include reversible bacterial adherence, irreversible adhesion, formation and maturation of matrix, and dispersal of cells. Generally, biofilm is formed on medical devices; or in the tissue of the host; or on fresh fruits and vegetables; or on agricultural products used for food consumption. Biofilm provides a strong platform for interaction and communication among the individuals present in the colony. Biofilm's can withstand antibiotics, disinfectants, immune defenses, high temperatures, nutritional changes, changes in pH etc.

Formation of Biofilm in *H. pylori*

Several studies reported the ability of *H. pylori*, components of biofilm and the role of several factors to form biofilm [15–21]. Stark et al. [15] reported that strain NCTC 11637 of *H. pylori* has the ability to form biofilm. Stark et al. [15] noticed biofilm at the air-liquid interface when continually grown in a fermenter. Later, Cole et al. [16] reported that *H. pylori* strains have the ability to form biofilms on glass surfaces. Role of outer membrane vesicles (OMVs) (22 kDa proteins) in formation of biofilm on glass surfaces was also well studied in *H. pylori* [17, 18]. Extracellular DNA (e-DNA) and mannose-related proteoglycans (proteomannans) are part of extracellular polymeric substances (EPS) of *H. pylori* biofilm [19, 21]. Grande et al. [19] revealed that e-DNA is a component of EPS of biofilm and its role in stabilizing the structure of biofilm. Yang et al. [21] showed that proteomannans are the components of EPS; and are involved in the formation of biofilm. At the same time the role of neutrophil-activating protein A (NapA) in formation of biofilm was established. Expression levels of NapA influenced the formation of biofilm [21]. Expression of NapA is more in cells of biofilm when compared to planktonic cells. This difference leads to different phenotypic biofilm as observed in *napA* deficient mutants [21]. Grande et al. [20] observed that biofilms with multiple *H. pylori* strains are more virulent than biofilms with single strains. The reason for virulence can be genetic exchange facilitating the production of strains which are more dangerous.

Biofilm and Quorum Sensing in *H. pylori*

The cells in the biofilm have an effective communication system known as quorum sensing (QS) [22, 23]. The signaling molecules which are part of the communication system are known as autoinducers (AIs) [24, 25]. AI-1 (N-acyl-L-homoserine lactone (N-AHL) molecule), AI-2 (cyclic furanones), oligopeptides and diffusible

signal factors (DSF) are the QS molecules which were well characterized [26–28]. AI-2 and DSF were reported and well characterized in *H. pylori* [27]. AI-2 initiates formation of biofilm whereas, DSFs help in communicating between two microbes in the biofilm. LuxS produces molecules akin to AI-2, which perceives signal via chemoreceptor TlpB to initiate quorum-sensing. Lux S plays a role in methyl cycle and cell-to cell signaling; thereby controlling the motility of *H. pylori* by modifying transcription and biosynthesis of flagella; and formation of biofilm. These changes supported by Lux S increases the survival of bacteria [27, 29–32]. DSFs control transition of *H. pylori* into a sedentary state (resistant coccoid form); and autoaggregative behavior initiating the formation of biofilm [28]. Inhibition of quorum signaling molecules AI-2 and DSF of quorum sensing system (QSS) in *H. pylori* is known as quorum quenching and inhibitors are known as quorum quenchers or quorum sensing inhibitors [23]. Although literature reports many quorum quenching molecules (qqm), no specific qqm were reported for *H. pylori*. Therefore, there is a need to explore and identify new specific qqm's for *H. pylori*.

Biofilm and Antibiotic Resistance in *H. pylori*

Literature reports resistance of several antibiotics or antimicrobial agents towards bacterial biofilms [33–37]. Cells in the bacterial biofilm showed 10–1000 times resistance towards antimicrobial agents. Biofilms when exposed to antibiotics showed several phenotypic changes and alteration in signaling pathways leading to partial antibiotic resistance. The phenotypic changes leading to partial antibiotic resistance include changes in biofilm structure, cell morphology, growth rate, induction of extracellular DNA and bacterial membrane vesicles (BMVs). Biofilms require certain signaling mechanisms like cyclic dimeric guanosine monophosphate (c-di-GMP) signaling, oxidative stress response, quorum sensing, SOS response and starvation stress response. These signaling mechanisms are altered when biofilms are exposed to antibiotics leading to partial antibiotic resistance. In conclusion, literature reports different mechanisms of antibiotics resistance like (i)

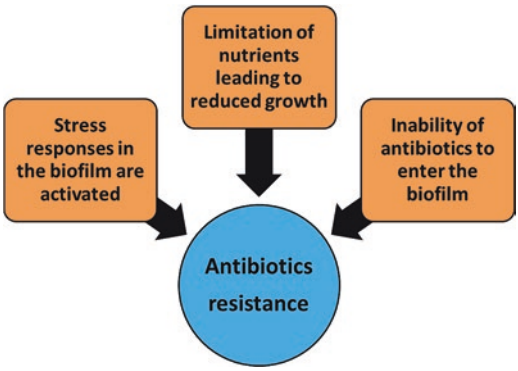

Fig. 1 Strategies followed by bacterial biofilms that lead to antibiotic resistance

the inability of antibiotics to enter the biofilm, (ii) limitation of nutrients leading to reduced growth and antibiotic resistance, and (iii) activation of stress responses in the biofilm leading to antibiotic resistance [33, 38–42] (Fig. 1). Antibiotic resistance is an increasing problem for failure of eradication of *H. pylori* infections. However, there are few reports on antimicrobial resistance of *H. pylori* biofilm. *H. pylori* biofilms resistant to antibiotics clarithromycin, amoxicillin and either or metronidazole – (CAM) were identified. The resistance towards antibiotics CAM by the *H. pylori* cells in the biofilms was more when compared with planktonic cultures. The CAM resistance of the clinical isolates in the biofilm ranged between 10% and 30% [43].

Biofilm and Immune Evasion by *H. pylori*

People infected with *H. pylori* showed an increase in activated dendritic cells (DCs) and macrophages in the gastric mucosa. B-cells, DCs and macrophages form the antigen presenting cells (APC) to internalize and process antigen. However this antigen is presented via class II MHC molecules to CD4+ T-cells leading to initiation of T-cell response which is antigen specific. Interleukins (IL-6, IL-1β and IL-12) and tumor necrosis factor are produced by activated macrophages causing inflammation (gastritis). Further, inflammation is prolonged and augmented leading to ulcers and gastric cancer [1, 2, 44]. There are several strategies adapted by the pathogen both in the planktonic and biofilm mode to avoid immune system of the host. *H. pylori* in the planktonic cell mode adapt a number of immune evasion strategies to protect itself from immune system of the host. The strategies adapted by *H. pylori* in the planktonic cell mode to evade immune system are hindering the perception of the innate immunity; averting the actual T-cell response; modulating adaptive immunity; and avoiding humoral response.

Generally biofilms use a number of strategies to withstand host defense mechanisms. The key strategies reported in the literature to evade host immune system are (1) limiting the penetration of leukocytes into the biofilm, (2) QS increasing resistance to leukocytes, (3) decreasing leukocytes adeptness to engulf biofilm (4) suppressing the activity of leukocyte, (5) controlling genetic switches of biofilms, [45] (6) dysfunctioning or destroying macrophages, and (7) pathogens forming affective biofilm shields (Fig. 2). Though, there are detailed reports on mechanisms of immune evasion used by biofilms of *S. aureus* and *P. aeruginosa*, very few reports were available on immune evasion used by biofilms of *H. pylori*. Biofilms in general can be formed by monospecies or polyspecies or single strains or multiple strains. When biofilms are formed by polyspecies or multiple strains, there are chances of recombination within biofilms [20]. There was enough evidence for existence of eDNA in biofilms of *H. pylori*. This existence of eDNA enables acquiring of eDNA from the matrix of biofilm via horizontal gene transfer leading to recombination among the strains in biofilm. As a result strains in the biofilm become more virulent

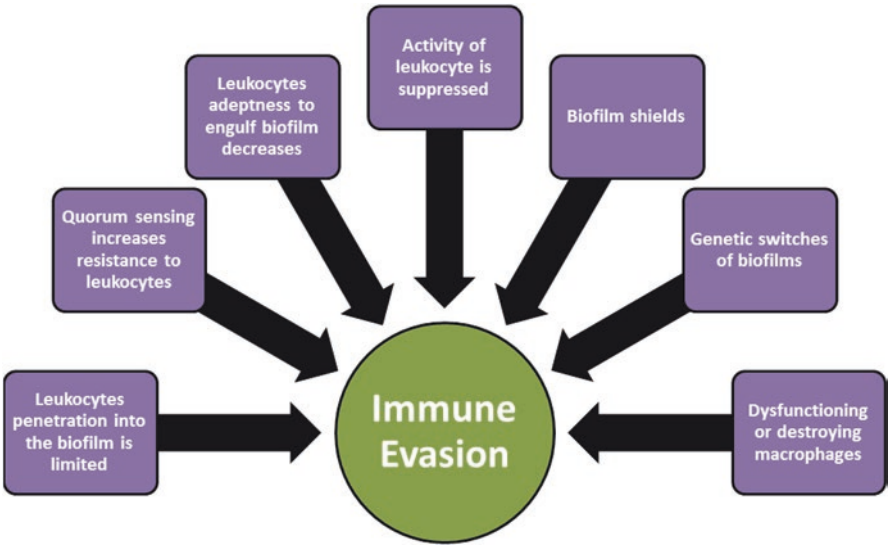

Fig. 2 Strategies followed by bacterial biofilms to evade hosts immune system

then earlier strains, and thereby increase persistence in the host. Thus, these biofilms can develop more tolerance to immune responses of the host.

Biofilm and Disinfectants Resistance in *H. pylori*

Literature reports resistance to disinfectants by several bacterial biofilms such as *E. coli, P. aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium fortuitum*, *M. marinum*, *K. pneumonia*, and *Staphylococcus epidermidis* [46]. Various disinfectants used to treat biofilms are benzalkonium chloride, cetrimide, chlorine, sodium hypochlorite, hydrogen peroxide, peracetic acid þ hydrogen, peroxide, chlorosulfamate, glutaraldehyde, chlorhexidine digluconate, silver nitrate, phenol, oregano, carvacrol, thymol, tea tree oil and eucalyptus oil. When treated with the above listed disinfectants, biofilms showed resistance. The mechanisms of resistance by biofilms towards disinfectants are (i). limiting the entry of disinfectants into biofilm, (ii). forming heterogeneous biofilm (multi-species or multi-strain composition of biofilm), and (iii). materializing three dimensional structure of the biofilm (Fig. 3). Though, there are detailed reports on resistance to disinfectants on several bacteria, there are no reports on resistance to disinfectants used against *H. pylori* biofilms. *H. pylori* often exists as biofilm in water and if biofilms of *H. pylori* develop resistance towards disinfectants which are used for treating water, then drinking water can be an important source for *H. pylori* infection. Therefore, there is a dire need to understand the resistance to disinfectants used against *H. pylori* biofilms.

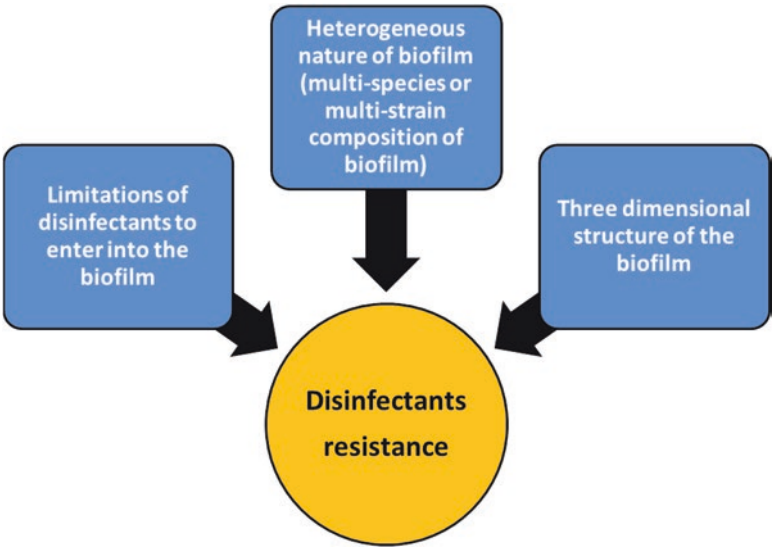

Fig. 3 Strategies followed by bacterial biofilms that lead to disinfectants resistance

Therapy for Preventing *H. pylori* Infection

The earlier approaches to prevent *H. pylori i*nfection include using antibiotics against planktonic cells of *H. pylori*. Rising antibiotic resistance to efflux pumps is reported against *H. pylori* [1, 2]. In order to counter antibiotic resistance many groups were successful in identifying new or alternative drug targets [47–53], developing new drug combinations. The present approach or therapy for preventing *H. pylori i*nfections includes treating cells in biofilm and treating bacterial biofilms is still under development. Literature reports that biofilm can be eradicated possibly by decreasing formation of biofilm; inhibiting adherence of bacteria; decreasing the production of the EPS; disrupting mature biofilms; and inhibiting quorum signaling molecules [54–57]. This is possible in the presence of a known antibiofilm agent N-acetylcysteine (NAC). Cammarota et al. [12] in *invitro* studies was successful in preventing and disturbing *H. pylori* biofilms using NAC a known mucolytic, antibacterial, and a thiol-containing antioxidant agent. NAC competitively inhibits cysteine or as it might act in response to proteins as it contains a sulfhydryl group.

Conclusion

H. pylori has the ability to form monospecies biofilm with single or multi-strains either in water system or gastric mucosa of the host. The matrix of the biofilm (EPS) consists of eDNA, OMV's and proteomannans which help in initiation, formation

and stability of the biofilm. The AI's (AI-2 and DSF) of *H. pylori* act as the QS molecules and help in communication among the cells of the biofilm. The inability of antibiotics or disinfectants to enter into biofilm leads to resistance to both antibiotics and disinfectants. More detailed studies are required for understanding immune evasion by *H. pylori* and in preventing *H. pylori* biofilms.

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Authors Contribution CS and NNR initiated the review, participated in writing and revised the manuscript.

Conflict of Interest Statement The authors declare that there is no potential conflict of interest.

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Quorum Sensing and Biofilm Disassembly Process in *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Xanthomonas campestris*

Santosh Kumar Singh

Abstract Quorum sensing (QS) is a cooperative activity among bacterial cells that is mediated by extracellular cell signaling biomolecules and regulates multiple social traits like biofilm. Similar to QS, biofilm formation is also a cooperative activity among bacterial cell population that leads to formation of extracellular matrix in which bacterial cells are living embedded. Multiple findings intuitively indicate that QS may regulate biofilm formation when cell density of bacterial populations reaches at threshold levels.

However, a group of studies provide convincing evidences that QS initiates in established biofilm and leads to maturation and dispersion of biofilm. This chapter will explain the emerging concepts that QS regulates biofilm disassembly process using three pathogenic bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus* and *Xanthomonas campestris*) as example.

Keywords Bacteria · Biofilm · eDNA · Quorum sensing · Bacterial adhesins

Introduction

Bacteria are the unicellular organism and they have capacity to grow, divide and sense environmental signals independently. Though they have abilities to perform these process independently, they communicate with their neighboring cells for their better survival through specific signal molecules. This cell to cell communication is called as quorum sensing (QS). Bacteria identifies their cell density through QS by quantifying accumulation of specific molecules that their community secrets. When bacterial cell density is high, sufficient signaling molecules accumulates in extracellular environment and initiates the signaling. Generally, QS signaling molecules are low molecular weight biochemical which belongs to wide variety of classes eg. cis-unsaturated fatty acids (DSF family of signaling molecules), specific peptides, acyl homoserine lactones (AHLs) and furanosyl borate diesters (AI2) [1–3].

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Biofilm formation is the common process that bacteria does in cooperative manner. Biofilms are the microbial communities that are embaded in the extracellular matrix and grow by attaching on a substratum [4]. Inside biofilm, bacterial communities are protected from adverse environmental threats like desiccation, immune system attacks, antimicrobials and protozoan ingestion. Biofilm formation is three step process that includes (1) Attachment of bacteria on substratum (2) Growth, division and production of extracellular matrix and (3) Dispersion of bacterial cells from biofilm. The question worth asking is that at which cell density QS comes in picture for regulation biofilm formation. It is intuitive that accumulation of QS signaling molecule do not occur at the step of substratum attachment because it involves single swimming bacteria. It may occur at latter steps when bacteria grow, divide, forms microcolonies, produces extracellular matix and extracellular secreted molecules started accumulating the environment that may be enough to initiate maturation or dispersion of biofilm [4].

Recently multiple studies presented evidences that variety of bacterial species use their QS mechanism to initiate the disassembly of biofilm. Biofilm dispersion has important role in bacterial life because it promotes bacteria to get released from existing colony and form their new niche for better survival and nutrient supply.

Since QS signaling network is complicated and involves multiple genes those encodes for products which are required at different stages of biofilm assembly and dispersion. In this chapter, multiple observations from different studies have been collected which demonstrate that QS act as regulatory network for biofilm dispersion in three bacterial species viz. *Pseudomonas aeruginosa, staphylococcus aureus and Xanthomonas campestris*.

QS and Biofilm Development in *Pseudomonas aeruginosa*

Biofilm formation and Quorum sensing mechanism has been deeply studied *Pseudomonas aeruginosa* which often causes serious infections like urinary tract infections (UTIs), *pneumonia* and other forms of bacteremia in patients who were hospitalized for more than a week. In *P. aeruginosa*, QS machinery is composed of two AHL loops viz. LasI/LasR and RhlI/RhlR where LasI/LasR loop is upstream to RhlI/RhlR loop. Both of these QS systems are consist of LuxI type synthases (LasI and RhlI which synthesize AHLs) and LuxR type receptors (LasR and RhlR). At higher cell density, accumulated AHLs binds specifically with LuxR type transcription factors. LuxR type proteins folds and stabilizes after binding with AHLs start regulating the transcription of target genes. Many times, AHL bound LuxR type proteins also induces the transcription of *luxI* gene and provides signal amplification via feed forward auto-induction circuit [3, 5]. The link between QS and biofilm formation was first reported in 1998 [6]. This study demonstrated that LasI/LasR QS loop was required biofilm differentiation process but it did not play any role in substratum attachment [6]. Later, multiple in vitro studies demonstrated the role of QS machinery in *P. aeruginosa* biofilm differentiation process but these results

remained anomalous. The variation in these studies were mainly due to differences in culture conditions and different biofilm model used [7]. The evidences of these studies in *P. aeruginosa* that demonstrated the dependence of biofilm formation on QS was mainly due to involvement of different factors which is required for specific stages of biofilm differentiation. QS mediated extracellular DNA (eDNA) release is one such factor that provides structural stability to biofilm [8]. Swarming motility, which is regulated by QS, is associated with early stages of biofilm formation mainly because swarming directs initial attachment to the substratum [7]. A group of initial studies observed that LasI/LasR activated the transcription of *pel* genes whose products synthesizes glucose-rich exopolysaccharide (PEL) that forms biofilm matrix [9]. On contrary, another study found that Las directed QS inhibits the production of PEL [10]. This study demonstrated that LasI/LasR induces the expression of tyrosine phosphatase TbpA. TbpA regulates the activity of diguanylate cyclase (TbpB) that in turn decreases the levels of c-di-GMP. C-di-GMP is required to bind on c-di-GMP receptor PelD which is required for PEL biosynthesis. Thus LasI/LasR QS decreases PEL synthesis through TbpA. The production of rhamnolipids is regulated by AHL and PQS loop of QS, also play important role in *P. aeruginosa* biofilm development [11]. Rhamnolipids are biosurfactants that was demonstrated to play influential role in late biofilm formation and maintains the channel between mushroom shaped biofilms. These channels are essential gateways for distribution of nutrients and oxygen, and removal of waste products [12]. *rhlAB* operon that is responsible for rhamnolipids production lies in the stalk of mushroom shaped structures [13], but they play crucial role in the formation of mushroom cap by regulating the twitching mobility of bacteria [14]. A remarkable study demonstrated that appropriate amount of rhamnolipids is crucial for proper biofilm formation [15]. This study found that overproduction of rhamnolipids results into the detachment of biofilm. Exogenous addition of rhamnolipids from *P. aeruginosa* resulted into detachment of wild type *P. aeruginosa* and other microbial biofilms [16, 17]. Above studies presents compelling evidence that QS direct biofilm dispersion in *P. aeruginosa* by reducing the biosynthesis of one of the major exopolysacchharide, PEL and by elevating the synthesis of rhamnolipids surfactants molecules. The observation of elevated eDNA production that is a component of biofilm matrix by QS appears contrary to the knowledge that QS induces biofilm dispersion. However, the production of eDNA was mediated by cell death and cell lysis of bacteria in the biofilm, which is directed by QS. Rhamnolipids, in addition to biofilm dispersion, also provide protection to released bacteria from innate immune defense of PBMCs [18].

