Transition of solitary to biofilm community life style in bacteria: a survival strategy with division of labour

SUBHADEEP CHATTERJEE*1, BISWAJIT SAMAL1,2, PRASHANTEE SINGH1,2, BINOD B. PRADHAN1 and RAJ K. VERMA1,2

1Centre for DNA Fingerprinting and Diagnostics, Uppal, Hyderabad and
2Graduate studies, Manipal Academy of Higher Education, Manipal, India

ABSTRACT Multicellularity is associated with higher eukaryotes having an organized division of labour and a coordinated action of different organs composed of multiple cell types. This division of different cell types and organizations to form a multicellular structure by developmental programming is a key to the multitasking of complex traits that enable higher eukaryotes to cope with fluctuating environmental conditions. Microbes such as bacteria, on the other hand, are unicellular and have flourished in diverse environmental conditions for a much longer time than eukaryotes in evolutionary history. In this review, we will focus on different strategies and functions exhibited by microbes that enable them to adapt to changes in lifestyle associated with transitioning from a unicellular solitary state to a complex community architecture known as biofilm. We will also discuss various environmental stimuli and signaling processes which bacteria utilize to coordinate their social traits and enable themselves to form complex multicellular-like biofilm structures, and the division of labour operative within such communities driving their diverse social traits. We will also discuss here recent studies from our laboratory using a plant-associated bacterial pathogen as a model organism to elucidate the mechanism of bacterial cell-cell communication and the transition of a bacterial community to a multicellular-like structure driven by the complex regulation of traits influenced by cell density, as well as environmental sensing such as chemotaxis and nutrient availability. These studies are shedding important insights into bacterial developmental transitions and will help us to understand community cooperation and conflict using bacterial cell-cell communication as a model system.

KEY WORDS: quorum sensing, biofilm, adhesion, extracellular polysaccharide, heterogeneity, cheating, bet-hedging, fitness

Introduction

Bacteria have been generally considered as unicellular, and therefore solitary organisms that are associated with given environments such as soil, plants, animals and water. They have served as excellent model systems to study fundamental biological processes such as replication, transcription, translation and basic physiology. Most bacterial studies are done in broth cultures under laboratory conditions of presumably homogeneous cultures with uniformly dispersed cells. The concept that bacteria can modulate their behaviour at high cell density came with the study of bioluminescence in the Gram-negative marine bacterium known as Vibrio fischeri, which forms a symbiotic association with some marine animals, such as Euprymna scolopes (Nealson et al., 1970; Nealson 1977). The bacteria inhabit the light organs of Euprymna and emit light due to the activity of luciferase enzymes. Researchers observed that production of light by V. fischeri was density dependent as the bacterium at low cell density in broth culture was unable to produce bioluminescence. However, when the culture density increased to very high concentrations in broth culture (similar to the cell density attained in symbiotic association), the bacterial cells exhibited bioluminescence. The phenomenon of a coordinated response at a particular high cell density was coined as “quorum sensing (QS)” (Engbrecht et al., 1983; Fuqua et al., 1994). With further studies of bacterial behaviour and understanding the mechanism of quorum sensing in the last decade, the QS mediated communication system that is utilized by the unicellular organism to perform and...
coordinate complex tasks similar to multicellular organisms has been elucidated (Miller and Bassler, 2001; Ng and Bassler, 2009). This also opened the field of sociomicrobiology (the connection between quorum sensing and biofilm formation) and researchers started looking at microbial communities in natural habitats or under laboratory conditions which mimic the natural environment (Parsek and Greenberg, 2005; Turovskiy et al., 2007). The study of microbes on different surfaces in nature revealed that bacteria often formed highly organized structures known as biofilms. Microbial biofilms consist of either single or multiple layers of bacterial cells that adhere to various surfaces and form robust structures that provide protection from different environmental stresses such as antimicrobial compounds, low nutrient availability and changes in the temperature and pH of the surrounding environment (O’Toole et al., 2000; Palková, 2004; Fleming et al., 2007; Nadell et al., 2009; López et al., 2010). In this review, we will discuss the mechanism of formation of biofilms in bacteria and how they utilize these multicellular-like structures to perform complex social tasks. We will also discuss the mechanisms of the reverse process of biofilm dispersal which is also highly dynamic and reversible in nature. Finally, we will elucidate the complexity of the QS response and biofilm formation that often involves the emergence of cheaters and the interplay of coordinated and heterogeneous social responses that generates phenotypic diversity in an otherwise genetically identical bacterial population or community.

