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Decoding the mystery of how bacteria "talk": Among Gram-negative microorganisms

Tan Wen-Si[1,2]*, Jodi Woan-Fei Law[3], Vengadesh Letchumanan[3], Kok-Gan Chan[4,5]

[1]Illumina Singapore Pte Ltd, Woodlands Industrial Park E1, Singapore
[2]Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
[3]Novel Bacteria and Drug Discovery (NBDD) Research Group, Microbiome and Bioresource Research Strength Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway, Selangor Darul Ehsan, Malaysia.
[4]Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[5]International Genome Centre, Jiangsu University, Zhenjiang 212013, PR China

Abstract : To date, microbial diversity is still the least well understood component of biodiversity. Bacteria are the most abundant microorganisms where most species are often found ubiquitous. Microorganisms such as bacteria are diverse in their impacts such as in spreading of infectious diseases or play a valuable role in biotechnological purposes. Hence, it is interesting to gain a look upon the ways where bacteria regulate their daily processes in the environment. Bacteria communicate with each other through extracellular signalling molecules or also known as autoinducers (AIs) that are produced, detected and show response. This process is termed as quorum sensing (QS) which indicates that bacteria do communicate in order to perform various physiological activities. QS enable bacteria to have the advantages that are unattainable as individual bacterial cell. This review emphases on the characteristics of quorum sensing (QS) and its benefits in understanding different kind of bacterial QS-dependent activities. This fundamental insight from QS system will enable us to manage bacterial activities by targeting their communication circuit.

Keywords: Communication; quorum-sensing; signalling molecules; *N*-acylhomoserine lactone

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***Correspondence:** Wen-Si Tan, tmarilyn36@gmail.com. Illumina Singapore Pte Ltd, Woodlands Industrial Park, 757716 Singapore.

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Introduction

Bacteria are always recognized to exist as isolated and anti-social lives. They can be present as free-living, associated with decaying material or attached to surfaces of rocks, stones, sand grains or aquatic animals as part of biofilm[1–8]. Researches over the past decades had disclosed that bacteria are able to communicate via chemical signalling system in order to communicate within species had led to the realization that bacteria are able to behave in a much more complex patterns[9]. Specifically, bacteria release signaling molecules which are also known as autoinducers; then detect and respond to the accumulation of those molecules[10]. Bacterial communication or coiled as "quorum sensing (QS)" by Fuqua and colleagues to describe the process where bacterial communication achieved depending on the density of bacterial cell population where it is synchronize with the concentration of signal molecules in the extracellular environment[11,12]. In a simplest guise, QS benefits bacteria as a community rather than as an individual. This review emphases on the characteristics of quorum sensing (QS) and its benefits in understanding different kind of bacterial QS-dependent activities.

Quorum Sensing (QS)

The paradigm shift of understanding the ability of bacteria in conducting complex patterns of co-operative behaviors had lead us into translating QS into four essential steps[13]. First, bacterial cell population density and concentration of autoinducers in the external environment increase simultaneously. Once the bacteria sense the threshold, the signal will diffuse into bacterial cells and bind by receptor proteins which hence causing an activation of signal transduction cascade (Figure 1). The autoinducer-receptor protein complex will bind with targeted promoter to induce an auto-regulatory mechanism to either up-regulate or down-regulate certain bacterial phenotypes[13,14].

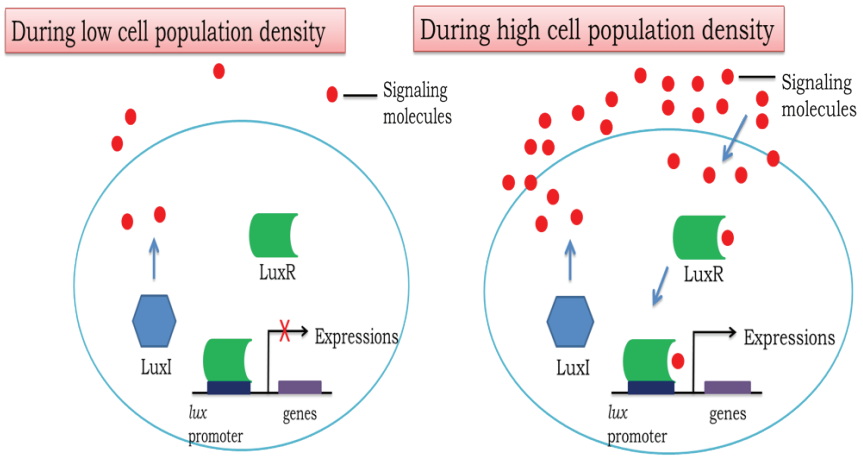

Figure 1. Two QS regulatory components in Gram-negative bacteria: LuxR (transcriptional activator protein) and LuxI (autoinducer synthase). Signal molecules accumulated in a cell-density-dependent manner until a threshold level is reached. No gene expression is driven at low bacterial cell density but at high bacterial cell density, gene expression will be activated.