QS and Biofilm Development in *Staphylococcus aureus*

QS mediated biofilm formation in *S. auerus* is dependent on Agr system [19, 20]. Agr system comprised of membrane bound protein AgrB, QS peptide AgrD and two component signal transduction system AgrC and AgrA. AgrC is sensor histidine

kinase that phosphorelates its cognate response regulator AgrA. When AgrB modifies AgrD QS peptide, the cell membrane became impermeable to this peptide. As a result, AgrD binds to membrane bound protein AgrC that autophosphorelates at conserved histidine residue. Consequently, AgrC phosphoryltes its cognate response regulator, AgrA. Phosphorylated AgrA initiates the activation of its own transcription along with the target genes [1, 21] (Fig. 1). Initial studies on the role of Agr on biofilm formation demonstrated that *agr* mutants were hyperactive on biofilm development [19]. Since Agr system was known to produce extracellular protease, it was assumed that that less extracellular protease production was helpful in enhanced biofilm formation [20]. Recent advancement in the field demonstrated that the function of Agr system is more complex that production of extracellular protease. Agr system also plays role in the production of biofilm matrix compounds. In *S. aureus* two type of biofilm matix were found. One is exopolysaccharide PIA/PNAG and other is surface adhesion proteins. Agr system does not have any role in the production of PIA/PNAG whereas, it downregulates the expression of surface adhesion molecule viz. fibronectin binding proteins (FnBPs) and protein A [22]. FnBPs and protein A, under specific environmental conditions induces the formation of proteinaceous biofilm [23–26]. Another recent study identified additional role of Agr system in biofilm dispersion. In *S. epidermidis* and in *S. aureus*, secreted phenol soluble modulins (PSMs) showed surfactant like properties which mediates

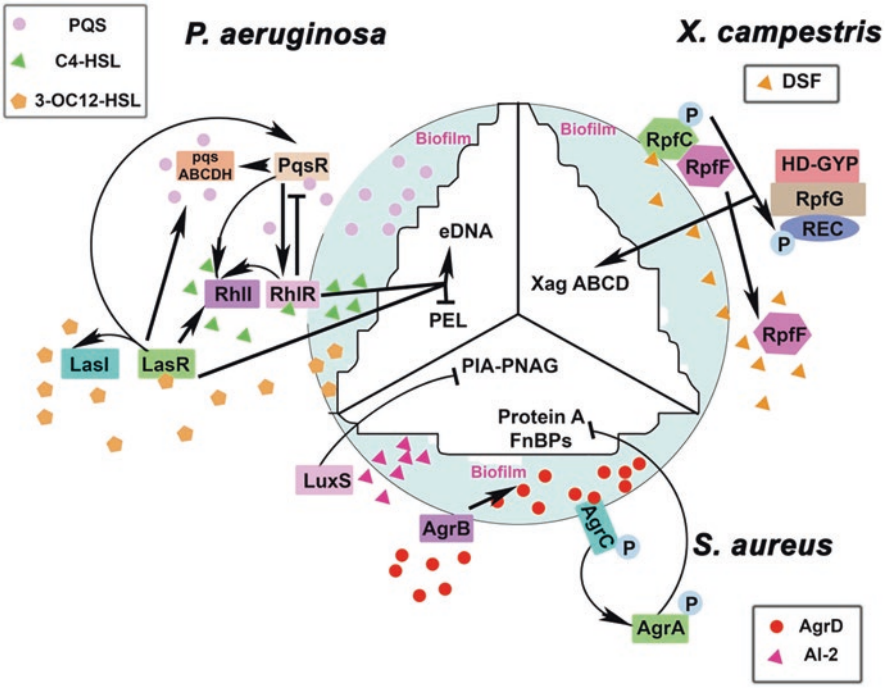

Fig. 1 Schematic representing the regulatory network between QS and Biofilm development

the biofilm dispersion process through Agr system. Transcription of PSM operon is under strict regulation of AgrA. Agr mutants were unable to produce PSMs. Laser confocal scanning microscopy of *agr* mutant's biofilm revealed that PSM is important for biofilm development, biofilm thickness, volume, roughness and channel formation. In specific growth conditions, PSMs polymerize to form amyloid-like fibers [27]. These amyloid-like fibers play important role in biofilm formation under certain conditions and mutants lacking PSMs were unable to form biofilm. These results demonstrated the dual role of PSMs. In its monomeric state, it exhibits surfactant properties which are required form biofilm dispersion whereas in polymerized state, it promotes biofilm development. The environmental conditions which regulate the switch between monomeric and polymeric state are still not known.

A recent study identified a functional *luxS* gene in *S. aureus* that produces AI-2. *luxS* mutants showed elevated levels of biofilm formation. AI-2 activates the expression of IcaR, which is negative regulator of PIA/PNAG [28]. Since the potential AI-2 receptor was not identified in *S. aureus* was not identified, the regulatory mechanism that connects AI-2 with IcaR expression remained enigmatic.

QS and Biofilm Development in *Xanthomonas campestris*

Diffusible signal factor (DSF) family of proteins constitute QS system in *X. campestris pv campestris*. DSF was initially characterized as *cis*-11-methyl-2-dodecenoic acid in *X. campestris* [29]. Biosynthesis of DSF in *X. campestris* was dependent on *rpfB* and *rpfF* genes that encodes a putative long chain fatty acyl CoA ligase and crotonase enzyme respectively. Additionally, *rpfC* gene product functions as DSF sensor and regulate DSF biosynthesis. At low cell density, RpfC remains complexed with RpfF in unphosphorylated form and limits DSF production. At high cell density, accumulated DSF molecules induces autophosphorylation of RpfC that lead to release of RpfF and thus increases the production of DSF [30, 31]. Additionally, RpfC along with RpfG constitutes two component regulatory system. RpfG degrades c-di-GMP into two molecules of GMP. Phosphorylated RpfG exhibits its phosphodiesterase activity reduces the levels of c-di GMP molecules. *rpfF, rpfC, rpfG* mutants exhibits cell aggregation when grown in liquid medium. Addition of DSF in these cellular aggregates leads to dispersion of these aggregates in *rpfF* mutants, whereas aggregates of other mutants did not show any dispersion. These observations indicated that DSF induces dispersion of biofilm in *X. campestris* through RpfC/RpfG two component system. The DSF mediated effector of biofilm dispersion was identified as endo-β-1,4- mannanase that is extracellular enzyme and encoded by *manA* gene. endo-β-1,4- mannanase disrupts the biofilm generated by all possible *rpf* mutants. Studies indicated that there are additional DSF induced biofilm disrupters apart from ManA protein [32]. Recent studies identified that repression of *xagABC* (encodes putative glycosyltransferase) transcription by RpfC/RpfG system also disrupts biofilm in *X. campestris*. *xagABC* product is responsible for the synthesis of exopolysaccharide which is essentially required for biofilm

Table 1 List of QS regulated biofilm dispersion mechanisms in *S. aureus, P. aeruginosa and X. campestris*

Organism	Quorum sensing system	Biofilm dispersion mechanism		
		Surfactants	Matrix inhibitors	Matrix degraders
P. aeruginosa	PQS, LasI/R, RhII/R	Rhamnolipids	Pel	–
S. aureus	AI2, Agr	PSMs	PIA/PNAG	Proteases
X. campestris	DSF	–	XagABC	ManA

formation [33]. In addition, cyclic-AMP receptor like protein (Clp) is also responsible for DSF mediated biofilm disruption by altering c-di-GMP levels.

Overall, it can be concluded that QS in *X. campestris* act as regulatory system in biofilm dispersion mainly through ManA and XagABC levels (Table 1).

Conclusion

Understanding bacterial biofilm development and dispersion is of enormous interest because the underlying mechanism is highly instrumental for designing and developing novel drugs for bacterial pathogens. Initial studies mainly focused on the earlier steps of biofilm development and identified cell surface adhesins which are required for interaction with suitable substratum. Later, studies deciphered the mechanism and regulation of biofilm matrix exopolysaccharides. Recent observations demonstrated the role of QS in biofilm maturation and dispersion. Dispersion of biofilm will be daunting task for individual bacteria. This scenario was concluded from observation when antimicrobial drug was administered against QS signaling system that failed in biofilm dispersion whereas QS mimicking molecule leads to dispersion of biofilm. Above evidences also demonstrated that many bacterial species uses surfactant molecules for disassembly of biofilm. It will be interesting to ask a question that does the combination of surfactant molecules along with antimicrobial compound will be effective treatment for eradication of biofilm.

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Part V
QS-Regulated Behaviours in Gram-Positive Bacteria

Quorum Sensing Mechanisms in Gram Positive Bacteria

Veer S. Bhatt

Abstract Quorum sensing (QS) is a form of intercellular communication that enables bacteria to coordinate gene expression in a density-dependent fashion. Bacterial signaling molecules called autoinducers are central to this process. When released into the surrounding environment they bind to signaling receptors on the surface of neighboring bacteria, and upon reaching a threshold level activate quorum sensing genes. Gram-positive bacteria employ small post-translationally modified peptides called autoinducing peptides (AIPs) as signaling molecules. AIPs are often integral elements of a histidine kinase two-component signal transduction system. In certain cases the secreted AIPs may be imported back into the cell after release. They are then identified by cytoplasmic transcription factors. In this system, extracellular proteases process the secreted precursor-AIP into mature AIP. Upon return to the cell, the mature AIPs bind to, and alter the activity of the corresponding transcription factors. Some examples of such transport are know to be critical in sporulation, competence, and enzyme production in *Bacillus subtilis*. A large gamut of peptides is secreted from bacteria by de novo biosynthesis and proteolytic degradation. These peptides include pheromones that modulate expression of specific genes of Gram positive bacteria to regulate biosynthesis of quorum dependent proteins such as virulence factors in addition to serving critical roles in a myriad of bacterial life processes such as regulation of the bacterial competence, bacterial conjugation and bacterial virulence. Interestingly, bacterial cells can respond to the AIPs secreted by itself as well. The physiological effects of this 'self-sensing' have been studied rather recently in *Bacillus subtilis*.

Keywords Gram positive bacteria · Quorum sensing · Autoinducing peptides · Self-sensing · Bacterial virulence

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Overview

Bacteria, despite being unicellular organisms by conventional definition, can communicate with each other. This inter-cellular communication is orchestrated in a cell density dependent manner, and is thus called quorum sensing (QS). It was first reported in 1970 in the cells of *Vibrio fisheri* that exhibit bioluminescence upon reaching a relatively high cell density [53]. QS is involved in modulating many 'social' functions such as virulence, sporulation, competence, biofilm formation and even inter-species behavior [3, 12, 13, 30, 51]. Bacteria secrete certain signaling molecules called auto-inducers. As the cell density increases, the concentration of auto-inducers in the media and their corresponding uptake by the bacteria increase as well, eventually resulting in transcription-level regulation of one of the many social behaviors listed above. Whereas Gram-negative bacteria utilize acylated homoserine lactones as auto-inducers, Gram-positive bacteria mostly use specific peptides for this purpose with certain well known exceptions (such as AI-2 QS discussed below) and scant examples as seen in the marine water bacteria of the genera *Exiguobacterium* [8]. These peptides have variable sequences and are often modified post-translationally [1, 50, 74, 75]. Since, the current review is focused on Gram-positive bacteria, we will preferentially use the term 'auto-inducing peptides' (AIPs) as shorthand notation of auto-inducing molecules in Gram-positive bacteria.

QS in Gram-Positive Bacteria

Two Component Pathway

There are two major pathways of QS unique to Gram-positive bacteria. In the first pathway, AIPs are ribosomally synthesized as pro-peptides and then modified post-translationally. They are secreted via dedicated ABC transporters and oftentimes undergo cleavage by secreted proteases to mature into AIPs. Once the concentration of AIPs reaches a certain threshold, they are recognized by specific cell surface receptor kinases, in turn activating the kinase via phosphorylation on a conserved His residue. The activated kinase subsequently activates a downstream intracellular regulatory receptor by transferring the phosphoryl group to an Asp residue. The activated intracellular regulatory receptor eventually regulates transcription of specific target genes as well as the those of AIP secretion pathway itself. Given that this pathway has two key elements, viz., the His kinase at the membrane and the intracellular regulatory receptor, it is commonly referred to as two-component pathway [74]. This pathway was first described in *Lactococcus lactis* and *Streptococcus pneumoniae* [32, 39] and later in many other Gram-positive bacteria [13, 35, 51, 67,

81, 82]. It is worthwhile to point out that as opposed to QS in Gram-negative bacteria where autoinducer molecules directly bind the cognate transcription factors, QS in Gram-positive bacteria predominantly involves an indirect regulation mediated via phosphorylation. This leads to signal integration in kinases as regulated by environmental cues. Thus, for example, a high cell density leads to sporulation only when it is concomitantly present with starvation conditions [7].

(i) Competence in *S. pneumoniae* and *B. subtilis* (Com system) [50, 84]. A dedicated ABC transporter transports the activated 17 residue AIP called competence stimulating peptide, that is initially synthesized as a 41 residue precursor peptide. Upon reaching a threshold, the AIP activates the corresponding receptor kinase. The activated receptor kinase is then able to undergo autophosphorylation. The phosphate moiety is then transferred to an intracellular regulatory receptor eventually activating the transcription of a specific **σ** factor needed for transcription of competence related genes. This system is needed for uptake of exogenous DNA in *S. pneumoniae.* Com system is also found in *B. subtilis* where the regulatory receptor forms homodimers to bind inverted repeat motifs of DNA.

(ii) Virulence in *S. aureus* (Agr system) [50, 82] and *C. perfringens* [55]. Agr (accessory gene regulator) locus contains several proteins, namely, AgrA, AgrB, AgrC and AgrD. The AIP is a precursor peptide encoded by AgrD that is proteolytically cleaved by AgrB to form a thio-lactone intermediate and then secreted. The AIP attains its final active form upon a subsequent cleavage in supernatant. Once a threshold concentration is reached after secretion, the corresponding sensor histidine kinase (AgrC) is activated. In turn, AgrC sets off a phosphorylation relay that eventually activates the intracellular regulator AgrA via phosphorylation. The activated AgrA increases the concentration of RNAII and up-regulates the transcription of all the *agr* genes. At the same time AgrA regulated RNA III transcription leads to expression of δ-toxin as well and results in expression of various virulence factors. A similar system was discovered in *Clostridium perfringens* that causes gas gangrenes in human tissue by producing extracellular toxins as well in some other species of *Clostridium* order [28, 50, 55, 80]. It should be noted that *Clostridium* order comprises of neurotoxin producing species such as *C. botulinum* and *C. tetani.*

(iii) Adherence in *E. faecalis.* Fsr QS system of *E. faecalis* was found to regulate a secreted metalloprotease called gelatinase (GelE). Gelatinase, in turn can cleave Ace. Ace belongs to the category of microbial surface component recognizing adhesive matrix molecules (MSCRAMM) from the surface of the bacterium. MSCRAMMs participate in adherence to host cells. Thus, disruption of Fsr and/or GelE was found to significantly improve adhesion to collagen [63].

Self-Signaling Pathway in RNPP (Rap, NprR, PrgX, and PlcR) Family

The second pathway can be thought of as self-signaling pathway. In this pathway the ribosomally synthesized and post-translationally modified AIPs may be secreted by SecA-dependent systems and activated by required modifications. The key difference, however, is that upon reaching a threshold concentration, these AIPs are transported inside the cell via an oligopeptide transporter system, unlike the two-component system where a receptor His kinase on the cell membrane is activated by the cognate AIP [49]. Phr (phosphate regulator) AIPs were the first ones to be described that belong in this class of AIPs [49]. The activated Phr AIPs were found to de-activate Rap-phosphatases and play important roles in competence and sporulation.

(i) Phr-Rap system [33, 37, 51, 60, 64, 73, 74] and a Phr-like system of *S. pneumoniae* [33]. Phr pro-peptides are ribosomally synthesized as precursor polypeptides carrying a secretion signal on N-terminus. They are then secreted into the *milieu* where they mature into the final active form upon proteolytic cleavage by secreted proteases. An oligopeptide permease (Opp) that also plays a role in nutrition [23] and belongs to ATP-binding cassette (ABC) transporter family transports the active Phr peptide back into the cell. Phr peptide can then inhibit regulator aspartate phosphatases (Raps) and thereby regulate gene expression. Raps are part of phosphorelay system involved in transcriptional regulation of several genes. A plasmid encoded Rap-Phr system was, for example shown to regulate production of secreted proteases in *Bacillus subtilis* [7, 36]. A set of Raps, namely RapA, RapB, RapE and RapH are responsible for suppressing signaling via phosphorylation. This suppression is relieved by inhibition of the Raps through their cognate Phrs, namely, PhrA, PhrB, PhrE and PhrH, thus playing an important role in stress response of *B.subtilis* in stationary phase as well as sporulation. Gohar et al. used DNA microarrays and other proteomics methods to further explore the regulon of PlcR and found 45 different genes controlled by 28 PlcR boxes [25]. Not only were the genes important in QS systems but also in nutrition and defense. A chromosomal RapC-PhrC system was found to mediate genetic competence, where PhrC inhibits RapC from acting on ComA [43, 73]. ComA along with ComP (ComP/A) regulate expression of several genes directly, and indirectly in combination with ComK. ComP is a histidine kinase while ComA is the response regulator. The locus named ComQXPA encodes two additional proteins: ComQ, an enzyme that is an isoprenyl transferase and ComX is a pre-peptide signal. Dogsa et al., reported a finding of 39 com-like predictions, and that 20 comQXPA-like loci could be present in addition to those predicted in *B.subtilis* and its close homologs [18].

(ii) Plc-Pap system [13, 22, 25, 28, 29, 33, 34, 54, 57, 60, 61, 64, 70, 71, 91]. PlcR regulates many virulence factors as well as Phospholipase C (*plcA* gene) at transcriptional level. In addition to positively regulating its own expression,

PlcR regulates expression of at least 45 genes many of which play key roles in virulence and proteolysis. The cognate peptide PapR binds PlcR on a tetratricopeptide repeat-type (TPR) regulatory domain, eventually driving conformational changes in PlcR to activate it, so that it binds to the target promoters. The activated PlcR binds DNA in a region known as PlcR-box and thus serves as a transcriptional regulator for the said genes. The cognate gene *papR* encodes a 48 residue long precursor polypeptide that is first secreted out of the cell and then re-imported inside the cell through OppABCDF system. The precursor pro-peptide is truncated to a oligopeptide (C-terminal heptapeptide of the precursor) version and becomes active, whereupon it activates PlcR [9, 26]. Despite most secreted virulence factors of *B.cereus* being controlled directly by PlcR, cerulide synthesis (ces) regulon has a different identity, namely, Spo0A-AbrB [45]. Interestingly, Frenzel et al., were able to show that both PlcR-Pap and Spo0A-AbrB have at least one common denominator, CodY [21]. Cody is a nutrient-responsive regulator that was reported to be involved with activation of genes in PlcR-Pap regulon while repression of ces operon.

(iii) PrgX-CF10. PrgX is a repressor found in *E. faecalis.* It can bind two AIPs, the chromosomally located gene product cCF10 and the plasmid located gene product pCF10. pCF10 acts as a co-repressor with cCF10 to repress PrgX and induce conjugation [87].

Rgg-SHP System

Rgg proteins, named after regulator gene of glucosyltransferase are also transcriptional regulators and have been a rather recent addition to the knowledge-base of bacterial QS (Gram-positive bacteria). They are widespread in the phylum *Firmicutes*. Whereas they are similar to the RNPP family in having a helix-turn-helix (HTH) motif important in binding to DNA, they do not share the tetratricopeptide repeat-type regulatory domain of the RNPPs [20]. The cognate peptides of Rgg proteins were characterized to have short hydrophobic regions and were called SHPs (short hydrophobic peptides) [11, 19]. Interestingly, LaSarre et al., reported the finding of dual Rgg protein regulation called Rgg2/Rgg3 important in biofilm biogenesis of *Streptococcus pyogenes* [41, 42]. The cognate AIPs, SHP2 and SHP3 were found to activate Rgg based QS in a dose-dependent manner.

Autoinducer-2 (AI-2)

AI-2 is a unique class of autoinducers in the sense that it is common to both Gram-negative as well as Gram-positive bacteria. This fact that has lead many researchers to call it a “universal language” of cross-talk extending to even inter-species communication [5, 6, 24, 79, 80]. 4,5-dihydroxy-2,3-pentanedione (DPD) serves as

common precursor molecule in AI-2 sensing and is synthesized by LuxS (S-ribosyl homocysteine lyase), a protein that is ubiquitous in bacteria. DPD spontaneously cyclizes and is reported to activate icaR pathway. icaR being a repressor for transcription of key proteins involved in N-acetyl glucosamine (UDP-GlcNAc) polymerization was unsurprisingly found to attenuate biofilm formation in several studies [86, 89] being regulated via ica operon. For example, Yu et al., proved that a *Staphylococcus aureus* ΔluxS strain could form stronger biofilms than the corresponding wild-type. It is worthwhile to mention that recently Trappetti et al., reported that AI-2 sensing was dependent on on FruA, a phosphoenolpyruvate-dependent sugar phosphotransferase system [78]. It was found that AI2-FruA mediated signaling could favor uptake of galactose as the primary carbon source, a situation found commonly encountered by pathogens such as *Streptococcus pneumoniae* in upper respiratory tract. AI-2 based QS has thus been demonstrated to be important in regulating many features in Gram-positive bacteria such as motility, virulence, luminescence and biofilm formation [27].

Biochemistry

Biochemical and structural aspects of many components in QS have been studied thoroughly now [2, 9, 16, 17, 22, 29, 30, 38, 57–60, 66, 83, 91]. Rap-Phr and PlcR-PapR will be discussed here to illustrate the importance of myriad 'conformational switches' present in QS systems (Figs. 1 and 2).

Rap proteins oftentimes employ either of the two strategies to regulate the cognate response elements: (i) they either utilize their phosphatase activity (RapA, RapB, RapE, RapH and RapJ) for dephosphorylation of the components of phosphorelay (e.g., Spo0F~P) [17, 38], or (ii) they directly bind (RapC, RapF, RapG, RapH, RapK) to their cognate response element to inhibit ability of the cognate response element to bind DNA [38, 72]. Notably, Rap H belongs to both categories [72].