**Bacterial quorum sensing enables social communication among individual members within populations of solitary cells**

Quorum sensing is a process by which bacteria communicate with each other via production and sensing of multiple types of secreted signaling molecules (Fig. 1). Several plant and animal-associated bacteria, including those inhabiting diverse environments exhibit quorum sensing (Fuqua et al., 1994; Parsek and Greenberg, 2005; Turovskiy et al., 2007; He and Zhang, 2008; Ng and Bassler, 2009). Diverse classes of quorum sensing signaling molecules have now been characterized that are involved in both intra-species as well as inter-species communication (Fuqua et al., 2001; Turovskiy et al., 2007; He and Zhang, 2008; Ng and Bassler, 2009). The most common and well studied quorum sensing system is that conferred by acyl-homoserine lactone (AHL) mediated signaling in several Gram-negative bacteria such as *Vibrio fischeri*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens* and *Erwinia carotovora* (Fuqua et al., 2001; Miller and Bassler, 2001; Ng and Bassler, 2009). The AHL mediated QS process has been studied as a model for the mechanism of production and perception of QS signals. In general, this involves an AHL synthase such as AhlI or LuxI that is involved in the production of QS signal. At low cell density, only a basal level of expression of the signal synthase is operative, leading to low levels of AHL signal accumulation in or near the cell. As cell density increases, signal production increases and this is either diffused or transported rapidly out of the cell. When the concentration builds up above a threshold level, the signal molecule (ligand) binds to the transcriptional regulator such as AhlR or LuxR (receptor), that thereby gets activated and binds to DNA and typically acts as a regulator of gene expression (Fig. 2) (Fuqua et al., 2001; Miller and Bassler, 2001; Ng and Bassler, 2009). In addition to AHL-mediated quorum sensing signaling, bacteria also exhibit QS mediated by other diverse signaling molecules such as furanosyl borate diester, fatty acid derivatives (3-Hydroxypalmitic acid methyl ester, diffusible signal factor) and cyclic peptide (thiolactone) (in Gram-positive bacteria) (Parsek and Greenberg, 2005; Turovskiy et al., 2007; He and Zhang, 2008; Ng and Bassler, 2009). In several bacteria, it has been shown that QS-mediated coordinated responses occur via synchronized regulation of gene expression leading to harmonious production and secretion of various extracellular products, often known as ‘public goods’, that are beneficial to the population as a whole (Greenberg, 1998; Palková, 2004; Darch et al., 2012; Pai et al., 2012). Several traits have been shown to be regulated by QS such as: (i) the production of extracellular polysaccharides, adhesions or attachment proteins that often play a role in biofilm formation, (ii) extracellular cell-wall hydrolyzing enzymes, (iii) iron chelating compounds known as siderophores, (v) virulence factors that are utilized for host colonization and infection, and (v) functions required for directional motility (Fuqua et al., 2001; Parsek and Greenberg, 2005; Williams et al., 2007; Ng and Bassler, 2009; Long et al., 2009; Darch et al., 2012; Pai et al., 2012).