The evolution of QS was originated from the discovery of controlling luminescence by *Vibrio fischeri*, a bacterium that forms a mutualistic light organ symbiosis with Hawaiian bobtail squid (*Eupryma scolope*)[15–17]. The cell-population density influenced luminescence portrayed by *V. fischeri* have been convincing many scientists to pursue on QS research for the past decade in defining the details of quorum regulation in this bacterium. Experimental analysis on dramatic pattern of light production in the squid portray the findings that autoinducer played a role in induction of luciferase[13,15,16]. A minute amount of *V. fischeri* is harbored in the light organ of the squid during daytime. As bacterial incubation hour increases, the production of signaling molecules at its concentration threshold trigger the luciferase expression. This bioluminescence was needed by the squid to counter eliminate its shadow and avoid predation at night. "Switching off" of luminescence occurred when the squid pumped out the bacteria pool from its light organ, hence bacterial population decreases discouraging the triggering of luciferase production due to insufficient signalling molecules production. The information emerged from *V. fisheri* system serves as a model for further discovery of quorum circuit in other species[17].

Auto-induction of luminescence is now recognized as a QS model with wide applicability in applied research on gene regulation and host association of bacteria. QS indeed not only involved in luminescence but also regulates a vast array of phenotypes. QS facilitates bacteria for adaptability and survival where it also appears as bridge for interaction of several different bacterial species with eukaryotic hosts. However, the life-threatening ability phenotypes that are regulated by QS causes many concerns. Biofilm formation, production of virulence factors and antibiotic resistance in several notorious pathogens such as *Pseudomonas aeruginosa*[18], *Burkholderia cepacia*[19], *Vibrio cholera*[20], *Streptococcus mutans*[21], *Clostridium difficile*[22], and *Erwinia carotovora*[23] were reported to be regulated by QS to attack different hosts ranging from human, plants and aquatic animals. Subsequently, the study into both QS systems and QS signal molecules are essential in various field from biotechnology, pharmaceutical and to agricultural industries, particularly targeting QS for establishment of novel antibacterial measures[13].

QS mechanisms have derived to three tracks; (i) *N*-acylhomoserine lactone (AHL)-based signalling system of Gram-negative bacteria (ii) oligopeptide-based system in Gram-positive bacteria and (iii) shared furanone-based system between Gram-negative and Gram-positive bacteria [24]. QS signal molecules are structurally diverse and ranged according to the needs of the bacterium itself. There are various types of QS signalling molecules discovered and documented such as the AHL, 2-alkyl-4(1H)-quinolones (AHQs), autoinducer peptides (AIP), DSF, palmitate methyl ester (PAME) and diketopiperazines (DKP) but they did share similarity such as small (< 1000Da) organic molecules or peptides with 5-20 amino acids and are highly diffusible[13,25–29]. Of all the signalling molecules documented and reported, AHL received the utmost attention by most research institutions. AHL is basically a group of signalling molecules which employed by most Gram-negative bacteria[14].

Quorom Sensing of Gram-Negative Bacteria

Quorum size is sensed by Gram-negative bacteria through AHLs production that accumulates in their surroundings as the cell population increases. To date, there are more than 100 species of bacteria reported to portray QS properties. These bacteria are found ranging from marine, soil and freshwater environment to plants and animals. Their presence play roles involving pathogens, symbiont, extremophiles and plant-growth promoting bacteria that varies according to its environment[30]. Many of these bacteria are able to produce multiple AHLs and contain more than one AHL synthases[12,13]. Some of the examples of AHL-dependent QS systems with the phenotypes controlled are summarized in Table 1. However, there are still an astronomical number of bacteria that are yet to be discovered and characterized whether they do communicate inter- or intra- species. Many researches should be carried on to understanding QS deeply; it will be the foundation for various areas in biotechnology, pharmaceutical and agricultural industries in such that QS is particularly the target of interest in antimicrobial purposes.

Table 1. Some examples of phenotypes controlled in AHLs-dependent QS systems in Gram-negative bacteria.