Rap proteins have two distinct domains: (i) an N-terminal domain that interacts with the cognate response element, which most often is a part of the two-component signaling cascade, and (ii) a C-terminal domain that binds the cognate Phr peptide. The N-terminal domain of three major members of RNPP family (NprR, PclR, and PrgX), in contrast to Rap proteins, adopts a characteristic helix-turn-helix type DNA binding motif [2, 58, 59]. Unlike the variation in N-terminal domain, the C-terminal domain of RNPP family is made of several tetratricopeptide repeat (TPR) domains. TPRs are defined by a pair of antiparallel α helices called A and B helices that are arranged in a helix-turn-helix manner [15]. Despite the lack of sequence conservation, they are oftentimes involved in mediating protein-protein interactions. The structures of apo- and binary complexes of RapF and RapJ were

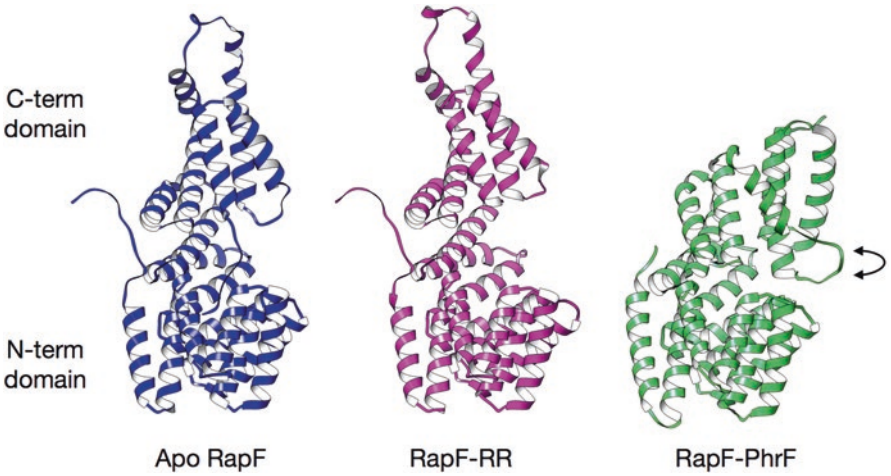

Fig. 1 Conformational changes in Rap proteins upon binding of the cognate Phr peptide. The degree of similarity between Apo RapF (blue ribbon; PDB id 4i9e) and RapF in a binary complex with its cognate response regulator (magenta ribbon: PDB id 3ulq) is quite high as reflected in root mean square deviation (RMSD) of 2.731 Å across all 376 pairs. On the other hand, RapF bound to the cognate peptide PhrF (green ribbon: PDB id 4i9c) undergoes considerable conformational changes and rearrangement of N-terminal and C-terminal domains. This is reflected in RMSD of 16.107 Å across all 372 atom pairs, when compared to the Apo structure. The proteins were superimposed using UCSF Chimera [62] and placed in the superimposed orientation to prepare the graphic using the same conformation

determined in two benchmark studies published in 2013 [22, 57]. It was found that PhrF allosterically induces conformational changes in RapF that prevent the binding of the cognate response element ComA [22]. These conformational changes are reflected in a ~155° rotation of N-terminal domain in RapF-PhrF complex relative to the apo form. Similar conformational changes were observed in RapJ-PhrC complex [57]. When compared to Rap proteins, the other members of RNPP family exhibit similar conformational rearrangements with the major difference that the degree of structural rearrangement is lesser in case of PrgX and PlcR than those in the Rap proteins [22]. Another key difference is that in contrast to Rap proteins, the other three members are known to directly bind DNA via their signature helix-turn-helix motifs. Grenha et al. determined the apo form of PlcR as well as its ternary complex with the cognate DNA binding region (PlcR box) as well as the cognate peptide, PapR [29]. The authors proposed that the inactive form of PlcR is activated in two sequential steps. The binding of the cognate peptide PapR unlocks the first lock formed the paired stacking interactions of Tyr64, a step that partially activates PlcR. The recognition of plcR-box brings about the opening of the second lock formed by the paired stacking interactions of Ile68.

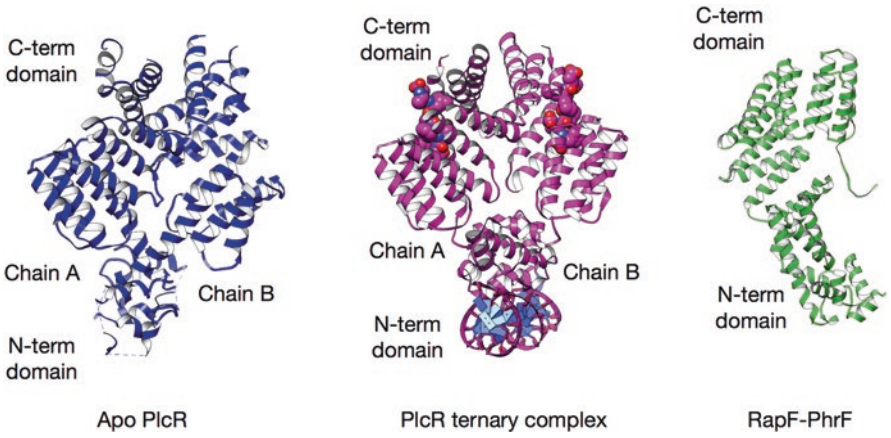

Fig. 2 Conformational changes in PlcR proteins relative to Rap proteins. The N-terminal domain of Rap proteins binds to the cognate response regulator whereas the N-terminal of PlcR proteins binds directly to the cognate region in DNA called PclR-box to directly regulate gene expression. Binding of the cognate peptide (PapR) to PclR results in conformational changes that eventually unlock the latter while allowing it to bind to PclR box (apo PclR is depicted as blue ribbon (PDB id: 4fsc); ternary complex of PclR with PapR (spherical atoms) and PclR box is depicted in magenta (PDB id: 3u3w). Another key difference is the multimeric arrangement of PclR vs possibly monomeric arrangement in Rap proteins (depicted as green ribbon, PDB id: 3ulq). The proteins were superimposed using UCSF Chimera [62] and placed in the superimposed orientation to prepare the graphic using the same conformation

Cross Talk, Quorum Quenching (QQ) and Inhibition

In nature, bacteria co-exist with other micro- and macro- organisms. How do QS systems of each bacterial strain respond to QS system of a different bacterial strain or a different species altogether? The opposite also poses an interesting question. How do non-self species react to QS system of a given bacterium. Indeed there may be intra-species crossing of a pathway with a QS pathway. For example, Multidrug resistance efflux systems and QS systems may share the same export machinery [85]. But multiple bacterial species could eavesdrop on a common QS signal as discussed in an excellent review by Atkinson and Williams [1] and reported by Zhou et al. [90] among others. Zhou et al. found that the 'cheaters' may use the common QS signal to cooperate but QS-null mutants are not effective at this. Cooperation in absence of non-self species could be expected. For example, pigment production by *Chromobacterium violaceum* is seen irrespective of the source of the QS signal [48]. The interaction between multiple strains has been reported several times as well. For example, multiple groups of Agr system of *S. aureus*, may exhibit cross-activation, cross-inhibition as well as little to no effect on each other [1]. Tashiro et al. reported that addition of a QS signal from *Pseudomonas aeruginosa,* Pseudomonas quinolone signal (PQS) to *E. coli* K-12 resulted in an increased production of membrane vesicles [76]. In a different study, Rella et al. found that

P. aeruginosa exhibits anti-fungal activity against *Cryptococcus spp.* that is mediated yet again by PQS [65]. Kumar et al., were the first to report and example of a QS signal that participates in inter-species cell death [40]. In this study QS signals called extracellular death factors in *P. aeruginosa* and *B. subtilis*, that could trigger mazEF-mediated death of *E. coli*.

Inhibition of QS systems has been referred to as Quorum Quenching (QQ). QQ is often a result of inter-species interaction. Czajkowski et al. discuss the quenching of acyl-homoserine lactones (AHLs) among Gram-negative bacteria as well as that of AIPs in Gram-positive bacteria [14]. Teasdale et al. reported QQ of several Gram-negative strains such as *Vibrio harveyi* mediated by a Gram-positive bacterium *H. salinus* [77]. Han et al. also reported a similar finding where a Gram-positive bacterium (called LQQ) quenches the QS signaling in Gram-negative bacteria [31] and so did Chu et al. [12]. On the contrary *P. aeruginosa* was found to produce an antibiotic called C12-TA that can dissipate membrane potential as well as pH gradient in Gram-positive bacteria thereby killing them [44].

Given the role of QS is virulence and persistence (e.g., by biofilm formation), its inhibition has also been a constant endeavor in scientific community [47]. Oftentimes, this inhibition could be brought about by natural compounds produced by species from other kingdoms. Essential oils obtained from *L. alba* were found to be inhibitory towards QS in *Chromobacterium violaceum* and *S.aureus* [56]. Ambuic acid, a fungal metabolite was found to inhibit QS in *E.faecalis* [52]. Rather recently, Manifold-Wheeler et al. found that apolipoprotein B can potentially inhibit the above described Agr QS system [46] Plant extracts have also been found to inhibit QS [68, 69]. Indeed synthesized anti-bacterial chemicals have also been shown to inhibit QS [3, 10].

Self-Sensing

In addition to the *milieu* concentration dependent effect of AIPs and other signaling moieties of various pathways, the local concentration of the secreted AIP could also influence various life-processes in a quorum independent manner. Given recent developments in this area and that QS has been the major focus of the current discussion, utilization of QS components to invoke non-QS responses is quite intriguing. This "*secrete-and-sense*" phenomenon has been studied in an engineered yeast by Youk and Lim, and could be considered to be more inclusive than QS only by allowing cells to respond in an asocial manner as well [88]. More recently Bareia et al. investigated the presence of this "*self-signaling*" in *B. subtilis*, a Gram-positive bacteria [4]. One of the key results they obtained was that in a mixture of cells that secrete AIP and those that do not secrete AIP, the AIP-secreting cells could elicit a stronger "QS response" when compared to the non-secreting cells. Using a quantitative approach, the authors first established that this difference is indeed due to self-signaling and then provided evidence that this kind of self-signaling can contribute to an elevated survival advantage in presence of antibiotics.

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Novel Insights on the *Bacillus* Quorum Sensing Mechanism: Its Role in Competence, Virulence, Sporulation and Biofilm Formation

S. Anju, Y. Aparna, Bhukya Bhima, and J. Sarada

Abstract A large number of *Bacillus* sps are ubiquitous and can modulate in diverse environments. The QS response in *Bacillus* sp. involves expression of adaptive extracellular factors like food-degrading enzymes, virulence factors, antibiotics, or biosurfactants. They produce QS signals as small peptide molecules i.e. autoinducer peptides (AIP) processed from their oligopeptide precursors. Members of the Rap-Phr family of QS systems in *Bacillus subtilis* are involved in regulation of competence, sporulation and biofilm formation. Moreover they possess a typical Com QXPA QS system which controls the expression of nearly 200 genes, including both extracellular and intracellular factors. However in *B. thuringenesis*, the virulence expression, sporulation and nectotropism are strongly regulated by NprR/NprX signal regulators which belong to RNPP family. NprR/NprX QS system was identified to regulate the expression of pathogenesis in *B.anthracis*, which causes fatal pulmonary infection. Two established QS systems PlcR/PapR and NprR/NprX for virulence regulation were found in different species of *Bacillus*. Current review emphasizes on the comparative study of different QS systems in Bacilli which control the pathogenesis and development processes. Despite the fundamental biological importance in medicine and industry, *Bacillus* QS molecules can serve as potential biomarkers.

Keywords Quorum sensing · *Bacillus* · Com QXPA QS · PlcR/PapR · NprR/NprX

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Introduction

Bacterial cells have mastered the art of quorum building for successful proliferation in an ecological niche. They possess a network of complex integrated processes that have enormous diversity in genetic expression. There are many regulations inside a bacterial cell that coordinate expression of genes, which are controlled by quorum sensing circuits [40]. Signalling molecules of quorum sensing play a vital role in proliferation and metabolic activity of the cell. Study of outstanding role of signalling molecules, gives the insight about the coordinated network among different species. Bacteria are most successful ubiquitous and easily adapting organisms to extreme unfavourable conditions. To adapt in diverse conditions, they have evolved with devices for sensing, that are regulated by gene regulations. Every set of system is tuned to respond specific conditions and hence it would be appropriate to assume that, each process has unique sequence of regulation [36]. In this context, elucidation of quorum sensing mechanism can be instrumental to figure out regulation of competence, sporulation, virulence, formation of biofilms and horizontal transfer mechanisms in the genus *Bacillus* [24].

Bacillus genus is a biggest prominent group of bacteria that exists in diverse ecological niches. They belong to the group firmicutes that are aerobic, gram positive, sporulating, among which some are facultative anaerobes. *Bacillus* is known to produce enzymes, antibiotics, surfactants as well as exopolysaccharides. The genus is prominent with many species that are known to utilize peptides as QS signals, thereby eliciting a variety of behaviours, that include secretion of diverse molecules. Among them, *B.subtilis* and *B.polymyxa* have been identified to produce medically useful antibiotics like bacitracin and polymyxin B. Some of the species of *Bacillus* associated with plants synthesize antimicrobial substances like bacillaene and macrolactin. These antibiotic like substances inhibit bacterial and fungal pathogens that cause infections in plants. Apart from the members that produce exotic secondary metabolites, this group also includes a common contaminant, *Bacillus cereus* found in canned foods and pathogens like *Bacillus anthracis* and *B.thuringiensis*. Ability of *Bacillus* to adapt with different adverse environments and continue proliferation by competing for resources makes their role very distinguishable [6].

QS system controls and regulates all most all developmental processes. Unlike the Gram-negative QS systems, Gram-positive bacteria usually use two component regulatory systems to sense the external pheromone and to control the transcription of downstream genes [35]. *Bacillus* produce QS signals as small peptide molecules i.e. autoinducer peptides (AIP) which are processed from their oligopeptide precursors. AIP's are secreted during growth, accumulate till signal threshold concentration is reached and get activated by specific histidine kinase receptors [4]. Knowledge on the QS mechanisms or circuits would not only provide their role in regulation, but also unfolds the probable ways of manipulation for medical and commercial applications.

QS Signalling in Competence

Competence is a condition in which the bacteria are transformed into an unusual state where they become capable of, taking up DNA into the cell from the surrounding medium. Competence is developed in *Bacillus* during the change over from logarithmic to stationary phase of growth. *Bacillus subtilis* is known to have a definite DNA binding along with uptake system with five loci, comC, comE, comF, comG and nucA that are together referred as late competence operon. DNA uptake is achieved by a protein similar to pilin, consisting of a number of proteins that is product of comG operon [33]. Previous reports indicate the probable role of comC in assembly of the operon structure. The comE operon encodes polytopic transmembrane protein. This protein forms a pore that guides the DNA inside the cell, where it can associate with the DNA *Helicase* resembling protein encoded by the comF operon. It is found that there is no nucleotide specificity for DNA binding and DNA uptake. Therefore a competent *Bacillus* cell can incorporate a plasmid DNA, phage DNA or chromosomal DNA. DNA uptake is catalyzed by the endo nucleolytic cleavage catalyzed by NucA that is membrane bound. As a result, linear fragments of DNA not more than 20 Kb in size are formed. These single- stranded DNA fragments after the uptake by the competent cell get linked with bacterial recombination proteins RecA and AddAB. Upon association with recombination proteins, the foreign DNA is enabled to integrate into the host genome [33].

Bacillus subtilis has a quorum sensing (QS) referred as ComQXPA that is capable of controlling various factors of a cell, thereby regulating the development of competency in a cell [18]. This unit of QS is characterized by four proteins: the isoprenyl transferase – comQ, the signal peptide-comX, the histidine kinase comP and the response regulator comA. The signalling peptide comA is a deca peptide, synthesized as a 55 residue propeptide before being processed. After a hydrophobic modification on the tryptophan residue by isoprenyl transferase- comQ, it is secreted to initiate the signalling. Once isoprenylated comX reaches a critical concentration, it triggers the auto phosphorylation of the membrane bound comP. The comQ facilitates comX peptide production. A two component comP/comA signal kinase response regulator senses the high level concentrations of comX signals. Phosphorylated comA activates the expression of the *comS* gene. comS plays a very important role in maintaining the competence factor protein, comK concentration. ComS prevents the proteolytic degradation of comK, which is a transcriptional activator that regulates the competence development in *Bacillus subtilis*. Increased concentrations of comK controls the late competence operons, that results in synthesis of proteins required for DNA uptake, processing and integration into the genome [2].

Phosphorylation by comP in presence of high concentrations of response regulator comA occurs during the development of competence in a cell. Phosphorylation of comA results in DNA binding activity, leading to the expression of several genes and ultimately results in the expression of late competence genes.Com A is allosterically inhibited by response regulators like Rap F and Rap C proteins. Until a high concentration of corresponding pheromone is reached, they block the DNA binding

domain thereby delaying activation of competence genes. One of the studies has reported a conspicuous polymorphism in the comQXP loci among the *Bacillus* species. This polymorphism was assumed to be the reason for the specific patterns of activation and inhibition of quorum sensing response [43]. Hayashi et al. [25], reported the production of unique pheromone ComX natto – a hexa peptide by *Bacillus subtilis sp. natto*. This strain was used as starter to make one of the Japanese fermented food from soya beans as Natto. As this pheromone stimulates the production of γ-PGA, it has commercial importance as additive.

Signalling Circuits in Sporulation

Bacillus is known to sporulate under environmental stress or at unfavourable conditions like nutritional deprivation. The metabolic transformation of vegetative cell into spores is regulated by quorum sensing circuits and specific genes involved in sporulation. Low cell density results in starvation and poor sporulation in *Bacillus sps*. Only at a required quorum size, in response to environmental signals sporulation is triggered. Sporulation in *B.subtilis* is coordinated by the well studied Rap–Phr QS circuit, which responds to the environmental signals and is identified to regulate the sporulation. This system is also known to regulate competence and biofilm formation apart from sporulation in *Bacillus*. Rap-Phr circuit is a two component system which has signal peptide pheromones (encoded by phr genes) and a regulator molecule called as Rap (regulator aspartate phosphate phospahtase), which control the on and off switch for sporulation (*Spo0 A*).

Pheromones of *Bacillus sps* are oligo peptides in nature and are encoded by *phr* genes. These are synthesized from their specific genes as pre peptides and secreted outside the cell. These precursor peptides are processed and modified extracellularly into penta or hexa peptide signalling peptides. Matured peptides gets transported back into the cell by OPP complex (oligopeptide –permase complex) and interact with their cognate Rap proteins. These regulator or connector proteins in turn modulate the response regulators by removing the phosphate moiety on aspartate residue by their phosphatase action or by blocking them physically. Rap proteins are also known to be inhibited by specific Phr peptides. Genes encoding Rap proteins are found to be located downstream of *phr* genes. In *Bacillus subtilis,* Rap proteins were identified to control the response regulators involved in the developmental process like sporulation (Spo0F), competency (Com A) and virulence (Deg U) [5]. Rap proteins function only in the free peptide form and have two domains, one with a tricopeptide repeat (TPR) which can interact with the signal peptide and the other domain either exhibit its phosphatase activity or as contact dependent inhibitor of response regulator. Binding of signal peptide to its specific Rap protein leads to its inactivation, which in turn results in derepression of its response regulator [5].

Competence and Sporulation Factor known as CSF, is the second signalling pentapeptide, known to regulate the sporulation in B.subtilis. CSF is derived from the C terminus of secreted polypeptide Phr C protein and it is imported back into the

cell by OPP complex –ABC. At low concentrations CSF inhibits Rap C protein, while at high intracellular concentration it inactivates the Rap B which in turn causes dephosphorylation of sporulation response regulator protein Spo F~P. This dephosphorylation results in synthesis of another sporulation master regulator Spo 0A~P in the cell. Spo0A gets the phosphoryl group through the phospho relay system from – Spo0F. During the transition to stationary phase, enough signal peptide gets accumulated inside the cell as the cell density increase so that it can bind efficiently to the Rap protein and displaces it from the response regulator (Fig. 1). This phosphorelay initiates the sporulation process in the cell. Spo0F being the component in Spo0A activation phosphorelay, is known to be repressed by Rap A, RapB, Rap E, Rap H, Rap I and Rap J [19].

Phosphorylation of the response regulators Spo0A and Sop0F is mediated by two sensor kinases Kin A and Kin B. These kinases are known to be involved in interpreting the environmental signal and transducing the signal into autophosphorylated kinase. Spo0B phosphotransferase transfers the phosphoryl moiety reversibly

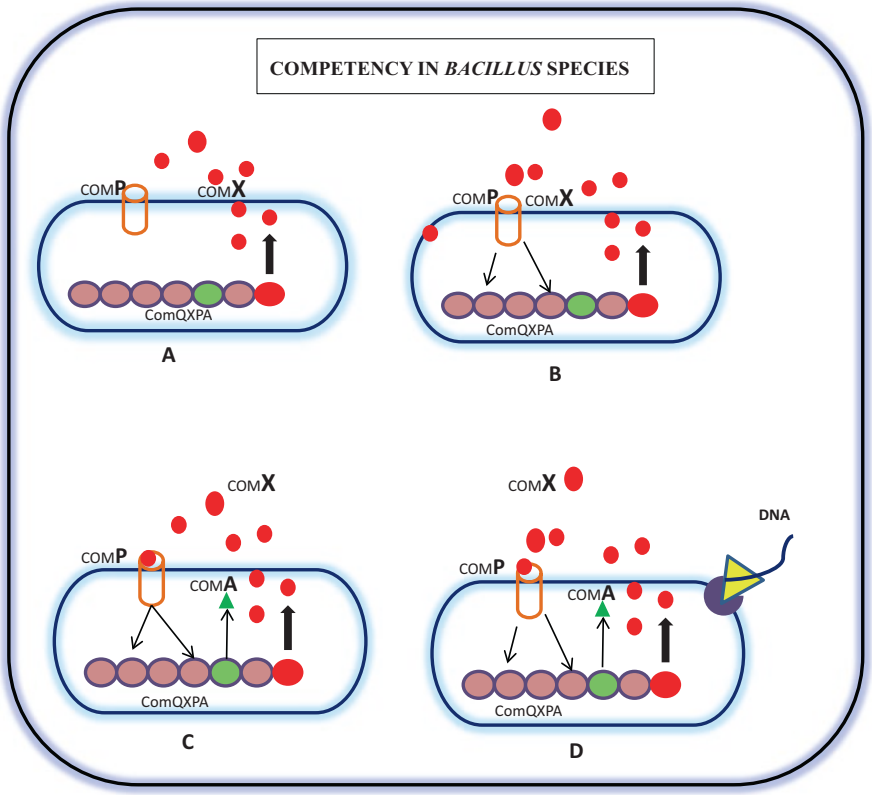

Fig. 1 Mechanism of competency in Bacillus species. (**a**) ComX signal peptide production. (**b**) Binding of ComX signal peptide to histidine kinase ComP (**c**) Production of response regulator ComA. (**d**) Target gene expression

between the two response regulators. These kinase have distinct role to play during sporulation and regulated by different environmental signal inputs. Kin B is mostly found during exponential phase where level of sporulation is low because cells are in vegetative growth. However, Kin A is important for initiation of sporulation and observed at stationary phase [28].

CSF at higher concentration forces the cell to undergo sporulation by two ways i.e. by activating the components of sporulation phosphorelay and by inhibiting the synthesis of Com K which results in switching of the competence machinery inside the cell. Another way of inhibiting the competence machinery is by binding of CSF to Com P and inhibiting its phosphorylation. This leads to the decreased synthesis of competence factors. Hence, CSF exhibits a concentration dependent regulation of sporulation and competence in *B.subtilis* [3].