Our laboratory uses the *Xanthomonas* group of phytopathogens as model organisms to study quorum sensing, cooperation and social behaviour in bacterial community. This review will focus on *Xanthomonas* QS biology and will elaborate on how *Xanthomonas* coordinates the expression of multiple social traits via coordination of cell-cell signaling and environmental sensing to achieve multicellular-like social tasks. The *Xanthomonas* group are phytopathogens that cause disease in several economically important crop plants such as rice, tomato, cabbage, citrus etc (Niño-Liu et al., 2006; Büttner and Bonas, 2010; Mansfield et al., 2012).
Among Xanthomonads, Xanthomonas oryzae pv. oryzae (Xoo) and Xanthomonas oryzae pv. oryzicola (Xoc) are important rice pathogens (Niño-Liu et al., 2006; Mansfield et al., 2012). In the early 2000s, while working on isolation of virulence-deficient mutants of Xanthomonas by genetic screening, at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India, we isolated a genetic mutant defective in a gene in a gene cluster known as “regulation of pathogenicity factor (rpf)” (Chatterjee and Sonti, 2002). Our group, along with scientists at the John Innes Centre in Norwich, UK independently showed that the rpfF gene is involved in the production of a diffusible signal factor that is involved in the virulence of Xanthomonas (Fig. 2) (Barber et al., 1997; Chatterjee and Sonti, 2002). Further characterization of the signaling molecule and characterization of various mutants revealed that Xanthomonas produces an unusual fatty acid QS signal molecule (cis-11-methyl-2-dodecenolic acid) known as “diffusible signal factor (DSF)” (He and Zhang, 2008; Deng et al., 2011; Ryan and Dow, 2011). With the appreciation of the novelty of the DSF family of signaling molecule, it has now increasingly become evident that several groups of bacteria, such as members of the genus Xanthomonas, Burkholderia, Xylella, Stenotrophomonas all communicate using the DSF family of signaling molecules (He and Zhang, 2008; Chatterjee et al., 2008a,b; Deng et al., 2011; Ryan and Dow, 2011). In the rice pathogen Xanthomonas oryzae, DSF is involved in the positive regulation of biofilm formation and adhesin production (required for attachment), and in the negative regulation of motility and production of cell wall hydrolyzing enzymes (Rai et al., 2012; Rai et al., 2015). Characterization of the DSF-mediated signal transduction process in the Xanthomonas group of phytopathogens revealed that the regulation of virulence-associated functions by DSF mediated signaling is a complex process involving multiple sensors and response regulators that act in parallel and with complex regulatory interactions with each other (Chatterjee et al., 2008a,b; Rai et al., 2015). In Xanthomonas oryzae, DSF not only promotes the transition of a solitary to a community lifestyle or biofilm, but it is also involved in the regulation of iron uptake, chemotaxis, and motility in a density-dependent fashion (Chatterjee and Sonti, 2002; Rai et al., 2012; Rai et al., 2015). Particularly when in a biofilm, the availability of scarce nutrients such as iron is limiting, the pathogen has to acquire and store iron from diverse environmental or host iron sources. QS coordinates the expression of multiple iron sensing regulators to achieve iron homeostasis in a cell density-dependent fashion that enables optimum growth and survival of the cells at high cell density and within biofilms that experience nutrient scarcity (Pandey et al., 2016; Pandey et al., 2017; Pandey et al., 2018).

Biofilms coordinated by quorum sensing (QS) represent a microbial multicellular transition from a solitary lifestyle

QS regulates biofilm formation in many bacteria. Biofilm provides a stable, safe structure for the survival of bacteria wherein they can perform multiple tasks such as nutrient acquisition, defense against host antimicrobial compounds, and stress tolerance (Costerton et al., 1994; O’Toole et al., 2000; Parsek and Greenberg, 2005; Danhorn and Fuqua, 2007; López et al., 2010). Xanthomonas species form multi-layered biofilms only at high cell density both under laboratory conditions as well as inside the host plant (Fig. 3) (Rai et al., 2012; Rai et al., 2015; Pandey et al., 2016). Us-