Formation of matrix producing cannibals is another interesting phenomenon noticed in *Bacillus* under extreme stress condition [3]. This is observed to be similar to programmed cell death (PCD) in which cells not involved in development process are killed by secreting toxic peptides like Sdp (sporulation delaying factor) & Skf (sporulation killing factor). When *Bacillus* is co existing with other bacteria, these two toxic peptides are secreted to eliminate the neighbouring non competent cells and release their nutrients under stress/depletion of nutrients in environment. Spo0A –the sporulation master regulator also controls the expression/synthesis of these Sdf& Skf. Cannabolism is also important during biofilm formation in *Bacillus*.

Signalling Circuit in Surfactin Production

Surfactin is a biologically active cyclic lipopeptide molecule with exceptional surfactant properties produced by *Bacillus subtilis*. It can reduce the surface tension of water by 45 mNm^{-1} at a low concentration of 20 mM. It is an amphiphilic molecule that has activity in hydrophobic and hydrophilic conditions. Known as a bio surfactant, it is established to have antibacterial, antifungal, antiviral, anti mycoplasmal and haemolytic activities. The structure reveals the presence of, a heptapeptide linked to a 13C–15C beta hydroxy fatty acid by means of a lactone bond. Surfactin molecules have a large spectrum of biological activity that is accounted by its ability to form a "horse saddle" conformation in solution. Many pathogens are prevented from adhesion to surfaces by surfactin due to its ability to penetrate the cell membrane and disrupt them. Surfactin also has a very prominent activity in inhibition of biofilm formed by bacteria, other than *Bacillus species* [42].

Surfactin production is catalysed non ribosomally by *surfactin synthetase* which is a large multi enzyme complex. A network of srfA operon and ComX-ComP signalling system together are involved in surfactin biosynthesis. The prenylated peptide ComX secreted by the *Bacillus*, activates the histidine kinase ComP (Fig. 2) [37]. As a result there is activation of ComA by ComP. ComP is a transcriptional factor of the srfA operon that is responsible for the surfactin synthesis. Recent studies have revealed surfactin as an extracellular signalling molecule apart from being

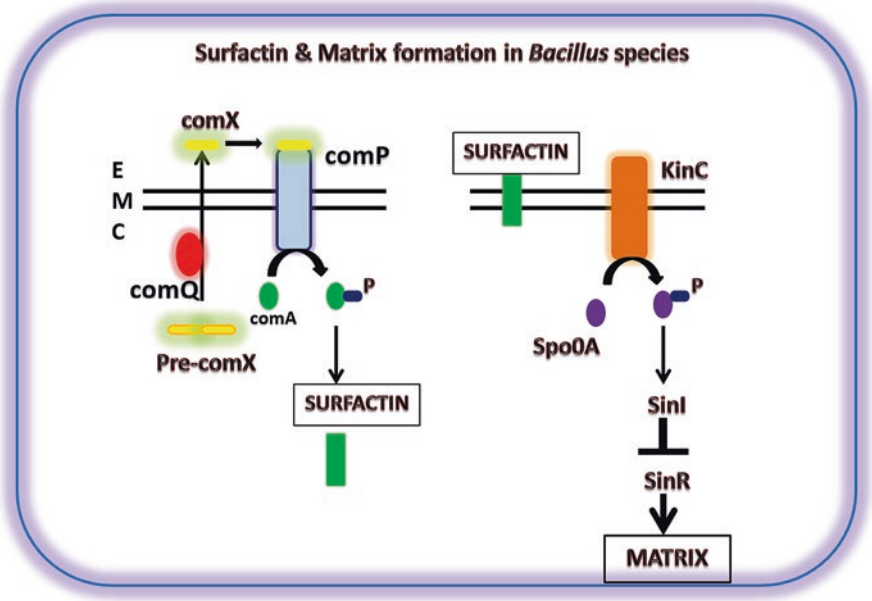

Fig. 2 Mechanism of surfactin and matrix formation in *Bacillus species*

a biosurfactant. The histidine kinase KinC is activated indirectly by surfactin which is responsible for phosphorylation of regulator Spo0A. This regulator mediates the signal pathway SinI-SinR that leads to production of extracellular matrix to form *Bacillus* biofilm. Studies have lead to conclude that not all the cells are activated to produce surfactin within a subpopulation. Only a few respond and transform to surfactin producers. It has been observed that this subset of cells producing the biosurfactant is the ones that undergo sporulation among the population. Surfactin gene expression is also found to be a requirement for competence. During the uptake of extracellular DNA by a competent *Bacillus* cell, surfactin is noted to have a role in facilitating the way in [23]. Surfactin production in *Bacillus* has a multi functional role in biofilm formation, competence and proliferation strategy in a mixed population.

Signalling Circuit in Biofilm Formation

Biofilm construction by bacteria is one of the fascinating phenomenons, exhibited by the cells that involve a variety of interactions within the species and among a number of different species. Bacteria in the biofilm community develop properties that enable them to acquire enhanced resistance to antibiotics [10]. Specific nutrient availability, chemotactic movement for attachment to surfaces, motility, release of

adhesins and surfactins influence the formation of *Bacillus* biofilms. Bacteria within a biofilm, communicate by a phenomenon, quorum sensing, by releasing chemotactic molecules or substances like pheromones. QS system results in the formation of this structured multicellular community of bacterial cells, biofilm [11]. Understanding the signalling, that results in a matrix bound population of cells that proliferate and have characteristic properties, helps in dealing with them strategically. Biofilm formation in *Bacillus* takes place in several steps, which include development, maturation and dispersal of the bacterial population [14]. At first, the cells which are motile become sedentary and commence producing extracellular matrix [34]. During this phase, cells form long chains and stick to each other by a matrix. In mature biofilms, cells secreting matrix undergo sporulation. Whereas in aged biofilms, cells are subjected to dispersal as some of them release substances such as polyamines and D-amino acids which cause break down of matrix [10].

Interplay of signalling molecules, results in the secretion of a matrix that is accomplished in biofilm formation. As part of QS system, an autoinducer AI-2, a furanosyl-borate-diester, leads to biofilm development. It is a product of LuxS, formed by transformation of ribose-homocysteine into homocysteine and 4,5-dihydroxy-2,3pentanedione (DPD). This compound cyclizes into a variety of furanones in the presence of water. *B. subtilis* possesses the forms different types of biofilms in various environments [48]. *B. subtilis* cells can sense different environmental as well as physiological signals, activate histidine sensor kinases which is responsible for phosphorylation of *Spo0A*. Transcriptional repressors AbrB and SinR, in the presence of Spo0A, are down regulated thereby, inhibits production of extracellular matrix, not allowing the biofilm development. Upon receiving the signal for biofilm formation during favourable conditions, cells are shifted from motile bacteria to bacterial chains which stick together by producing an extracellular matrix which provides an attaching source for other bacteria in the surrounding environment and therefore plays a crucial step in biofilm progression. The extracellular matrix formed consists of an extracellular polysaccharide and amyloid fibres. These principle substances forming the matrix are synthesized by epsA-operon [53].

The ComQXPA, QS system regulates the ECM production by means of two signalling molecules which are lipopeptide in nature. The signalling lipopeptide molecules ComX and surfactin promotes the synthesis of *EpsA-O* [13, 39]. Also these two components build up the *SpoA-P* which is involved in the down regulation of SinR. This in turn results in the repression of biofilm development in *Bacillus.* SinR is identified as pleiotropic regulator that binds to DNA in order to repress the expression of genes concerned with synthesis of matrix. Furthermore it controls process of sporulation and development of competence [51]. While there is a positive regulation mediated for ECM production to promote biofilm formation in the *Bacillus*, it is recognized that a few cells show the synthesis of surfactin (Fig. 3). Among the population certain cells, which are capable of responding to the surfactin are seen to initiate production of matrix. Eventually the cells that secrete matrix become unresponsive to surfactin [42]. Control of biofilm formation could be possible by switching off the LuxS QS system in *Bacillus*. Danielle Duanis-Assaf [15]

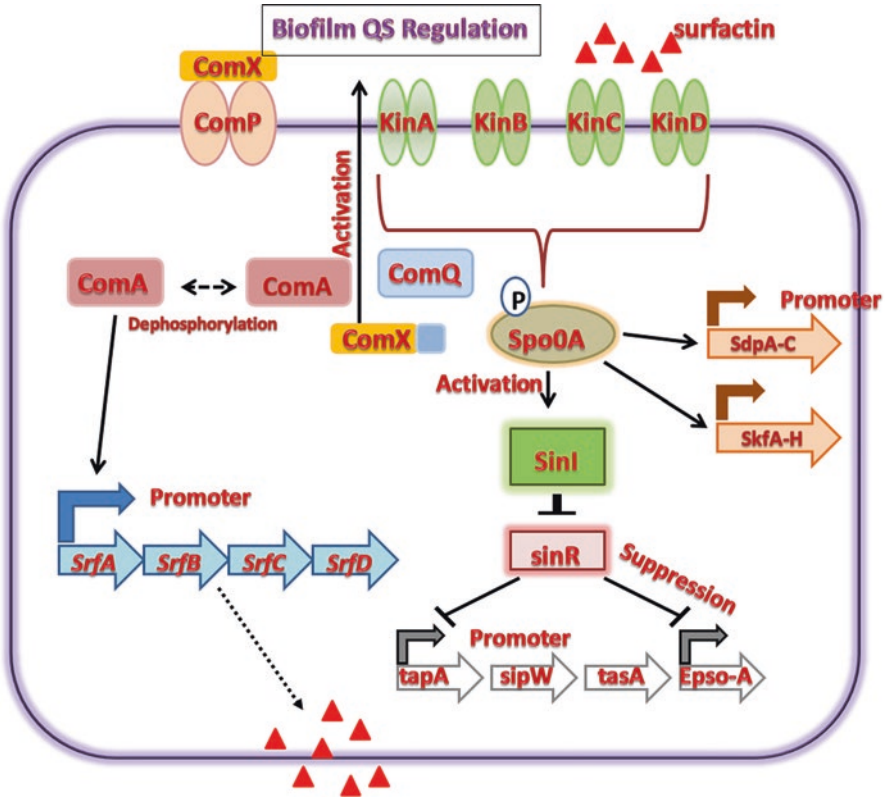

Fig. 3 Regulation of biofilm formation in *Bacillus* species

suggested lactose affect activation of LuxS system by activating of *Spo0A* which leads to biofilm formation by up-regulation of the extracellular matrix operons and *Spo0A* as the negative regulator of LuxS system.

Persistant contamination in food processing units is caused by *B. thuringiensis* which is efficient biofilm producer. These biofilms have heterogenous population of cells: virulent cells controlled by *PlcR*, Nectrophic cells dependent on NprR and cells undergoing sporulation controlled by *Rap* and *Spo0A*~ P. *Verplaetse, E* in 2017 analyzed cellular differentiation in these biofilms by growing them in a optimized sporulation medium. Interestingly no virulent cells were observed and two different routes led to the sporulation among these cells where majority of cells followed by NprR dependent and only few could follow NprR independent route [52].

Much has been understood regarding the *Bacillus species* biofilm development, from notable information derived from various studies involving a genetically homogeneous group. But in natural conditions the biofilms are composed of genetically heterogenous populations. In this perspective it would be appropriate to focus, future investigations on interactions and factors concerned in a mixed population for biofilm construction.

Signalling Circuits in Virulence

It is evident from the above mentioned mechanisms that quorum sensing is involved in regulation of competence, sporulation, surfactant and biofilm formation in *Bacillus* sps. However very few species of the genus *Bacillus* are known to infect humans, plants and animals. They belong to *B.cereus* group and involve species like *B.anthracis*, a potent animal and human pathogen*, B.thuringenesis*, insect pathogen and *B.cereus* [16]. They are known to cause intestinal and nonintestinal infections in animals and humans. QS regulated virulence is mostly studied in *B.cereus* where it is commonly associated with food poisoning [17]. It causes acute diarrhea which is mediated by expression of hemolysisns, phospholipases and toxins [7, 27].

In *B.cereus,* QS is mediated by transcription factor PlcR which is involved in regulation and expression of virulence factors. It binds to intracellular AIP which is produced from PapR protein [49]. *papR* encodes 70 base pairs and is found downstream of *plcR* gene. The PapR protein coded by this gene is about 48 amino acids long which contain a signal peptide at amino terminal region that targets this protein for secretory pathway [38]. Once it is secreted by the cell it binds with AIP forming PapR-proAIP complex. This complex is processed by another neutral protease B termed as NprB, which converts the inactive AIP to active AIP [44].NprB cleaves PapR-proAIP complex into smaller peptide fragments of length 5, 7, 8 and 11 aminoacids. All these are derived from carboxyl terminal region of full length PapR [44]. Among all the peptide fragments peptide with pentapeptide and heptapeptide fragments only activate PlcR protein, however heptapeptide is reported for maximum activation in in vivo [8, 44].

After processing, the PapR AIP complex is brought back into the cell by a specific system termed as oligopeptide permease system (Opp) [22]. Inside the cell, the AIP binds to *PlcR* transcription factor. Binding of AIP to *PlcR* brings a conformational change in *PlcR* DNA binding domain which facilitates its oligomerization there by causing binding to "*PlcR* boxes" [1]. This binding initiates the regulation of transcription of (Fig. 4) targeted genes [12]. *PlcR* is observed to be strong regulator for controlling the expression of nearly 45 genes. Some of them code for extracellular proteins like phospholipases, enterotoxins, proteases, hemolysins etc. [20, 21, 32]) while other regulatory circuits are involved in GGDEF containing transport systems, which is a two component system [21].

PlcR-PapR causes activation of nprR and nprX in *B.thuringenesis* [9]. The NprR-NprX complex in turn activates genes involved in necrotrophism, helping the bacteria to survive in the insect. NprR-NprX also activates the transcription of *papR*a, a gene encoding a peptide showing similarity with PapR. All these communication system control the fate and the physiology of the bacteria, thus, establishing a strong coordination between regulation of gene expression, cell development and infection. *B.anthracis* secretes tripartite toxin lethal factor, oedema factor and protective antigen encoded by *lef, cya* and *pag* genes which present on extrachromosomal plasmid pXO1. AI-2 secreted by *B.anthracis* is known to regulate the pathogenicity.

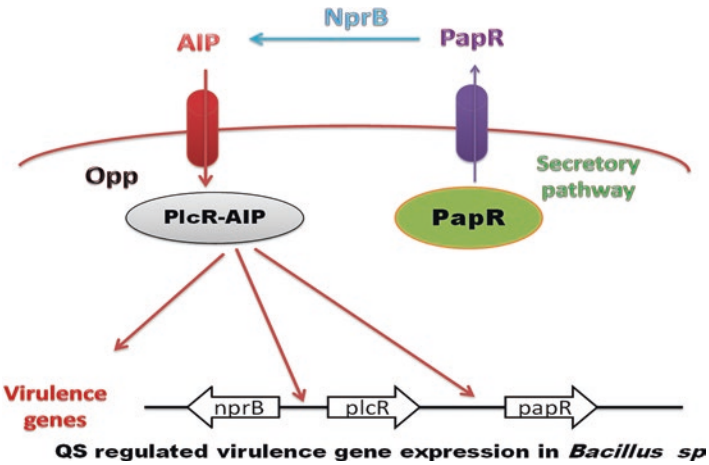

Fig. 4 Virulence expression in *Bacillus species*

Jones et al. in [31], demonstrated the role of luxS in secretion of toxins in *B.anthracis*. The concentration of AI-2 is found to be proportional to the bacterial cell density and toxin secretion. Interesting observations were made by [26], which states that deletion of *plcR* gene resulted in increased biofilm production stating a direct role of *plcR* in biofilm formation. But another point to highlight is though *plcR* is involved in virulence expression it did not have a control over all virulence genes expression as they are influenced by other QS circuit genes which includes *SpoOA* gene involved in sporulation, *CodY* gene, *FlhA* gene which is involved in motility, two component system etc. [29, 30, 47].

However diversity in sequences was observed in PapR-AIPs which enabled to classify the members of *B.cereus* into four major pherotypes. Which includes LPFE (F/Y), VP(F/Y), E(F/Y), MPFEF and LPFEH respectively. The same study also demonstrated that that there is very low cross reactivity observed among these pherotypes indicating that AIP and its PlcR receptor had coevolved [50]. Reports by Slamti and Lereclus [50]. Rocha-Estrada et al. [46] stated that these pherotypes are found in different species and there is a cross talk observed between different species. Also an interesting feature observed was that within the same species different isolates screened fall into different subgroups which prevented them from communicating with each other.

Future Perspectives and Conclusions

Knowledge in basic concepts and components of a QS system known in *Bacillus* sps is very limited, however the reasons for transition from the a simple ancestral QS system to a complex QS network which had an influence on QS regulated behaviour

in a bacterial community remains unexplored. This needs to be answered by further research in QS systems. Understanding of QS circuits and the mechanism of Rap inhibition gives the possibility of modifying Rap proteins, and paves the way for diversifying signalling pathways for biotechnological applications. Understanding the QS driven virulence factor regulation helps in designing kits for diagnosis which can help to identify the infections and also recognize targets for designing drug to combat infections. Quorum sensing molecules can serve as potential biomarkers under infectious conditions. *Bacillus* species are of major concern in the industries as they form biofilms in pipelines and on surfaces of machinery used in production and lead to major economic losses due to food spoilage. Regulation of Lux S QS system could be possible to control the biofilm formation. Another quorum quenching strategy is to combat bacterial pathogenicity by targeting Quorum sensing peptides (QSPs). There is requirement to show an immediate concern to infections caused by multi drug resistant bacteria and biofilms, for which developing antimicrobial peptides for prevention and treatment can give encouraging results.

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Quorum Sensing in Mycobacterium Tuberculosis: Its Role in Biofilms and Pathogenesis

Devanabanda Mallaiah and Pallaval Veera Bramhachari

Abstract Quorum sensing signaling molecules also called auto-inducers secretes from bacteria into its immediate extracellular environment and the molecules are concentrated as their bacterial population increases. At certain threshold concentration, auto-inducers regulate the expression of different types of genes and phenotypes, which includes virulence and formation of bio-films. Bio-films are responsible for 65% of bacterial infections. Mycobacterium tuberculosis (*Mtb*) causes one of the infectious disease named as tuberculosis (*TB*), infected one-third of world's population. In humans, following primary *TB* infection, *Mtb* enters into latent stage. Reactivation or re-infection by new *Mtb* soften and fragments the lung tissue leaving cavities. The success of *Mtb* comes from its ability to grow as pellicle, a bio-film like structure on the surface of such cavities. The *Mtb* bio-films are highly resistant to drugs and implicated in persistence. The presence of LuxR homologs and expression patterns of transcription regulator, WhiB3 suggests quorum sensing existence in *Mtb*. The involvement of nucleotide-based second messengers such as c-di-GMP in signal transduction gives another indirect evidence of quorum sensing mechanisms in *Mtb*. The present chapter reviews quorum sensing mechanisms and their importance in bio-film formation, regulation of gene expressions, virulence and pathogenesis of *Mtb,* which will provide basis for novel anti-tuberculosis therapy.

Keywords Quorum sensing · Virulence · Mycobacterium tuberculosis · Pathogenesis · Bio-film

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Introduction

Mycobacterium tuberculosis (*Mtb*) belongs to phylum actinobacteria, which is non-motile and rod shaped bacterium. It is also an acid-fast obligate aerobe which divides slowly for every 15–20 h. The *Mtb* cell wall contains three important components: arabinogalactan, peptidoglycan and mycolic acids. Based on the cell wall structure, *Mtb* considered as gram positive bacteria [2]. Tuberculosis (*TB*) is caused by *Mtb*, which affects one third of world's population annually. The disease caused by *Mtb* in any of the following ways: (1) production of virulence factors, (2) Colonization of host body and its persistence, (3) Invasion of host cells, (4) Expression of immunosuppressive compounds and (5) Expression of toxins [1]. The increased emergence of multidrug–resistant *TB* and its co-infection with HIV has become a major problem in anti-TB therapy [3].

Quorum sensing (QS) is a bacterial community phenomenon, which is mediated by secretion of small signaling molecules. The small signaling molecules are divided into three major classes: (1) N-acyl homoserine lactones (AHLs), which are produced by many gram-negative bacteria, (2) Oligopeptides, which are employed by gram-positive bacteria, (3) Autoinducer-2, DPD (4,5-dihydroxy-2,3-pentanedione) are used by both gram-positive and negative bacteria [4]. The indiscriminate and over use of antibiotics increases the chances of drug resistance in bacteria, which has become a serious health concern globally. The emergence of drug-resistance bacteria decreases the effectiveness of current treatment modalities. Therefore, novel strategies or development of compounds against drug resistance bacteria is needed immediately. Various studies have been reported that QS is responsible for the regulation of biofilms formation and pathogenicity in both gram positive and negative bacteria. Since QS is linked in regulation of biofilm formation and virulence in many pathogenic bacteria, it has been proposed that QS will become a new potential target in the development of novel antibacterial therapies. The compounds with anti-QS activity are known as quorum quenching (QQ), in which the molecules do not kill, but attenuate the pathogenic bacteria. Some studies reported that anti-QS molecules increased the bacterial biofilms sensitivity to antibiotics in both in vitro and in vivo [5]. The present chapter describes about QS mechanisms in *Mtb* and its role in biofilms formation and pathogenesis.

The Concept of QS and Its Evidence in *Mtb*

Communal behaviors of various gram-positive and gram-negative bacteria are coordinated by the mechanism called QS. In the QS of different bacteria, many specific genes are involved in the regulation in response to bacterial population density. Coordination between the size of bacterial population and expression of specific genes are achieved by specific signaling molecules called auto-inducers. Bacteria sense their population density by releasing these diffusible auto-inducers. Small

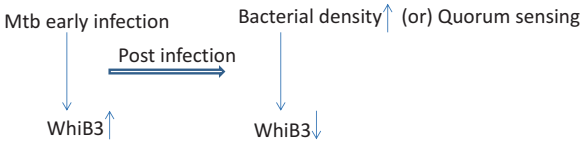

Fig. 1 Expression of putative transcriptional regulator at early and post infection. ↑ Increase, ↓ decrease

amounts of auto-inducers are released and diluted in the surrounding environment by basal level expression at low bacterial population density. These signaling molecules gradually accumulate as the bacterial population expands until a certain threshold concentration is reached. At beyond a threshold concentration, auto-inducers bind to receptors present on the cell membrane or cytoplasm [6].

Different QS signaling mechanisms have been reported in both gram-negative and positive bacteria. In gram-negative bacteria, regulatory proteins such as LuxI and LuxR are involved in QS. These regulatory proteins activate the synthesis of autoinducers such as acylated homoserine lactone (AHL). The secreted AHL binds to its cognate receptor and forms autoinducer-receptor complex, which in turn binds to promoter region and regulates the expression of genes. In gram-positive bacteria, modified oligopeptides as autoinducers activates the sensor kinase in the cytoplasm, which in turn phosphorylates the response regulator proteins. Finally, the response regulator protein binds to target promoter and regulates the expression of genes [7]. Bacteria exploit QS to exhibit certain phenotypes such as bio-film formation, extracellular matrix and virulence factors production. These phenotypes are important for establishment of strong associations between pathogenic bacteria and with their specific hosts [8].

In gram positive mycobacteria, there is no clear evidence of QS reported. However, some evidences of QS mechanism have been revealed in *Mtb*. In one bioinformatics study, they have shown that the presence of LuxR-like regulators in mycobacteria, which are important players in QS [9]. Some indirect evidences revealed that the putative transcription regulator called WhiB3 gene was linked in QS regulation of *Mtb* recently. The expression patterns of WhiB3 correlated with the changes in bacterial density, which suggests the existence of QS in *Mtb* [10]. The expression of WhiB3 will decrease with the increase in bacterial density after post-infection by *Mtb* (Fig. 1). Other indirect evidences of QS also shown that the nucleotide based second messengers are involved in the regulation of various physiological processes of mycobacterial species [11].

Role of QS in *Mtb* Biofilms Formation

Biofilms are surface adherent, multicelluar bacterial communities formed by different types of stresses such as nutrient limitation, heat shock and exposure to antibiotics. Biofilms are also described as structured aggregations of microorganisms develop on non-biological (medical devices) and biological surfaces. Biofilms are one of the survival strategies of bacteria, responsible for 65% or more of all

infections and highly resistant to host immune mechanisms and also to other conventional anti-bacterial therapy [12].

Formations of biofilms are common in many different types of micro-organisms such as bacteria, archaea and fungi. Initially, bacteria attach firmly to surfaces and proliferate in their numbers. As the number increases to certain concentration triggers the activation of QS circuits, which endogenously regulates secretion of signal molecules. When secreted molecules reach the certain critical concentrations, they are taken up and activate regulatory mechanisms. The secreted signal molecules regulate the transition from susceptible planktonic to adaptively resistant multicellular biofilm formation. Some cells dissociate from mature biofilm structures and spread, colonize other new surfaces, where they develop very rapidly against to stress signals in order to avoid adverse effects of stress signals. This planktonic-biofilm transition is a highly regulated and complex process [13]. A biofilm program'is activated by differential expression of specific genes even before a structured biofilm is formed [14].

The infectious disease *TB* is not much virulent in humans when compared with animals. Primary *TB* infection regresses within a few weeks in humans due to immunity and enters into latent stage, where it is not completely sterilized. In Primary *TB* infection, granulomas are formed and spread systemically and also to lymph nodes. Reactivation of dormant (or) re-infection by new *Mtb* causes cavities on the soft lung tissues. In these cavities *Mtb* grows massively and form the structure called pellicle, which separates from host immune response to prevent from lymphocytes penetration and also increases drug tolerance like that of many other bacteria [15].

There are studies reported the formation of biofilms by *Mtb* H37Rv in the laboratory conditions [16]. But when, where and how *Mtb* forms biofilms in vivo remains to be determined. The existence of three genetic loci such as pks16, helY and pks1 suggested that their role in *Mtb* biofilm formation [17]. Among several protein kinases, PknG was shown that it regulates the formation of biofilms in *Mtb* by redox sensing pathways [18].Some studies shown that biofilms formation by *Mtb* depends on keto mycolic acids [19]. Another studies also reported that QS is responsible for the formation of biofilms by *Mtb*, where it is mediated through variety of small molecules such as c-di-GMP [11]. Bacterial biofilms are developed by many factors such as contact surface, pH, nutrient availability, contact time with surface, growth stage, surface hydrophobicity and textures of surface, temperature, humidity etc.,. Among these factors temperature, pH and nutrient composition are suggested to be important for the growth of mycobacterial biofilms [20].

Role of QS in the Pathogenesis of Mtb

Biofilms formations as well as other physiological processes such as persistence and pathogenesis of *Mtb* are regulated by QS (Fig. 2). Many virulence factors of mycobacteria were identified such as: sigma factors, proteases, lipids, secretion

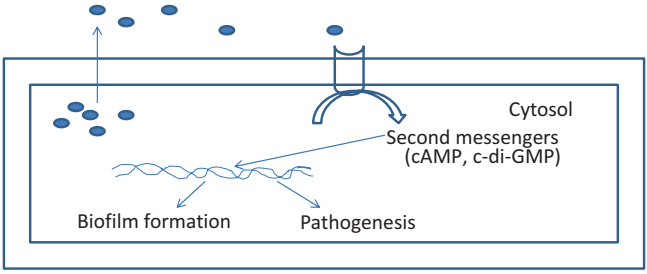

Fig. 2 Role of second messengers in Mycobacterium tuberculosis biofilm formation and pathogenesis. Autoinducers (●)

systems, regulators etc. Depending on the function, the virulence factors have been classified into following groups: (1) Lipid metabolism, (2) cell envelop proteins, (3) protein kinases, (4) Proteases, (5) metal-transporter proteins, (6) proteins inhibits anti-microbial effectors of macrophages, (7) regulators of gene expression, (8) unknown function proteins, (9) other virulence proteins [21]. The *Mtb* cell envelope is characterized by complex lipids and glycolipids along with mycolic acids. Multiple methyl branched fatty acids are the components of *Mtb* cell wall lipids, which plays crucial role in pathogenesis [22]. The surface-exposed lipoglycan is lipoarabinomannan, suggested to be play as virulence factor. The *Mtb* cell envelope lipoprotein LprG also suggested that it is playing role in the normal expression of lipoarabinomannan, virulence and pathogenesis [23].

But, the clear signaling mechanism in pathogenesis (or) virulence of *Mtb* is largely unknown. The stationary phase is responsible for persistence and pathogenecity in mycobacteria. A second messenger such as cyclic-di-GMP is required for persistence in mycobacteria [24]. Some studies also suggested that high levels of cAMP in *Mtb* linked in the regulation of specific genes responsible for persistence and virulence [25]. Virulence factors are produced by QS in many pathogenic bacterial species [26]. The unusual cell wall of *Mtb* contains mycolic acid, which is a key virulence factor. Mutation in hadC, which encodes HadBC dehydratase results in dramatic change in mycolic acid structures, which causes loss of virulence in *Mtb* [27]. There are studies also reported that LuxR family regulator Rv0195 is responsible for the modulation of dormancy and virulence in *Mtb* [28].

Conclusion

Several studies reported that the involvement of QS in the regulation of biofilms formation and virulence in many gram positive and negative bacteria. However, some studies have been shown the indirect evidence of QS in *Mtb* biofilms formation and pathogenesis. In QS mechanisms multiple signaling molecules and their integration in the in vivo conditions produce various phenotypes by bacteria. But,

till now only few molecules have been reported in the QS mechanisms of Mtb. Understanding of clear signaling networks in the QS of Mtb and its role in biofilms formation and pathogenesis helps us for the discovery of new QQ molecules, which in turn improve the treatment of tuberculosis.

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Quorum Sensing in *Streptococcus pyogenes* and Their Role in Establishment of Disease

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Abstract The social behaviour of bacteria for the fulfilment of different physiological activities is defined as Quorum Sensing (QS). This ranges from conjugation, symbiosis, virulence, antibiotic production, sporulation and biofilm formation. *Streptococcus pyogenes* which is also named as group A streptococcus (GAS) is a Gram-positive bacteria, is reported to cause diseases strictly in human. The different QS mechanisms in GAS (group A streptococcus) reported till date include Rgg-SHP quorum sensing pathway, SilC (streptococcal invasion locus) quorum-sensing pathway, Lantibiotic regulatory systems, LuxS and AI-2. The proteins of Rgg family are conserved transcription factors, which is modulated by short peptides, thus involve in the biofilm formation and virulence of bacteria. The SilC mechanism involved in the invasive tissue disease and also in the biofilm formation, Lantibiotic regulatory systems aids bacteria in adopting different immune evasion strategies and thus allow them to persist in the harsh hostile environment. Lastly, LuxS and AI-2 are the common mechanisms in all the different bacterial species including streptococcus for the virulence, motility and bio-film formation. The current chapter focuses on the detail mechanism of all the four different pathways along with the role of Quorum Sensing for the establishment of disease in the host, the immune evasion strategies of bacteria using Quorum sensing (QS) and future clinical perspective with possible applications. This may help to increase our vision towards putative vaccine targets by exploiting the mechanisms involved in Quorum Sensing.

Keywords Quorum sensing · *Streptococcus pyogenes* (GAS) · Virulence · Immune evasion

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Introduction

Group A *Streptococcus pyogenes* (GAS) are gram positive, non motile bacteria. It resides mainly in oropharynx [1]. GAS is clinically important bacteria. GAS is responsible for various human infections within the range from benign to life threatening. It causes infections like pyoderma (skin infections), tonsillitis, pharyngitis (strep throat), impetigo, streptococcal toxic shock syndrome (STSS), scarlet fever, endocarditis and necrotizing fasciitis [2]. GAS is a very clever micro-organism, it has the potential of host immune modulation and evasion [3]. There has been various post immune sequelae has been reported. The GAS classified as "**nephrotogenic"** is responsible for the cause of Acute poststreptococcal glomerulonephritis (APSGN). It is described as disorder arises due to deposition of immune complexes, which affects the kidney. The average death cases reported in a year is approximately 5000 and 47,000 APSGN patient has been estimated [4]. The another class of post infection upshot is the repeated episodes of GAS causes post-inflectional sequel coined as Rheumatic heart disease (RHD). The anticipated global burden of disease caused by GAS is 18 million cases per year and near about 517,000 deaths [5]. Perhaps, the bacteria are very sensitive to penicillin [6]. In many cases, the diagnosis of disease becomes too late which results in severe pathological conditions. These squeal of disease is not handled by simple penicillin thus, resultant of infections may not be manageable in few cases. Thus, understanding the mechanism involved in bacterial infections is of great importance.

Box 1: Clinical Symptoms and Signs of Various Diseases Caused by Group A Streptococcus (GAS)

S.No.	Disease	Type	Clinical signs and symptoms	Associated M types	References
1.	Impetigo	Superficial	Skin pustules that mature into honey colored scabs	33,41,42,52,53,70	[7]
2.	Scarlet fever	Superficial	Deep red rashes on skin, strawberry tongue, Pharyngitis		[8]
3.	Pharyngitis	Superficial	Sore throat and fever	1,3,5,6,12,14,17,19,24	[2, 9, 10]

(continued)

Box 1 (continued)

S.No.	Disease	Type	Clinical signs and symptoms	Associated M types	References
4.	Acute rheumatic fever	Sequelae	Carditis, polyarthritis, syndehman chorea (jerky movements), subcutaneous nodules	1,3,5,6,11,12,14,17,18, 19,24,27,29,30,32,41	[3, 11–13]
5.	Rheumatic heart disease	Sequelae	Mitral valve and aortic valve get affected, regurtation and stenosis occur. Difficulty in breathing	1,3,5,6,11,12,14,17,18, 19,24,27,29,30,32,41	[12, 14–17]
6.	Acute poststreptococcal glomerulonephritis	Sequelae	Complement deficiency, immune complex deposition, urinary sedimentation, hypertension and edema	1,4,12,49,55,57,60	[18, 19]
7.	Bacteremia	Invasive	High fever, vomiting and nausea		[20]
8.	Puerperal sepsis	Invasive	Abdominal pain in pregnancy, fever and chills	28	[21]
9.	Cellulitis	Invasive	Tender and swollen part of skin		[22]
10.	Necrotizing fasciitis	Invasive	Tissue destruction, vomiting, skin lesions, fever	1,3,28	[22]
11.	Streptococcal toxic shock syndrome	Invasive	High fever and multi-organ failure	1,3	[23]

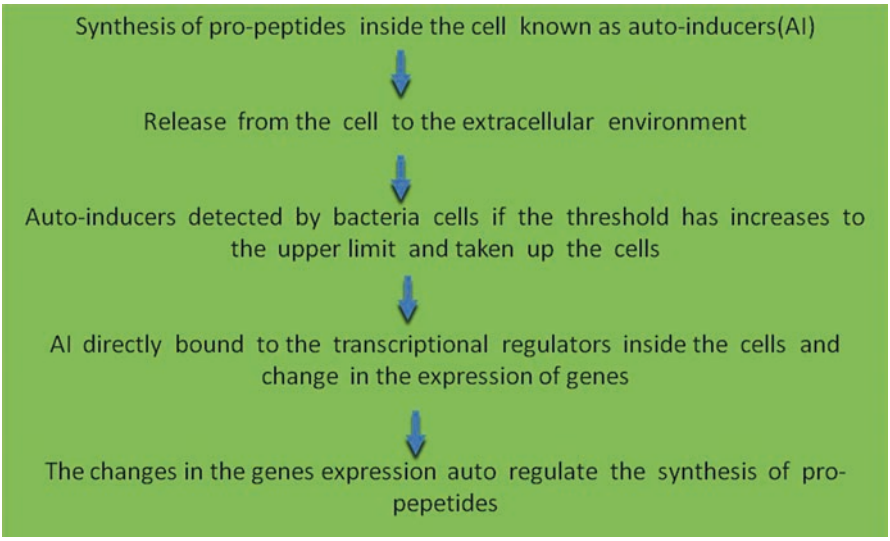
Epidemology, Mechanism and Mode of Infection

The GAS isolates has been distinguished on the basis of emm protein. The emm typing is done from the 5′ region of the gene encoding the emm protein. This region is variable among all isolates, so far there are 200 types of strains has been identified [24, 25]. The GAS colonize in the throat on the epithelial surface, but sometimes also resides in the vaginal and anal linings. It has been reported that GAS resides on the epithelial surface but also able to breach the lining and propagate insides the organs [26, 27]. The GAS is spread through skin to skin infection and saliva, it become prevalent in overcrowding areas and has been observed more in school children and aboriginal population [2]. GAS has the capability to perform antigenic mimicry which help them to sustain its growth and maintain its virulence against the immune system of host. Various strategies has been reported in case of GAS immune evasion. It has been reported that cytoslin streptolysin (SLO) which is defined as the pore forming bacterial protein inhibit the fusion of lyosome during the phagocytosis event and thus escape from the harsh acidic mileu [28]. The SLO protein has also induces the death the cell death while residing inside the macrophages and neutrophils [29].

Biofilms Formation and Quorum Sensing

Apart from targeting the host immune cells, this bacteria also modulate its own gene expression inorder to survive in the harsh hostile environment and prevail the infections in the host. Biofilms formation has been reported as one of the pathogen modulatory exercise in *Streptococcus pyogenes* (GAS) [30]. Biofilms are the enveloped structures consist of sessile bacteria enclosed in a matrix of polymeric substance. Biofilms are formed by bacteria using the well defined phenomena which are stated as **Quorum sensing (QS)** [31, 32]. QS Is used by bacteria for fulfilling their various activities which includes the collective traits such as virulence, biofilm formation, swarming, conjugation. QS is reported in gram positive and gram negative bacteria both, for the persistent of infection and evasion of immune responses of host. Socialization among bacteria includes biosynthesis, secretion and detection of ligand named as auto inducers. Production of these chemical substances in enormous amount triggers the cascade of QS, results in the altered gene expression. The auto-inducers in gram positive and gram negative bacteria behaves differently. In gram-negative bacteria N-acyl homoserine lactones derivatives act as auto inducers which diffuse freely to and fro from the cells and directly interact with intracellular regulatory proteins. In gram positive bacteria, the pro-peptides are formed which processed and form ligand which binds with the receptors like as the membrane bound sensor which have the histidine protein kinase activity (refer to Box no. 2) [33]. Quorum sensing in *Streptococcus pyogenes* has been classified under four system.

Box 2 Steps of quorum sensing in gram positive bacteria



1. Rgg-SHP quorum sensing pathway: Rgg are the class of regulatory proteins which are poorly studied in case of gram positive bacteria. After the name of Regulator gene of glucosyl-transferase in *Streptococcus gornodii*, the RGG termed was coined. In *Streptococcus pyogenes* paralogs of Rgg has been identified, such as RopB (Rgg) (spy49_1691), Rgg2 (spy49_0415), Rgg3 (spy49_0449c) and ComR (Rgg4) (spy49_0032). Rgg2 and Rgg3 are located in opposite directions to each other in the close proximity of SHPs genes (Short hydrophobic genes). Study was done in the Rgg2 and Rgg3 mutant strains, and its role in the quorum sensing has been demonstrated. It has been shown that the Rgg2 with Rgg3 are involved in the biofilm formation and also in the positive feedback mechanism by expressing the SHPs genes. [34]

   RopB: RopB is a dimeric protein primarily engaged in the positive and negative regulation of most of the genes. SpeB i.e. Streptococcal pyrogenic exotoxin are the proteins involved in extracellular matrix formation, role of SpeB in the virulence has been reported [35]. RopB belongs to the member of Rgg family and curbs the expression of n numbers of genes, out of them SpeB has been reported as the most studied one. Studies reveal that RopB dependent SpeB expression is regulated with the density of GAS and the peptides releases by the bacteria regulates the virulence activity.

   Rgg2 (spy49_0415) and Rgg3 (spy49_0449c): Biofilm formation and lysozyme resistance are the main functions of these proteins. These are cytoplasmic in nature and formed for the pro-peptides forms such as SHPs. The exportation of SHPs is done by a transporter named as PptAB. The ABC transporter PptAB is conserved among all *firmicutes* but their cognate substrate and functions among different species is remain uncleared. Recently in 2016, study was done

by creating PptAB mutant GAS, and it has shown that this specific ABC transporter is important for the SHPs protein translocation [36]. Biofilm synthesis by Rgg 2/3 sensing pathway in GAS has been reported.

To understand the mechanism involve in biofilm formation, processed pheromones from bacterial supernatant were isolated, bioluminescent reporters were tagged and mass spectrometry were used to detect and characterize it. SHPs of different length were detected and there synthetic peptides has been used to validate the biofilm growth assay. Rgg SHP interactions are validated by using fluorescence polarization assay [37]. Thus, Rgg2/3 serve as a cytoplasmic receptor for SHPs and PptAB act as transporter for such proteins which involve in biofilm growth and virulence in *Streptococcus pyogenes (GAS)* [36].

ComR (Rgg4) (spy49_0032): ComR is the another class of Rgg member involved in the horizontal gene transfer. Competence is the state where the bare DNA is uptake from the outer environment. The uptake of the foreign sequence is regulated by the series of genes and the expression of these genes is master regulated by the cascade of molecules. In streptococcus species the master regulator defined as SigX has been identified. But not all class of streptococcus is naturally competent. In GAS, the type II ComRS quorum sensing has been identified and the novel pheromone which regulates this pathway has been identified. The stability of SigX has been identified and the another class of protein called cytoplasm protease (ClpP) regulates its stability. Figure 1 explains the proposed model for competence gene regulation in GAS. It was hypothesized on the basis of previous findings in *S.mutans* that the expression of sigX is regulated by ComRS. The secretion of ComS is done by bacterial cell, its further processing and maturation is done by an unknown process which forms a sigX-inducing peptide (XIP).The released XIP in the extracellular environment get imported with the Opp transporter. Inside the cytosol XIP binds with ComR, thus results in the dimer. This dimer binds with the P1 promoter of which is upstream of comS and sigX, thus activates there expression. The accumulation of SigX is

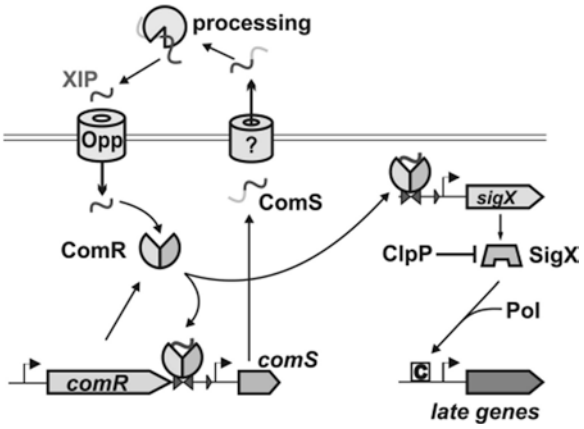


Fig. 1 The proposed mechanism of ComR in *streptococcus pyogenes.* (Source: Mashburn-Warren et al. 2012)

depend upon the ClpP protease. SigX and RNA polymerase together bind with promoter region named as CIN box which activates the transcription of late competence genes. While this study explains the presence of competence genes in GAS, and these genes express on the density depended manner. It gives us the clear report of ComR rgg regulator in *Streptococcus pyogenes* but still the in vitro transfer of DNA in GAS is not possible. Using radio labelled DNA it has been demonstrated that the transformation is blocked at particular stage. [38]

2. **SilC (streptococcal invasion locus) quorum-sensing pathway:** The Sil system is the first Quorum sensing network explained in *Streptococcus pyogenes*. The Sil is a locus which gets activated by the pheromones defined as SilCR. The overall cascade is run through the two- component system termed as SilA-SilB. In vivo virulence genes were identified in the GAS with the help of polymorphic-tag-lengths transposon-mutagenesis (PTTM).The transposon is inserted in the locus termed as Sil locus, which results in low virulence in mice model. The movement of the strain from skin to spleen is also get attenuated. The Sil consist of five genes named as Sil A-E. The two component system is encoded by silA and silB, while the ABC transporters are encoded by silD and silE. There is an ORF next to combox promoter which is called as silC [39]. The DNA promoter region required for the activation of SilA pathway has been characterized. It is consist of two direct repeats of 10 bp with 11 bp of spacer. With wide array of bioinformatic analysis, 13 bacteriocin genes were identified that are under the regulation of SilA. Using the GFP accumulation, the SilCR signalling has become more clear. Using a little amount of synthetic SilCR, the autoinduction in GAS demonstrated the ability of naturally producing SilCR [40]. The overall study suggest the role of Sil in colonization and virulence.
3. **Lantibiotic regulatory systems:** During the establishment of infection, colonization and niche formation, bacteria meet to high nutrition demand and competition. To minimize this competition, bacteria have evolved with various strategies which help them to successfully establish the infection without the interference of host immune system. Thus, the Lantibiotic system helps bacteria to adopt various immune strategies and successfully survive in harsh hostile environment. The bacteria synthesize bacteriocins, which is defined antimicrobial peptides (AMP) which kill the bacteria of same or different species. These AMP kills its neighbouring bacteria with different modes such as pore formation, cell wall synthesis inhibitors. Lantibiotics are the class I bacteriocin identified in *Streptococcus pyogenes* and also in some other class such as staphylococcus. Lantiobiotics are synthesized in unprocessed form define as with the leader sequence located in the N terminal while the C terminal is involved in post translational modifications. The enzymatic chain of reactions i.e. the dehydration of serine/threonine in the C terminal region leads to the formation of 2,3-dehydroalanine (Dha) and 2,3-dehydrobutyrine (Dhb). The further addition of thiol group from the nearby cysteine group results in the formation lanthionine and methyllanthionine respectively. These specialized stable thioether rings are important for biological activities. The recognition of substrate is done by leader peptide [41]. The another class of bacteriocin define as classIIb has been

identified in M18 *S. pyogenes* strain, the promoter region upstream to class II b has been identified using recombinase-based in vivo expression technology (RIVET) system, the activity of the promoter has been studied in mouse. It has been shown that the peptides named as SpbM and SpbN, both are essential for antimicrobial activity. It has been also shown that the *S.pyogenes* immunity genes are encoded downstream of spbN [42]. It has been reported that *S.pyogenes* has evade immune response by hiding inside the macrophages, SalY which is homologous to lac-operon of lantibiotic has been identified in *S.pyogenes* and its mutant study has revealed that the SalY is crucial for bacteria survival inside macrophages [43]. While lantibiotic act as a AMP for their neighbouring microorganisms, it also has proven for adopting immune evasion strategies such as dwelling inside the macrophages (as discussed above). The lantibiotic as described is the crucial system for niche establishment is also the type of quorum sensing mechanism, where the production of lantibiotics occurred through density depended manner. The auto-regulator are the lantibiotics with the promoter region responsible for the synthesis of peptides [44]. The overall setup is arranged in the operon. The production of lantibiotic occurs in propeptide form which further processed and transported out of the cell. The mature lantibiotic is also sensed by the two regulatory system (TCS), thus the production of lantibiotic is sensed and regulated. In one of the study in *S.pyogenes* SF370, role of TCS in case of quorum sensing has been established. Since SF370 is a strain which lacks bacteriocin synthesis was co-cultured with nisin A and demonstrated SrtRK TCS (of SF370 strain) is susceptible to ex-bacteriocin, thus regulating the ABC transporter SrtFEG. It has been also demonstrated that TCS is also crucial for the *S.pyogenes* when cultured with nisin A-forming *Lactococcus lactis* [45]. Streptin and streptococin are the other two classes of lantibiotics studied in case of GAS [46].