Fig. 2. A model depicting the basic mechanism of quorum sensing signal transduction. (A) Diffusible signaling molecule (DSF) is made by the enzymatic action of QS signal synthase. The DSF signal concentration increases inside the cell and diffuses out into the extracellular space. As the cell number increases the production of DSF signal increases and the concentration of DSF signal builds up above a threshold limit which is then detected by either membrane bound or cytoplasmic receptor. Binding of the DSF signal to the sensor leads to conformational change and induces auto phosphorylation of sensor kinase. The sensor kinase interacts with response regulator by phosphate-transfer and the response regulator may bind to target promoters to induce gene expression. In case of Xanthomonas QS system, the RpfF (DSF synthase) makes DSF signaling molecule which binds with the sensor RpfC and other sensors which activates the response regulator RpfG. RpfG is a cyclic Di-GMP hydrolyzing protein which degrades cyclic Di-GMP regulates gene expression and modulates social behaviour. (B) Representative picture of a typical Xanthomonas colony on a laboratory medium. The bacteria produce extracellular polysaccharide and DSF signaling molecule in a density dependent fashion. Production of signal and regulation of virulence associated function are important for causing disease on rice plant (Shown from left to right are the leaves of a rice plant that are infected with the wild type and the DSF-deficient mutant strains of Xanthomonas oryzae pv. oryzae respectively).
ing both confocal laser scanning microscopy in conjunction with probes for the study of biofilms revealed that the formation of biofilms in *Xanthomonas* is a stage-specific process that requires both cell-cell attachment as well as cell to surface attachment (Fig. 3; Fig. 4) (Das *et al.*, 2009; Darsonval *et al.*, 2009; Gottig *et al.*, 2009; Rai *et al.*, 2012; Rai *et al.*, 2015). Since the infection process in the host involves both active directional motility to enter the host via specific portals followed by the migration and colonization the interior host tissue, such as xylem vessels, synthesis of biofilm-forming factors is coordinated in a density-dependent fashion (Rai *et al.*, 2012; Verma *et al.*, 2018). At low cell densities when DSF levels are low, the lack of a QS signal promotes chemotaxis or directional motility, enabling *X. oryzae* to enter the rice xylem vessel through small openings on the leaf surface known as hydathodes. Once inside the hydathodes, the pathogen spreads and forms microcolonies inside the xylem vessels, and different sets of attachment proteins known as “adhesins” (that are induced by both QS and environmental condition-dependent manner) are produced. The in *vitro* environmental conditions for such production mimic conditions inside the host (Fig. 3; Fig. 4) (Pradhan *et al.*, 2012; Rai *et al.*, 2012). It is logical to think that if all attachment proteins are made simultaneously, it will interfere with the systemic spread, since the colonization process involves entry, migration, spread and disease progression (Fig. 4). We have characterized several adhesins (attachment proteins) in *Xanthomonas* that are required for virulence, biofilm formation and attachment such as XadA, YapH, and XadM. Study of the dynamics of biofilm formation, and the expression patterns of these virulence factors both inside the host plant and under host mimicking *in vitro* conditions revealed that the adhesins such as XadM are expressed in a density-dependent fashion within the bacterial community (Fig. 4) (Ray *et al.*, 2002; Das *et al.*, 2009; Pradhan *et al.*, 2012; Pandey *et al.*, 2016). In addition to various adhesins, *Xanthomonas oryzae* also produces extracellular polysaccharide (EPS) that is composed of xanthan (a complex polysaccharide) and glucan carbohydrate. EPS provides protection to the bacteria against harmful plant defense molecules, as well as acts as an extracellular matrix (Flemming *et al.*, 2007; Kakkar *et al.*, 2015). Attachment and biofilm formation studies revealed that (i) the production of EPS is also regulated by DSF-mediated QS, and (ii) EPS, together with surface-exposed adhesions such as XadM is involved in the process of attachment of bacterial cells to various surfaces (Pradhan *et al.*, 2012; Kakkar *et al.*, 2015; Rai *et al.*, 2015). EPS also plays a role in the suppression of plant defense responses that protect bacterial cells during host colonization (Kakkar *et al.*, 2015).

Interestingly, one of the components of EPS is a cyclic glucan that is both secreted and cell associated. Recent studies indicated that glucan is involved in iron homeostasis as it sequesters iron from the environment and also suppress harmful plant defense responses when bacteria are colonizing the host plant xylem vessels (Kakkar *et al.*, 2015; Jawvadi *et al.*, 2018). In *Xanthomonas oryzae*, DSF-mediated signaling negatively regulates chemotaxis, thus contributing to biofilm stability (Rai *et al.*, 2012; Rai *et al.*, 2015). In *Xylella fastidiosa* (an important insect transmitted plant pathogen and a close relative of *Xanthomonas*), it has been shown that mutants that exhibit hyper-motility are deficient in biofilm formation (Chatterjee *et al.*, 2008a,b). Recent study to understand the role of the chemotaxis system in *Xanthomonas oryzae* revealed that chemotaxis mutants that are deficient in directional motility, form biofilms even better than the wild type strain (Verma *et al.*, 2018). Study of the regulation of biofilm formation by DSF-mediated signaling (influenced by chemotaxis-specific nutrient availability) revealed that high cell density triggers biofilm formation, and there is a fine tuning of motility and chemotaxis that coordinates the transition of solitary to biofilm lifestyle in *Xanthomonas* (Rai *et al.*, 2015; Pandey *et al.*, 2016). Biofilms also enable bacteria to survive under nutrient-limiting conditions, wherein many essential nutrients such as iron, sugars are limited in amount to support microbial growth at high cell density (Cassat and Skaar, 2013). In addition to cell-cell signaling mediated by DSF, the regulation of iron uptake and metabolism also cross talks with QS signaling in *Xanthomonas* (Chatterjee...
Coordination of social trait in bacteria: unicellular to multitasking transition