4. **LuxS and AI-2:-** The autoinducer -2 (AI-2) was first reported in gram negative bacteria called *Vibrio harveyi* and later it has been identified its involvement in QS depended mechanism for the production of luciferase activity. LuxS is an enzyme identified for the production of AI-2. The LuxS enzyme has been identified in both gram negative and gram positive bacteria. As LuxS is the key component of this system which diverts the interest for the identification of lux/AI in gram positive bacteria. The mechanism involved in this type of QS is widely differing as explained in the former types of QS. This type of communication skill of bacteria is not peptide depended. The AI-2 synthesis pathway is adjunct with a metabolic pathway coined as activated methyl cycle (AMC). This AMC pathway main focus is to utilize the S-adenosylmethionine (SAM) and decompose its toxic by-products. SAM is an important provider of methyl groups required for the processing of building blocks such as DNA, RNA, protein and other biological activities of an organism. During the course of such events the toxic in between product like *S*-adenosylhomocysteine (SAH) is formed, which further degraded by the nucleosides to form S-rybosylhomocysteine (SRH) and adenine. The break-down of SRH to homocysteine and 4,5-dihydroxy 2,3-pentanedione (DPD) is carried out by LuxS. Pro-AI-2 molecules are formed

by the spontaneous cyclization of DPD which further reacted with borate leads to the signal identified by Vibrios [47]. LuxS and AI-2 role in causing virulence, biofilm formation has been studied in various bacteria. To examine the role of LuxS in *S.pyogenes* biological activities, a LuxS mutant was designed of an M3 serotype. Functional characterization of the mutant explained its internalization by HEp-2 cells with greater efficiency as compare to the wild type strain. Expression of genes such as speB (streptococcal pyrogenic exotoxin B), hasA (hyaluronic acid synthesis) which are known to involve in the internalization of SP268 strain were checked in case of wild type and mutant strain. There was increase in mutation of emm3 and reduced level of expression in case of speB in mutant strain. Previously, it was considered that SpeB and M3 proteins are involve in internalization by epithelial and endothelial cells. Later, it was cleared that only M3 helps in internalization while the SpeB protein impede the GAS uptake. Thus, the work which was performed in LuxS mutant strain gives the more light on the LuxS/AI-2 pathway and its importance on GAS internalization mechanism [48]. In another study conducted on M1 and M19 strain of *Streptococcus pyogenes* explains the involvement of the luxS/AI-2 pathway in the metabolism and adaptation of the bacteria in tough host environment. It has been reported that the expression of LuxS and AI-2 get reduced at low pH and thus explains its adaptation capability under stress condition. In order to mimicking the host environment *S.pyogenes* were grown in RPMI with 10% serum and decreased level of both the genes were observed. It has been also observed that LuxS mutant *S.pyogenes* strain can be successfully enters and survive inside epithelial cells and macrophages [40]. Thus, suggesting that this QS type helps bacteria to communicate in such a way which results the organism to sustain its life within its targets. Its aid in internalizing, releasing proteins for adopting different immune evasion strategies.

Conclusion and Perspective

The *Streptococcus pyogenes* is responsible to cause many types of disease in human. Understanding the pattern of bacterial survival and retention inside the host is essential. Like other bacterial species, it has been seen that the *S.pyogenes* is also a social micro-organism which sustain its life inside biofilms and colonize in its host [1]. The wide clear picture of QS in *S.pyogenes* is portraited by Sil system which explains the role of QS in virulence and colonization. There are other type of QS circuits that has been explained in S.pyogenes called as type II ComRS quorum sensing which involved in control of DNA transfer. Lantibiotic, a class of bacteriocin has also been identified in this species which shows the antimicrobial activities and form the AMP such as Streptin and streptococin. Its role for insulting the host immune system attack was explained. LuxS/AI-2 pathway an another class of QS and its importance during the course of internalization and establishment of the infection has been clearly explained. Though, the work related to QS system in

S.pyogenes has been established, the information in this area is very little for clinical applications. The clear pathogenesis of this bacteria and its QS system is needed to be explored more. From the past research evidences, it has been clear that QS involves in the establishment of infection such as biofilm formation, colonization and adhesion. It has also been clear that this bacteria adopt various immune evasion strategies such as hiding inside macrophages by exploiting the QS system [40, 43]. It is apparent that this species as similar to other bacteria perform their various biological activities by using QS. To understand the molecular pathways and there component may help to target and block there growth. The term which is well defined this phenomena is "Quorum quenching". Blocking of QS can be done by using enzymatic degradation of pathways components, using of inhibitors against signal molecules [49]. Thus, Quorum quenching would be the next possible therapeutic clinical step for clearing out the infection. In desire of finding out the therapeutic targets, the QS system in this species is need to be explored with greater extent as it may be the future promising treatment directions.

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Part VI
Other Related Topics on Quorum Sensing

Mathematical Model of Quorum Sensing and Biofilm

Sarangam Majumdar and Sisir Roy

Abstract Bacteria are unicellular microorganism, which are found in nature quite often. They talk to each other using chemical signaling process (quorum sensing) and ion-channel mediated electrical signaling mechanism. Quorum sensing is a density dependent bacterial collective behaviour and/or cell-to -cell communication mechanism. This widespread bacterial behaviour is related with biofilm formation, gene expression, swarming, virulence and bioluminescence. In a recent realization (experimental and theoretical study), it was observed that bacteria can also talk to each other through the wave of potassium and an oscillatory dynamics was noticed in bacterial biofilms. In this present chapter, we present two different mathematical frameworks of bacterial communication system. The first model is based on the bacterial density dependent behaviour with up-regulation and down-regulation of the production of quorum sensing molecules. Second model, we introduce two different types of the bacterial communication process within a mathematical framework, which is also related to the biofilm formation. This mathematical framework combine quorum sensing mechanism as well as electrical signaling process. We discuss different spatiotemporal patterns and chaotic behaviour in this communication system. Moreover, it gives a significant and the fundamental role of noise in the complex biological conversation system. Finally we propose some open problem in the last section of this chapter, which are helpful for the future research of the bacterial communication system.

Keywords Quorum sensing · Bioflim · Noise · Ion-channels · Quantum biology · Kinematic viscosity · Pattern formations

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Introduction

Nature is full of amazing organisms. Bacteria are one of them, which can exist everywhere in the world from the beginning of the life. It can be found from marine life to our everyday day life. The number of bacterial cells in adult human body is ten times the number of human cells. Bacterial cells are residing in host body and make a beneficial partnership between host and the guest, which is formally known as symbiosis. There is unity in diversity in the bacterial kingdom. Bacteria can fight together with their unique decision making technique. Now, we can make an analogy between bacterial and human behaviour. Human being can talk to each other using different languages. When we talk within a same community (e.g. Italian community, Deutsch community, English community), we use the same language. For example, Italian can talk to each other and understand Italian language. But when Italian is talking with English man/woman then he/she can use English language. Bacteria are also doing in a same way. Bacteria can talk to each other with chemical signaling molecules. They use same molecules for intra species communication (e.g. *Vibrio fischeri* use 3-Oxo-C6-HSL molecules for intra species conversation) and other type of signaling molecules for inter species communication. This bacterial communication process is known as **quorum sensing** (see Fig. 1).

What is the meaning of quorum sensing? Bacteria use very tiny biomolecules for there communication. These molecules are known as quorum sensing molecules (QSM) or autoinducers (AI). Bacteria are secreted out the QSM from the cell and the QSM is received by the other bacterium. When the threshold concentration of the quorum sensing molecules is achieved, then a coordinated change in bacterial behaviour is initiated. So, it is clear that bacteria sense by other cells present in their vicinity after attaining a certain threshold or quorum state [1–9].

Vibrio fischeri is a bioluminescent marine bacterium, where the quorum sensing mechanism was first observed [1]. This bacterium can be found in a free-living organism as well as a symbiont in the light-producing organ of an animal host, such

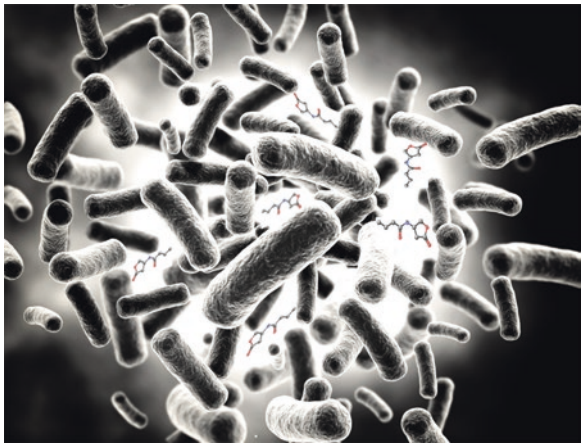

Fig. 1 Illustration of bacterial communication process through chemical signaling molecules

Table 1 List of quorum sensing bacteria with their quorum sensing molecules

Microorganism	Chemical signal
Aeromonas hydrophila, *Pseudomonas aeruginosa*	C4-HSL (an AHL)
Erwinia carotovora, *Pseudomonas aureofaciens, Yersinia enterocolitica*	C6-HSL
E. carotovora, *Vibrio fischeri*, *Y. enterocolitica*	3-Oxo-C6-HSL
Agrobacterium Tumefaciens	3-Oxo-C8-HSL
Vibrio harveyi	AI-2 (S-THMF-borate)
Straphylococcus aureus	Autoinducing Peptide (AIP)-I
Bacillus subtilis	Glu-Arg-Gly-Met-Thr (competence and sporulation stimulating factor)

as the Hawaiian bobtail squid. It was observed that *Vibrio fischeri* produced light in a batch culture, when the large numbers of bacterial cells were presented. In a free-living organism, *Vibrio fischeri* cannot produces light because of its low cell number densities. When the cell number densities are high enough then autoindusers become sufficient to induce transcription of genes (QS genes) that produce the enzyme luciferase, leading to bioluminescence [10–12]. Besides *V.fischeri* there are so many bacterium, which can also talk to each other using different types of signaling molecules (see Table 1).

Quorum sensing mechanism is attached with the biofilm formation. One can find biofilms in damp and wet environment and its play a significant role in a different infection. It is also associated to water treatment and remediation and many more [13]. From the point of view of medical science we can quote from the National Institutes of Health [14],

> Biofilms are clinically important, accounting for over 80 percent of microbial infections in the body. Examples include: infections of oral soft tissues, teeth and dental implants; middle ear; gastrointestinal tract; urogenital tract; airway/lung tissue; eye; urinary tract prostheses; peritoneal membrane and peritoneal dialysis catheters, in-dwelling catheters for hemodialysis and for chronic administration of chemotherapeutic agents (Hickman catheters); cardiac implants such as pacemakers, prosthetic heart valves, ventricular assist devices, and synthetic vascular grafts and stents; prostheses, internal fixation devices, percutaneous sutures; and tracheal and ventilator tubing.

So, we can say that the biofilms are dangerous and it is associated with different infections, which is by itself a leading cause of death in all around the world. The development of biofilm can be characterized as a multistage process (see Fig. 2).

Neurophysiology is one of the active research field in brain research, where ion-channel mediated neuronal signaling process gives us structural configuration of different ion-channel and its fundamental insight of human brain. In a more recent study, bacterial ion-channels provide fundamental and significant role of the structural basis of this signaling mechanism [16]. Bacteria have different types of ion-channels. The experimentally observed bacterial ion-channels are as follows

- Potassium ion-channel KcsA
- Chloride channels

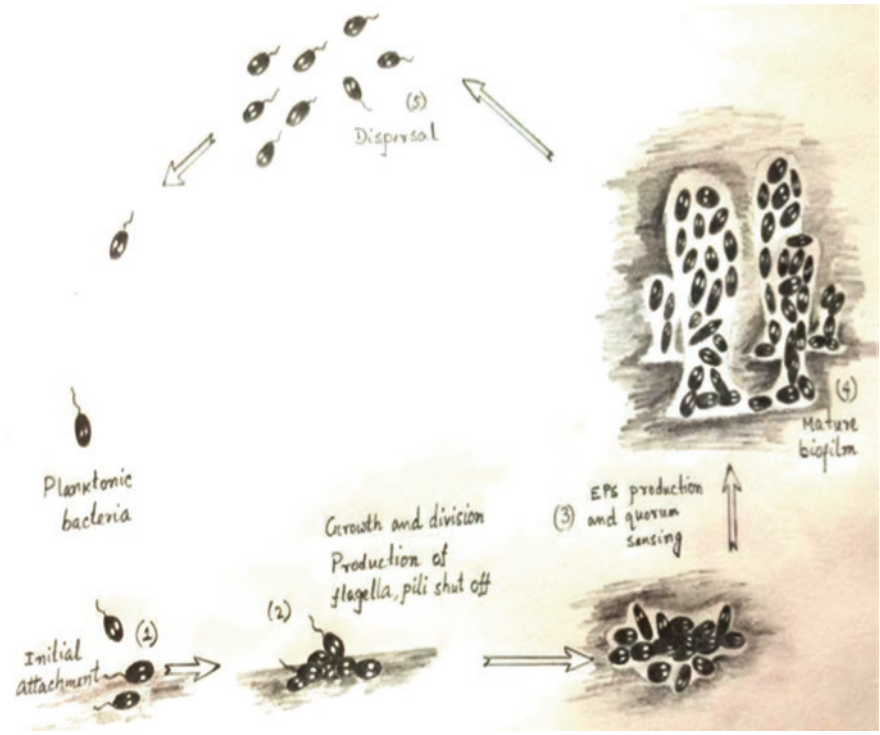

Fig. 2 Schematic diagram of the bacterial biofilm formation (multistage process). (Adapted from Majumdar and Pal [15])

- Calcium-gated potassium channels
- Ionotropic glutamate receptor
- Sodium channels

The above listed bacterial ion-channels are similar to those found in neuron. So one can think of investigating the bacterial ion-channels as a model. In this chapter, we are restricting ourself on potassium ion-channels (see Fig. 3). Now we are focused on some important experimental evidences of bacterial K^+ ion-channels and its structure and unique functional role in bacterial communication system and biofilms.

Recently, a great deal of effort has been devoted to understanding the unique function and structure of potassium ion channels of bacteria. G. M. Süel with his student and collaborators showed that potassium ion-channels conduct long-range electrical signals within *Bacillus subtilis* (gram positive bacteria) biofilm communities [17–20]. These waves form a positive feedback loop, in which a metabolic trigger induces release of intracellular potassium, which in turn depolarizes neighboring cells. This wave of depolarization coordinates metabolic states among cells in the interior and periphery of the biofilms (see Fig. 4).

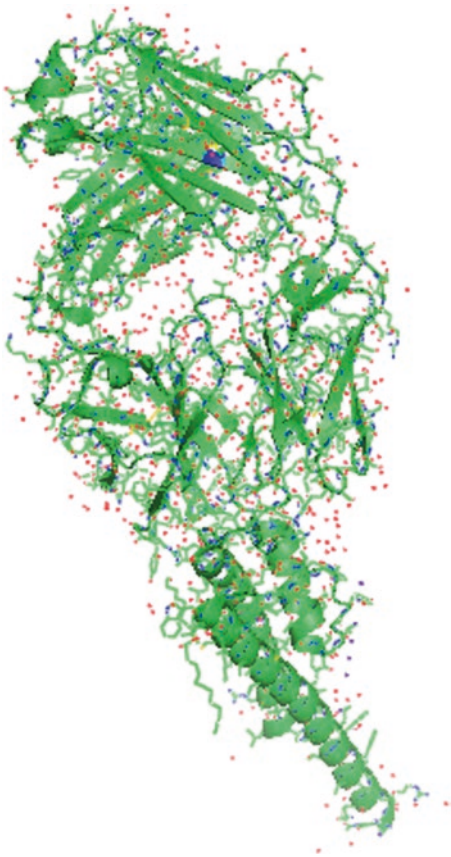

Fig. 3 Schematic diagram of potassium ion channel from PDB 1K4C

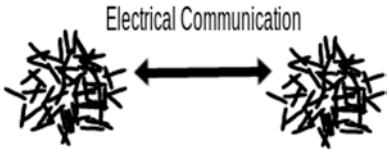

Fig. 4 Schematic diagram of electrical communication between bacterial biofilm

It has been studied that the metabolic oscillation of bacterial membrane is triggered by nutrient limitation. Adherent communities of *Bacillus subtilis* form biofilms and grow in interval of cycles once the colony reaches threshold size of population. These cycles arise when the cells present in the biofilms rundown of glutamate due to consumption of high amount of amino acid by peripheral cells. Glutamate starvation in the interior cells reduces the production of ammonium ions, which is required by the peripheral cells. As a result, the cell growth diminishes drastically [15, 21]. These findings raise the question of whether such extracellular signals could extend beyond the biofilm, resulting in long-range interactions that could affect distant bacteria that are not part of the biofilm.

Next, they studied the attraction of motile cells, which was due to changes in extracellular potassium generated during biofilm oscillations using the microfluidic device. They demonstrated that changes in extracellular potassium gradients are sufficient to direct motile cell behavior. The role of the potassium ion channel in motile cell attraction is experimentally verified and it shows that the potassium ion channels in biofilm cells play very important role in generating the electrical signal that attracts motile cells. Moreover, it shows that attraction also depends on the membrane potential- mediated sensitivity of the motile cells to the potassium signals generated by the biofilm [15, 21].

Time-sharing is a strategy, which is tropically employed in engineering and technological systems where users take turns consuming recourses. So the different systems are competing with each other. *B. subtilis* biofilm communities are engaged in collective growth-rate oscillations due to glutamate starvation. These oscillations are driven by a spatially extended negative feedback loop, where growth of the biofilms result in glutamate stress within interior and this stress in turn interfaced with biofilm growth. It has been reported that these biofilm communities undergoing metabolic oscillations become coupled through electrical signals, which cause in synchronizing their growth dynamics. Also, it increases the competition by synchronizing demand for limited nutrients. They confirm that biofilms resolve this conflict by switching from in phase to anti- phase. Different biofilm communities take turns consuming nutrients. Thus distant biofilms can coordinate their behavior to resolve nutrient competition through time-sharing. This is a very intelligent and efficient strategy to share the limited resources [15, 21–24].

In section "Mathematical Modeling of Quorum Sensing", we discuss a one mathematical model of the quorum sensing system of bacteria (*V. fischeri*) proposed by Ward et al. in 2001 [25] and in section "Mathematical Model of Electro-Chemical Bacterial Communication System" we emphasis the recent mathematical framework of bacterial two types of communication (chemical and electrical communication) which is proposed in [26] by Majumdar and Roy. In the final section "Open Problems", we shorted out some important and significant question for the future research in the context of bacterial communication.

Mathematical Modeling of Quorum Sensing

The cell number density of the bacterial population regulates quorum sensing mechanism or cell-to-cell communication system. J.P. Ward, J.R. King, A. J. Koerber, P. Williams, J. M. Croft and R. E. Sockett proposed the very interesting and useful mathematical model of quorum sensing in 2001 [25]. In this mathematical approach a system of ordinary differential equation is used to explain the cell growth and quorum sensing molecules production in a well-mixed population of cells.

In case of quorum sensing process of the *V.fischeri*, quorum sensing molecules (QSM) binds with the appropriate protein to form a complex, then this complex can bind to the *lux*-box part of the quorum sensing (QS) gene region of the chromosome

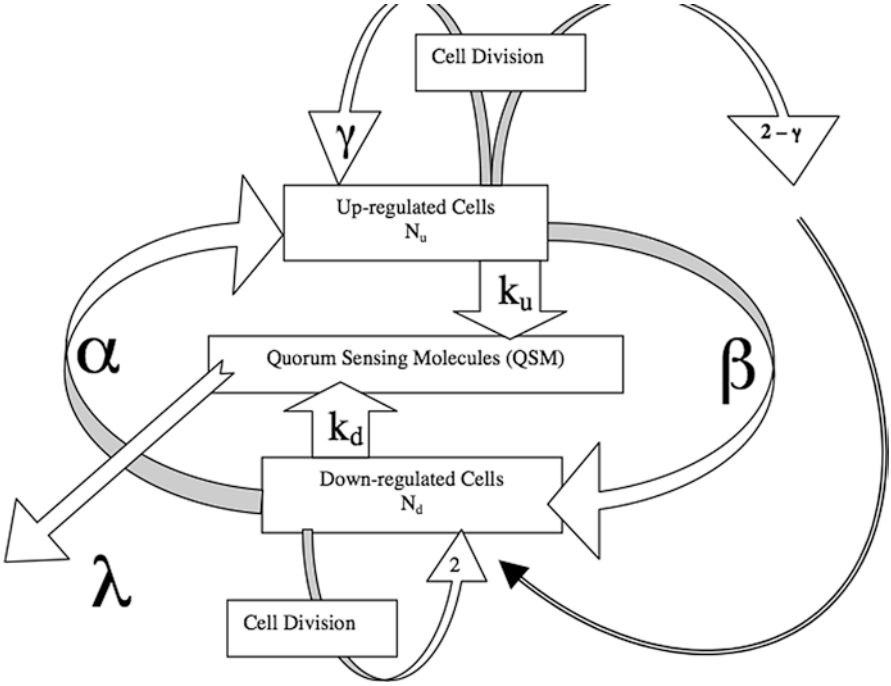

Fig. 5 Illustration of the down-regulation and up-regulation of the cells. (Adapted from [10])

and the binding of *lux*-box induces activation of the QS genes from a down-regulated state to an up-regulated state [25].

Model Assumptions

This mathematical model is based on some primary and compatible assumptions as follows (see Fig. 5)

- The bacterial population consists of up-regulated (density N_u, viewed as the number of cells per unit volume) and down-regulated (with density N_d) sub-population of cells, corresponding to bacteria with a complex-bound or empty *lux*-box respectively.
- The quorum sensing molecules are produced by up-regulated and down-regulated cells, at the rate k_u and k_d respectively with $k_d \ll k_u$.
- Down-regulated cells are up-regulated by quorum sensing molecules, with the rate constant a.
- Concentration A is changing.
- Down-regulated occurs spontaneously, due to breakdown of *lux*-box bound QSM-QSP complex at the rate β.

- Quorum sensing molecules can be broken down by the medium, and hence lost to the system, at the rate λ.
- Cell division of one down-regulated cells produces two down-regulated cells.
- Cell division of up-regulated cells produces on average γ up-regulated and $(2-\gamma)$ down-regulated cells (where $0 \le \gamma \le 2$) assuming that only a population of replicated chromosomes contain occupied *lux*-boxes. We anticipate that $\gamma \approx 1$, which indicates that division of one up-regulated cell produces one up-regulated and one down-regulated cell.
- Cell division rate of up-regulated and down-regulated cells are equal, being determined by the parameter r, where the doubling rate is $\ln(2)/r$ at low densities.

Model

Now we can write the dynamical system as follows (based on the above assumptions) [25]

$$\frac{dN_d}{dt} = r\left(N_d + (2-\gamma)N_u\right)F\left(N_d + N_u\right) - \alpha G(A)N_d + \beta N_u \tag{1}$$

$$\frac{dN_u}{dt} = r(\gamma - 1)N_u F\left(N_d + N_u\right) + \alpha G(A)N_d - \beta N_u \tag{2}$$

$$\frac{dA}{dt} = k_u N_u + k_d N_d - \alpha G(A)N_d - \lambda A \tag{3}$$

The above dynamical system has order three with nonlinearity. $F(.)$ is consider as a dimensionless bacterial growth function and $F(0) = 0$. The total density of the bacterial cells are quantify as $N_T = N_d + N_u$. Now we can add the Eqs. (1) and (2) we have,

$$\frac{dN_T}{dt} = rN_T F\left(N_T\right) \tag{4}$$

We can further assume that bacterial cell growth as a logistic growth with carrying capacity K. So we get, $F\left(N_T\right) = 1 - \dfrac{N_T}{K}$ and we can simplify this with an additional assumption that $F(N_T)$ is continuous with a single positive zero $N_T = K$, where $F'(0) > 0$ and $F'(K) < 0$, thus we have a stable and unstable steady state at $N_T = \mathrm{K}$ and $N_T = 0$ respectively.

Now we can focus on the function $G(A)$. As per the [25], one can describe the process of QSM-QSP complex formation and *lux*-box binding, as the units of which

being QSM concentration. Here we can consider the function $G(A) = A$ is linear. One can also use $G(A) = A/(1 + k_a A)$ at high concentration of QSM.

Discussion About Quorum Sensing Model

A bacterial quorum sensing mechanism is describing through this above mention model. Model is focused on the activity and the production of a single QSM and its subsequent effects on the bacterial population. The assumption of the model is quit natural and very much effective to describing growth and production within a batch culture. The solution of the model predicts that in quorum sensing mechanism there is a switching behaviour, which is also observed in the experiment. One can dimensionless the mathematical model and perform the linear stability analysis, steady state analysis and asymptotic analysis (see detail in [25]). From the point of view of stability analysis of the model, we can say that the stable solution and general solution of the model is compatible with the real solution, but it is not clear (from the analysis) what happens after the quorum is achieved [10].

Perspectives of the Model

This is a very simple model for the quorum sensing mechanism, where a single quorum sensing molecule and two different bacterial states are considered. As a matter of facts, the biological reality is not so simple. We can consider this mathematical framework as a first step towards more complex modeling approach. One can extend this investigation by introducing new important parameters with this model. The study of quorum sensing using this mathematical model gives us valuable insight into bacterial chemical communication system. We can implement this quantitative understanding for future research in medical science.

Mathematical Model of Electro-Chemical Bacterial Communication System

The densely packed bacterial populations develop a coordinated motion on the scales length (10–100 μm) in comparison to the size of a each single bacterium of the order 3 μm when the bacterial cell density reaches a sufficiently high value. Let us assume that the collective behavior of the densely packed bacteria inside the biofilm is similar to the behavior of the dense granular system. The dense granular system usually behaves like a fluid, which is quite different from the ordinary fluid. The finite size of the bacteria indicates the existence of an intermediate length scale,

which leads us to introduce a source of fluctuation, which is quite different than thermodynamic fluctuation. This new type of fluctuation can be considered as a non-local noise. The swimming induced stresses on the bacteria that can change the local arrangement of bacteria induce stress fluctuations. This stress fluctuation can lead to shear motion and hence is called non-local. Thus, two different type of noise are present in the bacterial communication system and dominance of one over the other depends on the force $\bar{F} = \frac{f}{\rho g}$ which is applied to the complex biological system where f be the volume density of the forcing and g is the acceleration due to gravity [27].

Viscosity and Non-local Theory

Let us consider the state space (ρ, v) of one component fluid, where ρ be the density and v be the velocity of the fluid. The stress tensor and/or the pressure term are the only constitutive quantity in this framework. We consider the higher order derivatives of the basic variable (density and velocity) to extend theory of usual hydrodynamics to weakly non-local hydrodynamics. Without loss of generality, the balance of mass and momentum can be expressed as

$$\rho' + \rho \nabla v' = \sigma_m \tag{5}$$

and

$$\rho v' + \nabla P' = \rho \vartheta \tag{6}$$

Here P is the pressure and ϑ be the force density. This is formally known as Cauchy momentum equation. Now, we can extend this framework by considering the state space spanned by $(\rho, \nabla\rho, v, \nabla v, \nabla^2\rho)$.

One can show that there exists a scalar valued function ϕ_v or non-local potential such that [26, 28]

$$\nabla.\sigma = -\nabla\phi_v \tag{7}$$

where ϕ_v is the course- grained potential or kinematic viscosity potential and σ_{ij} be shear tensor.

One can calculate the viscosity potential from the entropy density function

$$s\left(\rho, \nabla\rho\right) = -\nu \frac{\nabla\rho^2}{4\rho} - \frac{v^2}{2} \tag{8}$$

The non-local potential can be written as

$$-\frac{\nu}{2}\nabla^2\rho \tag{9}$$

where ν is kinematic viscosity and $\nu = \frac{\mu}{\rho}$ (μ is dynamical viscosity of the fluid). We define a kinematic velocity as $u_k = \frac{\nu}{2}\nabla \ln \rho$ [26], which is depends upon the cell density. Here we introduce the kinematic velocity in order to relate to a kind of fluctuations due to the existence of finite length scale associated to granular nature of the fluid. Finally (after some algebraic calculation), we get a general expression as

$$\nabla_t u + (u.\nabla)u = \nu\nabla^2 u + \nabla\eta \tag{10}$$

where $\nabla\eta = -\nu\nabla^2(\Delta u_k)$ and $\Delta u_k = u - u_k$.

The above Eq. (10) is known as noisy Burgers equation. We emphasize that the non-local hydrodynamical model (based on Ginzburg-Landau framework) can explain the quorum sensing phenomena in a consistent way. This noise gives rise to kinematic viscosity, which helps to understand the metastable states for quorum sensing.

This mathematical framework indicates a comprehensive view of an internal structure of the complex biological communication system and viscosity is the property which makes the bacterial cells stick together into clusters predicted by Zeldovich approximation, just mimicking gravitational effect on the smaller scales [27]. This approximation describes the general structure of this nonlinear biological phenomenon. It is to be mentioned that the origin of viscosity is traced back to the weakly non-local effects in the internal structure of the system. One of the present authors (SR) along with Llinas [27] showed that kinematic viscosity plays a vital role in forming the metastable states of the bacteria responsible for quorum sensing. Moreover, bacteria in biofilm form various types of patterns. Now we study the formation of patterns in Biofilms and the role of kinematic viscosity.

Kwak Transformation and Reaction- Diffusion Systems

The quorum sensing system is modeled by noisy Burger equation (Eq. 10). We can rewrite the Eq. (11) as

$$\nabla_t u = \nabla^2 u - (u.\nabla)u + h(x) \tag{11}$$

with $h(x) = \frac{\nabla\eta}{\nu^2}$. By using Kwak transformation $J(u) = \left(u,,u_x,,-\frac{1}{2}u^2\right)$ we can obtain a new system as

$$u_t = u_{xx} + w_{xx} + h(x) \tag{12}$$

$$o_t = o_{xx} + w_{xx} + h'(x) \tag{13}$$

$$w_t = w_{xx} + o^2 + u^2 o - uh(x) \tag{14}$$

The above Eqs. (12, 13, and 14) is a reaction- diffusion system, which gives the mathematical framework for the pattern formation.

Pattern Formations and Viscosity

In this multicellular system bacterial cells form different patterns based on chemical gradients of QSM signal that is synthesized by quorum sensing bacterial cells. The above theoretical analysis reveals that parameters like kinematic viscosity (associated to non-local noise) play most significant roles to form patterns over space and time. Furthermore, the mathematical approach is able to predict how the system behaves if we change the initial values. We emphasize that these are crucial physical parameters (kinematic viscosity and noise) of the system. It should be noted that the regulatory behaviors mentioned above are nontrivial consequence of the model. In our system, we observed that the quorum takes place in a certain range of kinematic viscosity $[0.01, 0.32] m^2/s$ which is considered as very small viscosity of the fluid (see detail in [29, 30]). We also use different numerical scheme and initial data to show the quorum sensing system behaviour. The behaviour changes with the initial data and system forms different wave patterns.

Electrical Communications and Non-linear Schrödinger Equation

The recent findings suggest that bacteria communicate through electrical signaling using waves associated to Potassium ions. One of the present authors (SR) along with Rodolfo Llinas showed that Potassium ions follow non-linear Schrödinger equation [31]. This equation can be written in the following form:

$$i\partial_t \psi = -\partial_{xx}\psi + \boldsymbol{h}'\left(|\psi|^2\right)\psi \tag{15}$$

where ψ is the wave function of Potassium ion. Now one arrives Complex Ginzburg-Landau equation by adding perturbation to the above non-linear Schrödinger equation following Melinkov approach.

$$\partial_t \psi = (1+\varepsilon)\partial_{xx}\psi - i\boldsymbol{h}'\left(|\psi|^2\right)\psi - \varepsilon g'\left(|\psi|^2\right)\psi \tag{16}$$

Here $\psi(x,t)$ is a complex field and $\varepsilon > 0$ while $\boldsymbol{h} = \boldsymbol{h}(\Xi)$ and $g = g(\Xi)$ are real analytic functions over $[0, \text{inf})$.

This non-linear Schrödinger equation is valid at the level of ion channel where the noise associated to opening and closing of the ion channel predominates. On the other hand the perturbation due to non-local noise becomes predominant at the cellular level. At the cellular level, the non-thermal fluctuation arises due to the presence of finite size of the cell or grain of the granular medium. This fluctuation gives rise to the perturbation on non-linear Schrödinger equation and we get generalized Complex Ginzburg-Landau (GL) equation. This Complex GL equation is used for the description of cellular communication through the chemical molecules and also needed to understand the generation of various patterns in biofilms.

Discussion

Following the above approach we state that the generalized complex Ginzburg-Landau equation is able to explain the quorum sensing phenomena as well as the electrical communication mediated by bacterial ion- channels. This mathematical framework gives us different type of phase transition, spatiotemporal pattern in the complex biological system.

Now we simulate one-dimensional complex Ginzburg-Landau equation in large domain with periodic boundary condition using pseudo- spectral method. This is in general a stiff problem in dynamics of this communication process evolving over both fast and slow timescales. Here, simulation is carried out by exponential time-stepping methods.

This approach captures near threshold behaviour of the quorum sensing system. Patterns are changing over space and time continuously and we notice an oscillation. This oscillation is triggered by nutrient limitation. Specifically, interior and peripheral cells compete for glutamate and as a result biofilm growth halts periodically. We call this phenomenon as **cooperative and completion in bacterial communities**. This oscillation increases when the bacterial community exceeds certain colony size. If we change the initial condition the patterns are also changing. Quorum sensing mechanism can be initiated periodically, when the number cell destiny reach a certain threshold. This chemical communication process completely depend on nonlocal noise, a range of kinematic viscosity and density values because they are inter related quantities and follow the equation $\nabla\eta = -\nu\nabla^2(\Delta u_k)$, $\Delta u_k = u - u_k$, $u_k = \frac{\nu}{2}\nabla \ln \rho$, $\nu = \frac{\mu}{\rho}$.

Spatiotemporal Disordered Regimes

In this bacterial communication system, we find some particular spatiotemporal patterns, when we are simulating our mathematical model in the following form

$$\partial_t \psi = \psi + (1 + i\bar{\alpha})\nabla\psi - (\bar{\beta} - i)|\psi|^2 \psi \tag{17}$$

where, $x \in [0, L]$ and $\bar{\beta} > 0$. Our model have plane wave solution as $\psi = a_k e^{i(kx+\omega_k t)}$ with $a_k^2 = \frac{(1-k^2)}{\bar{\beta}}$ and $\omega_k = \frac{1}{\bar{\beta}} - \left(\bar{\alpha} + \frac{1}{\bar{\beta}}\right)k^2$. These plane wave solutions are linearly stable with the condition $\bar{\alpha} < \bar{\beta}$, and $k^2 < k^2_{Eckhaus}$. On the other hand all solutions are unstable for $\bar{\alpha} > \bar{\beta}$ which is formally known as **Bejamin-Feir line**.

Here, the oscillatory state of the bacterial commutation system undergone a Hopf bifurcation and it is considerable importance of a spatially extended non-equilibrium communication system. Two different limiting cases arise one is dissipative ($\bar{\alpha} = 0$ and $\bar{\beta}$ tends to infinity) and other one is dispersive ($\bar{\alpha}$ tends to infinity and $\bar{\beta} = 0$). Dispersive case is equivalent to the integrable nonlinear Schrödinger equation. As a matter of fact, away from the intricacy of the bifurcation diagrams at small sizes ($L < 50$), there exists a large-size limit beyond which chaos becomes extensive and can be characterized by intensive quantities independent of system size, boundary conditions, and, to a large extent, initial conditions [26, 28]. Moreover, one can showed that the Lyapunov dimension is proportional to the system size L [26, 28]. We observed different disordered phase and spatiotemporal chaos, which play an important role for the statistical analysis of the disordered phases.

We observe a strongly disordered phase (see Fig. 6) above the BF line. This phenomenon is known as defect turbulence, which is characterized by a quasi exponential decay of the space-time correlation functions. It is very strong spatiotemporal chaotic phenomena in communication system. It indicates that the pulses of $|\psi|$ grow under the effect of dispersion term. The self focusing is stopped by the action of dissipation, breaking the pulse. Turbulence in the region is characterized by defects (points in space-time where $\psi = 0$). Pulses are the relevant objects to consider when approaching the nonlinear Schrödinger limit [26, 28, 32].

On the other hand, a weakly disordered regime is observed which we can call a phase turbulence (see Fig. 7). It can be defined by the absence of space-time defects. In this case ψ never reaches zero and the total phase is conserved. This is a form of chaotic behaviour, but the chaos is very weak in this regime. It indicates diffusive or sub-diffusive modes and describes the phase dynamics near the BF line.

Below the BF line another spatiotemporal disorder regime has been noticed (see Fig. 8). This regime is spatiotemporal intermittency regime, which consist of space-time regions of stable plane waves separated by localized objects evolving and interacting in a complex manner. This K^+ waves constitute the passive absorbing

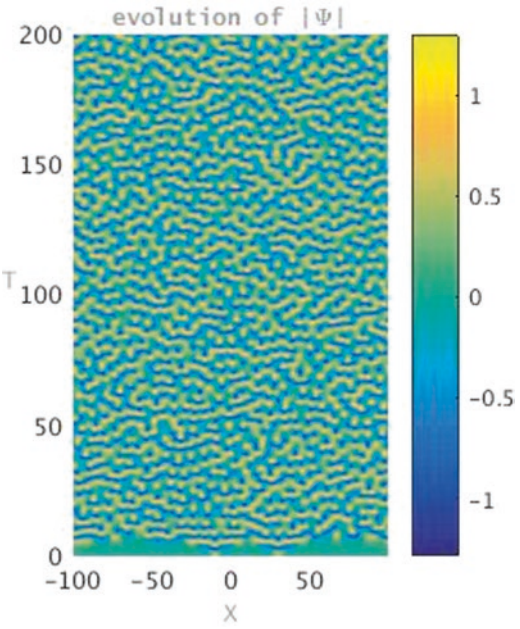

Fig. 6 Defect-mediated turbulence occur in bacterial communication system with $\overline{\alpha} = 2$ and $\overline{\beta} = -2$

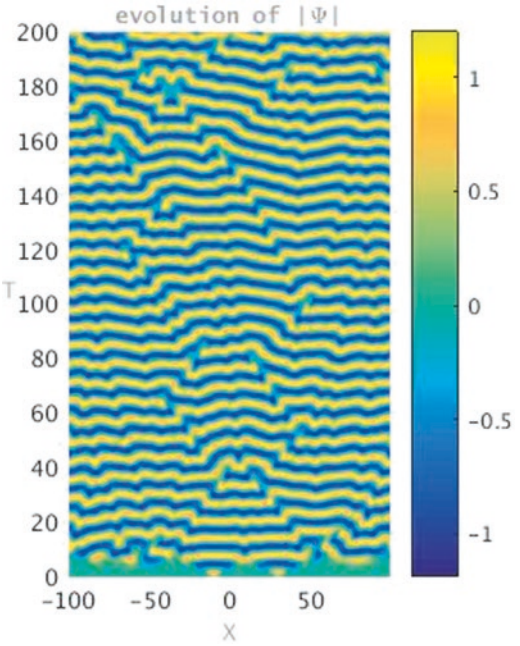

Fig. 7 Phase Turbulence with parameters $\overline{\alpha} = 2$ and $\overline{\beta} = -1$

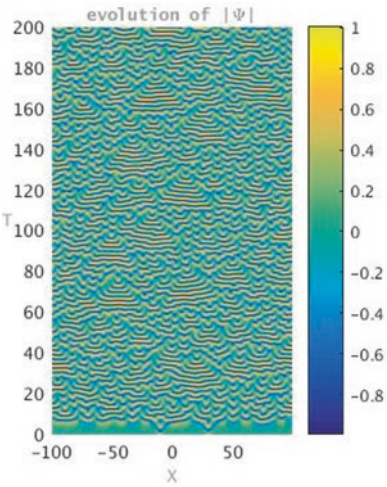

Fig. 8 Spatio-temporal intermittency with parameters $\bar{\alpha} = 0$ and $\bar{\beta} = -3$

state while the localized objects carry the spatiotemporal disorder. Here the defects don't appear spontaneously, the localized object carrying the disorder produces them. This localized structure is completely depending on the bacterial coordinated motion inside the biofilm.

Conclusion

It is clear from the above analysis that the bacterial communication at cellular level i.e. through chemical signaling the non-local noise and hence the kinematic viscosity plays significant role in understanding the quorum sensing of the bacteria in biofilm. Again the patterns in biofilm are generated for small range of values of kinematic viscosity. We use the non-local hydrodynamics as described by complex Ginzburg-Landau equation, which explain both quorum sensing and pattern formations in biofilm. Since it depends on certain range of kinematic viscosity this can be verified experimentally in the laboratory. The experimental observations clearly indicate that bacteria communicate also through electrical signaling. We show that the same complex Ginzburg-Landau equation describes the propagation of potassium ionic waves under certain condition. This particular complex Ginzburg-Landau equation can be recanted as Non-Linear Schrödinger equation. This is valid at the level of ion channels. As soon as we go up to the next level i.e.at the cellular level, the non-local noise perturbs this equation and we arrive at the generalized complex Ginzburg-Landau equation. This non-local noise or perturbation is negligible at the level of ion channel. So we have a single framework, which can explain both types of communications in a comprehensive manner. It is yet to be understood the significance of two paradigms i.e. one classical description at the level of chemical communication and one quantum paradigm at the level of electrical signaling for the same system. It will be studied in the subsequent works.