We have isolated a novel iron binding transcription factor named XibR (Xanthomonas iron binding Regulator) in Xanthomonas campestris pv. campestris that phenocopies many of the traits exhibited by QS regulatory mutants. These results indicate that bacteria employ complex sensing and signal transduction machinery to perform social task, such as biofilm formation that involves multiple regulators and sensors including those involved in sensing cell density and environmental conditions (Pandey et al., 2016).

Division of labour in bacterial social behaviour: non genetic phenotypic heterogeneity in QS response

It has been argued that multicellular-like behaviour induced by bacterial QS in high cell densities and the associated uniform response is beneficial to the cells. However, such a system can also exhibit a division of labour (Costa et al., 2006; Diggle et al., 2007; Sandoz et al., 2007; Xavier and Foster, 2007; Nadell et al., 2009). Evolutionary theory predicts that maintaining phenotypic heterogeneity in social traits can lead to bet-hedging survival strategies that can force in a division of labour within the bacterial community (Gardner et al., 2007; Davidson and Surette, 2008; Jacob and Schultz, 2010). In bacterial biofilms, the cells attach to form a multicellular-like structure that must be dynamic in nature in terms of phenotypic plasticity. Furthermore, the cells need to be free to migrate in search of new environments that might provide the needed nutrients (Costa et al., 2006). For that reason, phenotypic heterogeneity has been reported in diverse social processes such as chemotaxis-driven motility (Spudich and Koshland, 1976), persistence in the presence of antibiotics (Balaban et al., 2004), bi-stability of gene expression (Novick and Weiner, 1957) and induction of natural competence in Bacillus (Süel et al., 2006), that are often associated with biofilms. We have used Xanthomonas and Pseudomonas as model systems to address heterogeneity in QS response in microbial populations. Using single cell studies of QS dynamics via fluorescence activated cell sorting, live cell imaging, and competition experiments (with wild type and various QS mutants), we showed that bacteria exhibit reversible phenotypic heterogeneity in their QS response. Specifically, QS responding populations maintain a proportion of QS responding and non-responding cells in an approximately 80:20 ratio (Anetzberger et al., 2009; Pradhan and Chatterjee, 2014). Using competition experiments coupled with imaging of cells that formed aggregates from previously solitary cells revealed that the non-responders in a biofilm move away from the biofilm and therefore may contribute to biofilm dispersal and systemic spread (Pradhan and Chatterjee, 2014). In our recent study of Xanthomonas-plant interaction, we have used whole cell dual-biosensors that can track both bacterial localization and quorum sensing response in vivo and have studied the dynamics of heterogeneity in the QS response inside the host plant (Samal and Chatterjee, 2019). Our study indicates that division of labour consisting of QS-responsive and non-responsive populations gives stability to the bacterial community towards the initial successful
disease establishment, and the reversal of biofilm to planktonic cells contributes to the systemic spread of the disease within the host (Samal and Chatterjee, 2019). We proposed that this division of labour provides survival fitness to such a host-associated complex bacterial community under adverse conditions during its parasitic life cycle within the host.

Concluding remarks

Bacteria are increasingly being used as model systems to study social life. Quorum sensing acts as a signal for the transition from a solitary (planktonic) to a biofilm (multicellular) organization and vice versa within a bacterial community. It therefore can be used as a model system to study the evolutionary transition from unicellular life form to complex multicellular architectures. Since bacterial systems are amenable to genetic manipulation, and exhibit short generation times and is thus subject to rapid evolutionary changes, it can serve as good system to ask many questions such as: Do bacteria as a community have long or short term memory or program that enables phase-specific morphological and/or physiological adaptation? What triggers heterogeneity in performing social tasks? How are multiple signaling pathways coordinated at a cellular level with environmental conditions to mediate appropriate outcomes for the community? What other phenotypic or genetic switches are involved in QS and environmental sensing to coordinate the transition from unicellular life to biofilm cells? The LuxR-LuxI family of cell density-responsive transcriptional regulators of biofilm cells''.

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