Open Problems

In this section we have listed some open problems in the research field of bacterial communication as follows

- What is the exact role of noise in the bacterial communication system?
- What is the origin of noise in the bacterial communication system?
- Does noise driven oscillation takes place in this context?
- Is there any role of quantum noise?
- What is the meaning of quantum quorum sensing?
- Is it possible that bacteria have any intelligence?
- What information is processing by the talking bacteria?
- How bacteria process the information through electrical communication?
- What are the significant parameters in cell communication?
- Is there any kind of condensation (Bose-Einstein like condensation) at room temperature?
- What is the underling theory of this biological communication process?
- How can we design the new device to detect the microbial infection?

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Understanding the Bacterial Biofilm Resistance to Antibiotics and Immune Evasion

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Abstract Biofilm is a multicellular lifestyle for bacteria to survive in adverse environmental conditions. Biofilms withstand antibiotics, immune defenses, disinfectants, nutritional changes and high temperatures. The present chapter reviews information of biofilm and also provide insights on how biofilms are able to tolerate antibiotics and evade immune system.

Keywords Biofilm · Antibiotic resistance · Immune evasion

Introduction

Microorganisms thrive in nature by existing either as free living individuals (planktonic mode) or as community known as biofilm. It was assumed that the standard mode of growth for some bacterial species is formation of biofilms whereas the planktonic growth is an in vitro work of art [1]. The term biofilm was coined by William J. Costerton in 1978 to describe the 'surface-attached microbial agglomerations' [2]. The alternative description available according to Donlan and Costerton [3] is …." communities of microorganisms attached to a surface, producing extracellular polymeric substances (EPS) and exhibiting an alternate phenotype when compared with corresponding planktonic cells….". Biofilm is made up of water, bacterial cells, dead cells, and EPS [4]. EPS (referred as matrix) is 90% of the biofilm and EPS matrix consists of exopolysaccharides, DNA, proteins and other macromolecules [5]. The composition of the bacteria is different in the biofilm's. Bacteria form a biofilm either by recruiting the same bacterial species or by recruiting other bacterial species. If the bacterium recruits the same bacterial species then the biofilm formed is known as monospecies biofilm. Whereas, if the bacterium

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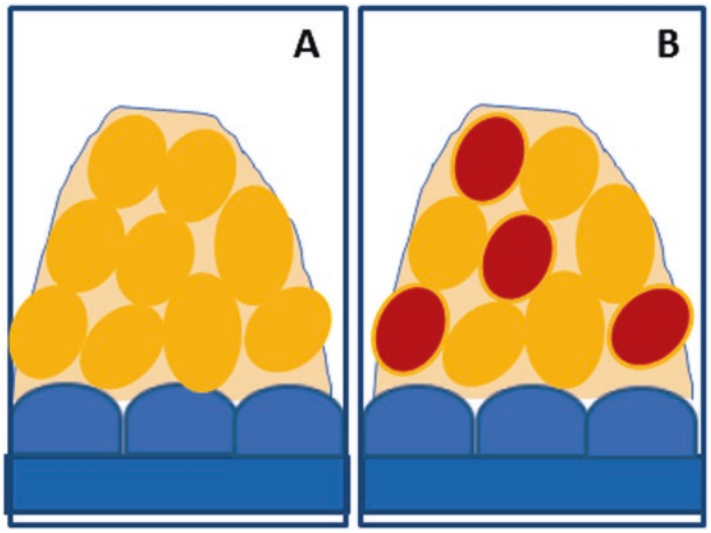

Fig. 1 Bacterial biofilm formed by the (**a**) same bacterial species (monospecies biofilm), (**b**) other bacterial species polymicrobial biofilm)

recruits the other bacterial species then the biofilm formed is known as polymicrobial biofilms (Fig. 1). Some available examples for polymicrobial biofilms are *Pseudomonas aeruginosa* mixed with *Staphylococcus aureus* [6]; *Prevotella* mixed with *S. aureus* [7]; and *Escherichia coli* mixed with *Bacteroides fragilis* [8]. Polymicrobial biofilms increase the rate of infection and survival of bacteria and thereby becomes recalcitrant [9]. *P. aeruginosa* and *S. aureus* biofilms [6]; and *Prevotella* and *S. aureus* biofilms [7] increased the infection rates of pathogens in a rat and mouse models respectively. *E. coli* with *B. fragilis* increased abscess formation in a mouse model [8].

Stoodley et al. [10] proposed a model to demonstrate how a bacterium like *P. aeruginosa* forms biofilm. The development of a biofilm (Fig. 2) includes the following five steps –

1. The first step includes initial or reversible adherence of bacterial cell to a surface in the host. This initial adherence of the bacterium to the surface is influenced by the factors like specific bacterial surface molecules (secreted adhesins and extracellular adhesive appendages), motility and chemotaxis. The forces acting or involved between bacterial cells and the surface of attachment are hydrophobic or electrostatic interactions.
2. The second step includes multiplication of the bacteria forming microcolonies. The microcolonies in the biofilm grow up both horizontally and vertically in size. The bacterial cells generate EPS on all sides of the microcolonies resulting in irreversible adhesion.
3. The third step includes development leading to formation of an early structure like matrix for biofilm.
4. The fourth step includes maturation of matrix leading to formation of biofilm. The mature biofilm is a either a “thick and mushroom-like or tower-like

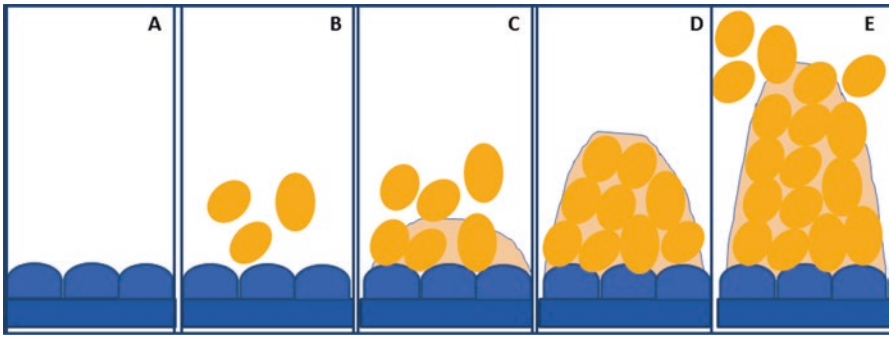

Fig. 2 The sequence of events involved in formation of a biofilm (**a**) surface/substrate for the formation of a biofilm, (**b**) bacterial cells adhering to the surface, (**c**) bacterial cells generating EPS resulting in irreversible adhesion, (**d**) development leading to formation of an early structure like matrix for biofilm, (**e**) maturation of matrix leading to formation of biofilm and dispersal of cells from the matrix of biofilm

Table 1 Biofilms related to devices

S. No	Devices	Reference
1	Orthopedic alloplastic devices	[11, 12]
2	Indwelling urinary catheters or urethral stents	[13, 14]
3	Intravenous catheters	[15]
4	Vascular prostheses	[16]
5	Cardiac pacemakers and prosthetic heart valves	[13, 17, 18]
6	Endotracheal tubes	[19]
7	Cerebrospinal fluid shunts	[20]
8	Peritoneal dialysis catheters	[21]
9	Biliary tract stents	[22]
10	Intrauterine devices	[23, 24]
11	Contact lenses	[25]
12	Tissue fillers	[26, 27]
13	Dentures	[28]

structures". The 3-dimensional structures filled with cells in groups as the number of bacteria increase. These structures form ducts between the groups allowing transport of water and nutrients; and removal of waste.

5. The fifth step includes dispersal of cells from the matrix of biofilm. Thereby biofilms display crucial disbanding mechanisms and release cells which are circulated to further sites. Fluctuation in oxygen, nutrient availability, other stress-generating situations, and toxic products are the factors persuading dispersal of biofilm.

Generally, biofilm is formed on medical devices; or in the tissue of the host; or on fresh fruits and vegetables; or on agricultural products used for food consumption (Tables 1, 2, and 3). Biofilm generally provides a strong platform for interaction

Table 2 Biofilms related to tissues

S. No	Disease	Pathogen	Tissue	Reference
1	Cystic fibrosis	*P. aeruginosa*	Lungs	[29]
2	Chronic obstructive pulmonary diseases	*P. aeruginosa*	Lungs	[30]
3	Tuberculosis	*Mycobacterium tuberculosis*	Lungs	[31]
4	Chronic wound infections	Invasive infectious agents like *Staphylococcus aureus*	Tissue with wounds	[32]
5	Chronic otitis media	*S. pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* and *S. aureus*	Ear	[33]
6	Chronic sinusitis	Viral or bacterial infection	Nasal passages (sinuses)	[34]

Table 3 Biofilms on fresh fruits, vegetables or agricultural products used for food consumption

S. No	Pathogen	Fruit/vegetable	Reference
1	*S. enterica serovar Saphra*	Cantaloupe melons	[35, 36]
2	*E. coli*	Apples	[37–39]
3	*E. coli* O157:H7	Lettuce and spinach	[40]
4	*Shigella sonnei*	Fresh parsley	[40]
5	*Shigella boydii*	Bean salad	[41]
6	*Shigella*	Parsley plants	[40]

and communication among the individuals present in the colony and also withstand antibiotics, immune defenses, disinfectants, nutritional changes, high temperatures etc., In this section, a detailed discussion on how biofilms tolerate antibiotics and evade immune system are given below.

Antibiotics Resistance

Though modification of the antibiotic molecule, reducing drug permeability, and modification of target binding sites are the known mechanisms for antibiotic resistance; formation of biofilm is another mechanism for antibiotic resistance. Biofilms when exposed to antibiotics show several phenotypic changes and alteration in signaling pathways. Changes in biofilm structure, cell morphology, growth rate, induction of extracellular DNA (e-DNA) and bacterial membrane vesicles (BMVs) are the phenotypic changes reported when exposed to antibiotic. The signaling mechanisms like Cyclic dimeric guanosine monophosphate (c-di-GMP) signaling, oxidative stress response, quorum sensing, SOS response and starvation stress response involved in the biofilm. These signaling mechanisms are altered when exposed to antibiotics (Fig. 3).

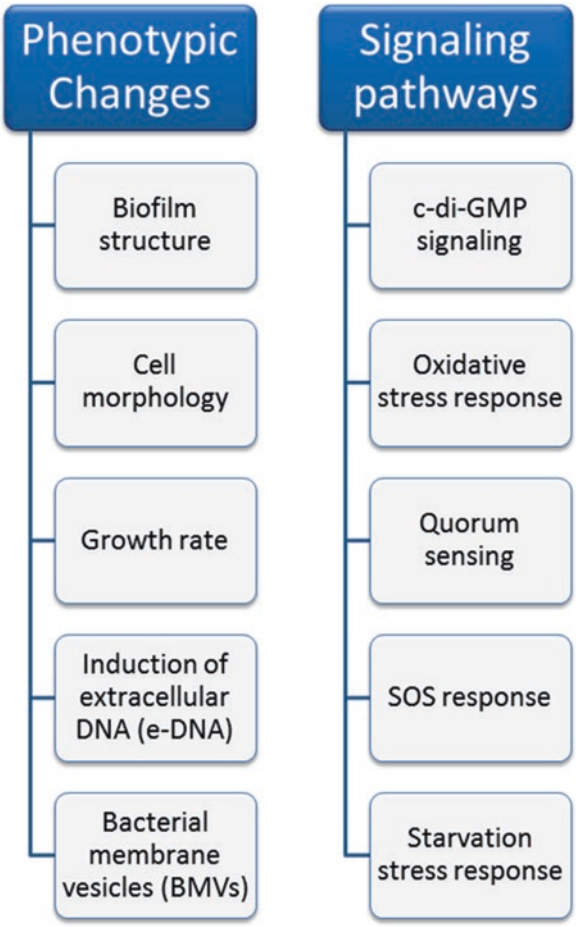

Fig. 3 Phenotypic changes and alteration in signaling pathways in a biofilm providing resistance to antibiotics

Alterations in bacterial cell morphology were reported in *Klebsiella pneumonia*, *E. coli*, and *Streptococcus mutans* when exposed to sub-lethal concentration of antibiotics and other compounds. *K. pneumonia* when exposed to carbapenem, imipenem, meropenem and doripenem; morphological alterations of *K. pneumonia* cell was observed. Round cells of *K. pneumonia* when exposed to carbapenem modified there size and shape through RpoS-dependent regulation [42]. When *K. pneumonia* was exposed to imipenem for 24 h significant cell shortening was observed, whereas significant cell lengthening was observed when *K. pneumonia* was exposed to meropenem and doripenem. *E. coli* when exposed to piperacillin or a combination of piperacillin and tazobactam, changed its morphology to filamentous form [43, 44]. *S. mutans* when exposed to xanthorrhizol (extract of *Curcuma* xanthorrhiza), changed its surface and contour of cell wall and membrane [45]. Thus, bacterial cells when exposed to antibiotics alter the shape with a possible connection to antibiotic response.

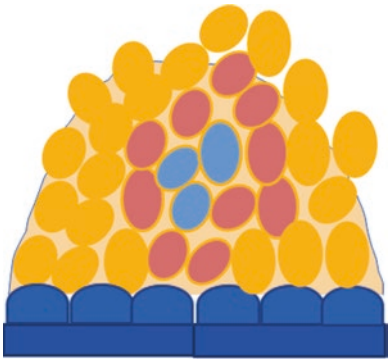

Fig. 4 Surface layer cells, middle layer cells and deepest layer cells of the biofilm

The change in the growth rate of cells in a biofilm when exposed to antibiotic is another notable feature. Cells in the biofilm can typically be classified as surface layer cells, middle layer cells and deepest layer cells (Fig. 4). Cells present at the surface, middle, and deepest of the biofilm are metabolically active, non-growing but alive, and dormant respectively. Cell surface cells of the biofilm are sensitive to antimicrobials, whereas middle layer cells acquire tolerance to some agents, and inner layer cells are tolerant to antimicrobial agents. The lowered metabolic activities of the middle layer cells; and zero metabolic activities in the inner cell layers of the biofilm are responsible for the resistance to antibiotics. Thus, biofilms when exposed to antibiotics exhibit reduced growth leading to antibiotics resistance.

eDNA is known for formation, sustaining and maintenance of biofilm [46–48]. The sources of eDNA can be external to the biofilm or can be one of the cells lysed in the polymicrobial species biofilm. This eDNA via horizontal gene transfer is absorbed by other competent cells of the biofilm leading to antibiotic resistance [49]. Further, eDNA binds to antibiotics [50, 51], or activates genes concerned with resistance leading to antibiotic resistance. Thus, role of eDNA in antibiotic resistance by various mechanisms is a fact.

BMVs have multiple roles like guarding the microbial cells from antibiotic stress, promoting biofilm formation; facilitating adherence; material delivery; retaining integrity of the cell membrane; and competing for growth factors. BMVs provide resistance to antibiotics such as polymyxin B, colistin, and melittin [52, 53]. In an experiment with *P. aeruginosa* biofilm, drug-binding proteins were identified in the BMVs; and this signifys a likely drug-sequestering consequence by content in BMVs [54, 55]. In another study, BMVs of *S. aureus* carrying protein lactamase showed resistance to ampicillin [56]. The other possible role of BMVs is acting as an interspecies communication system to transfer DNA, proteins, RNA, and toxins [57]. Another role of BMVs is to promote biofilm formation, where addition of BMV to *Helicobacter* planktonic culture initiated the formation of *Helicobacter* biofilm. Thus, vesicles allow microbial cells in the biofilms to thrive against antibiotics in addition to other roles.

Starvation of the middle and inner layer cells of the biofilm is known and biofilm induces response to this starvation. These starvation responses are known to protect

bacterial biofilm when exposed to antibiotics [58, 59]. Nguyen et al. [60] reported antibiotic resistance when nutrients are limited to biofilms and bacteria. The plausible explanation is that starvation response signal like RelA-SpoT mediates decrease in prooxidants and increase in antioxidants to protect biofilm from antibiotic. Thus, starvation responses have the ability to defend the biofilm from antibiotics.

SOS responses generated by bacterial cells in the biofilm were known to provide tolerance to antibiotics. DNA damaging agents or antibiotics increase the mutation rate leading to a "hypermutator phenotype". Hypermutators have an advantage in colonizing the host as well as in exhibiting virulence [61]. Hypermutator phenotypes also hinder recombination and generate SOS response. SOS response activates DNA repair and facilitates recombination, and as a result DNA repair mutants can acquire antibiotic resistance genes [62]. In *P. aeruginosa* MMR deficient mutators were to able adjust as a biofilm community, whereas planktonic cells were not able to adjust. Fluoroquinolones and ciprofloxacin induced SOS response in pathogens resulting in bacterial persistence [63, 64]. Though, the clear connection between SOS response and antibiotic resistance is not established; the above evidences are in favor of SOS response and antibiotic resistance.

Oxidative stress responses generated by bacterial cells in the biofilm were known to provide tolerance to antibiotics. Oxidative stress induces double-strand breaks in bacterial DNA and as consequence bacteria activates the DNA repair mechanism. The DNA repair mechanism facilitates recombination allowing the mutants to acquire antibiotic resistance genes [62]. Boles and Singh [65] revealed that oxidative stress induce mutations in the bacteria cells of biofilm leading to variants. And identified that activation of DNA repair have a tendency to increase antibiotic resistance in biofilms against gentamicin [65, 66]. Thus, oxidative stress responses generated by bacterial cells in the biofilm provide antibiotic resistance.

c-di-GMP signaling by bacterial cells in the biofilm bestows tolerance to antibiotics. c-di-GMP is the secondary messenger involved in regulating the formation of biofilm and persister cell [67]. Hoffman et al. [68] proved that signaling of c-di-GMP in *E. coli* and *P. aeruginosa* improved biofilm mass in the presence of antibiotic tobramycin. Thus, c-di-GMP signaling improves tolerance to antibiotics.

Quorum sensing (QS) facilitates antibiotics resistance to the bacterial cells in the biofilm [69]. QS signaling provided resistance in *P. aeruginosa* for antibiotics ceftazidime and colistin. LasR mutants of *P. aeruginosa* acquired beta-lactamase activity and showed resistance to ceftazidime. QS in *P. aeruginosa* is regulated and colistin-tolerant cells migrate to the upper layer of the biofilm using "type IV pili-dependent motility" [70]. This allows the biofilm to grow in size and helps the pathogen to persist even in the presence of antibiotics. Thus, QS signaling also have an important role in contributing resistance to antibiotics.

Immune Defenses

Biofilms use a number of strategies to withstand host defense mechanisms. Literature reports key strategies used by biofilms to evade host immune system. The strategies are (1) leukocytes penetration into the biofilm is limited, (2) QS increases resistance to leukocytes, (3) leukocytes adeptness to engulf biofilm decreases, (4) activity of leukocyte is suppressed, (5) genetic switches of biofilms, [71] (6) dysfunctioning or destroying macrophages, and (7) biofilm shields (Fig. 5). In this section we discuss in detail the immune evading mechanisms used by biofilms of *S. aureus* and *P. aeruginosa*.

Mechanisms used by biofilms of *S. aureus* to evade immune system are evading recognition of TLR2 and TLR9 [72]; skewing the immune response; dysfunctioning of macrophage; and impaired phagocytosis of leukocytes [73]. Though, leukocytes penetrate into biofilm they were not able to kill bacteria in biofilm due to impaired phagocytosis of leukocytes [73]. Although, macrophages were able to engulf immature or disrupted biofilm of *S. aureus* [72]; macrophages were not capable of engulf a mature biofilm. At the same time dysfunctioning of macrophages is due to release of products by biofilm. Therefore, the above mechanisms are used by *S. aureus* to evade the host immune system.

The alternative mechanisms used by biofilms to evade immune system are by developing protective layers around biofilms. *P. aeruginosa* biofilms to evade immune system have protective layers like exopolysaccharide alginate and rhamnolipids. The exopolysaccharide alginate in *P. aeruginosa* biofilms shields bacteria from leukocyte phagocytosis, whereas rhamnolipids form a "biofilm shield" and

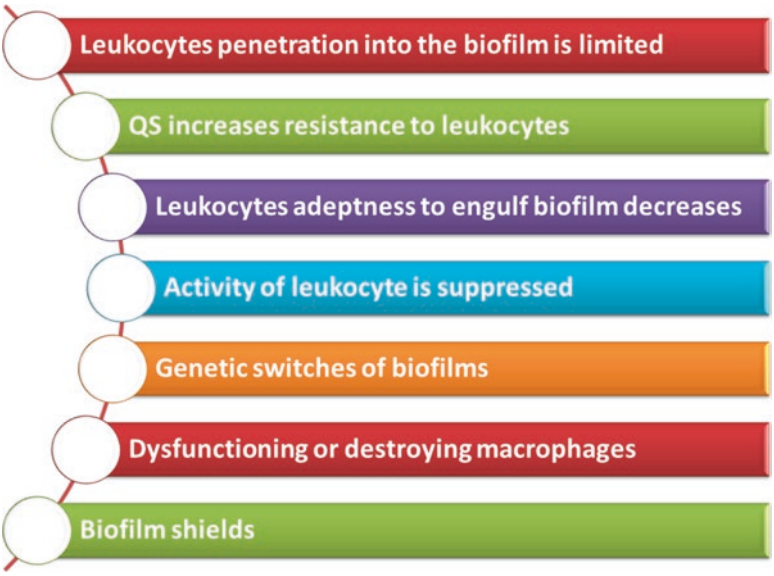

Fig. 5 Immunoevasion strategies used by biofilms to withstand host defense mechanisms

prevent the bactericidal activity of polymorphonuclear leukocytes (PMNs) [74]. Thus, biofilm shields prevent immune action against *P. aeruginosa* and protect it from host immunity.

Conclusion

Bacteria live in communities to provide a platform for interaction and communication among the individuals and also to withstand antibiotics, disinfectants, high temperatures, immune defenses, nutritional changes etc. Changes in biofilm structure, cell morphology, growth rate, induction of e-DNA, BMVs; and altered signalling mechanisms like c-di-GMP signaling, oxidative stress response, quorum sensing, SOS response and starvation stress response provide resistance to the biofilm. Limited leukocytes penetration into the biofilm, increased resistance to leukocytes, decreased leukocytes adeptness to engulf biofilm, suppression of leukocyte activity, genetic switches of biofilms, dysfunctioning or destroying macrophages, and biofilm shields form the important strategies of the biofilm to evade host immune system.

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Authors Contribution CS and NNR initiated the review, participated in writing and revised the manuscript.

Conflict of Interest Statement The authors declare that there is no potential conflict of interest.

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