Microorganisms	Major AHL(s)	Phenotypes	References
Acinetobacter baumannii	3-hydroxy-C12-HSL	Biofilm, virulence	Niu *et al.* 2008[31]
Aeromonas hydrophila	C4-HSL	Biofilms, exoproteases, motility, virulence	Jahid *et al.* 2013[32]
Aeromonas salmonicida	C4-HSL	Extracellular protease	Swift et al. 1997[33]
Agrobacterium tumefaciens	3-oxo-C8-HSL	Plasmid conjugation	Wang *et al.* 2014[34]
Burkholderia cepacia	C6-HSL, C8-HSL	Biofilms, virulence	Riedel *et al.* 2001[35]
Burkholderia glumae	C8-HSL	Protein secretion, oxalate production, swarming, virulence	Nickzad, *et al.* 2015[36]
Burkholderia pseudomallei	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-oxo-C14-HSL	Virulence, exoproteases	Ulrich *et al.* 2004[37]
Chromobacterium violaceum	C6-HSL	Exoenzymes, cyanide, pigment	McClean *et al.* 1997[38]
Enterobacter agglomerans	3-oxo-C6-HSL	Pectinase expression	Chalupowicz *et al.* 2009[39]
Erwinia carotovora	3-oxo-C6-HSL	Biofilm, virulence	Joe *et al.* 2015[40]
Nitrosomonas europaea	3-oxo-C6-HSL	Emergence from lag phase	Burton et al. 2005[41]
Pantoea stewartii	3-oxo-C6-HSL	Exopolysaccharides	Chug *et al.* 2015[42]
Pseudomonas aeruginosa	C4-HSL, C6-HSL, 3-oxo-C12-HSL	Biofilms, exoenzymes, exotoxins, swarming, virulence	Jakobsen *et al.* 2013[43], Jimenez *et al.* 2012[44], Williams 2007[13]
Rhizobium leguminosarum	C6-HSL, C8-HSL	Rhizome interaction	Lithgow *et al*. 2000[45]
Serratia marcescens	3-oxo-C6-HSL, C6-HSL, C7-HSL, C8-HSL	Biofilm formation, sliding motility and prodigiosin produciton	Horng *et al.* 2002[46], Rice *et al.* 2005[47]
Vibrio fischeri	3-oxo-C6-HSL	Bioluminescence	Ruby *et al.* 1998[17]
Vibrio harveyi	3-hydroxy-C4-HSL	Bioluminescence, virulence	Cao and Meighen, 1989[48], Manefield *et al.* 2000[49]
Yersinia pseudotuberculosis	3-oxo-C6-HSL	Motility and clumping	Atkinson *et al.* 2008[50]

AHL as Signalling Molecules

AHL signalling is highly conserved among the Proteobacteria and received the absolute attention in which intensive studies had been carried out[51]. A homoserine lactone ring with its β- and γ-positions remained unsubstituted, but has an α-position, the *N*-acylated with a fatty acid chain. This ring is highly conserved in all the AHLs documented and characterized (Figure 2)[51]. There are several structural differences that influence the characteristics of an AHL molecule which are (i) acyl side chain range commonly from 4 to 18 carbons where AHL usually carries an even number carbon chain (ii) reduction or oxidation carbonyl or presence of hydroxyl group at third carbon of the acyl side chain (iii) there is also possible for presence of unsaturated AHLs where double bond occurred in the 5 and 7 positions of a long acyl chain (12-14 carbons)[13,52]. The minimum acyl chain to be function as signal molecule is 4 carbons as in existence with lactone ring itself will be hydrolysed at pHs above 2 where 70% of *N*-propionyl-homoserine lactone is hydrolysed at pH 6[52]. The shortest naturally occurring AHLs was produced by several Gram-negative bacteria such as *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. AHL molecules was also found to interact with some other molecules such as cysteine, biotin and fluorescence that bring an effect on the binding affinity of modified AHL to its native AHL-receptor[53]. Besides that, alkali-driven rearrangement reaction can occur in 3-oxo-AHLs that lead to formation of corresponding tetramic acids, iron chelating compounds and antibacterial activities. In short, differences in substitution at predetermined sites on the AHL molecule confer its specificity and affect the functions to the cells

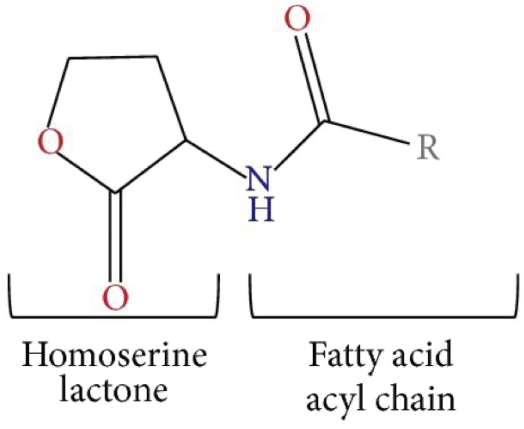

Figure 2. General structure of N-acyl homoserine lactone (AHL). R represents the fatty acid acyl side chains[51].

AHL diffusion and transportation through membrane is highly related with its structural features. AHLs tagged as amphipathic molecules, are wonted to diffuse freely from the internal cell environment to the external cell environment, and vice versa, demonstrated with *V. fischeri* and *E. coli* using a ^{3}H-labelled derivative[54]. However, this demonstration only proved using a short chain AHL, 3-oxo-C6-HSL. The hydrophobic characteristic is influence by the acyl side-chain length, the number of in-saturations and the nature of the C3 substituent (H, O or OH). On the other hand, the rate of AHL diffusion is correlated with the nature of acyl chain. If a long acyl chain AHLs diffuse through cell membrane, it would diffuse slower as compared with a shorter acyl chain AHLs[55]. Commonly, short acyl side chains are usually those AHL with acyl side chain lesser than C8 and can be passively diffused in and out the bacterial cell. It is to facilitate the transport of long acyl side chain AHL, an active transport mechanism. The presence of an active efflux of 3-oxo-C12-HSL in *P. aeruginosa* was evidenced, where the short-chain C4-HSL freely diffuses across the cell membrane[56,57]. Another active efflux is evidenced in *Burkholderia pseudomallei*.

Gram-Negative QS Circuits

The QS system in Gram-negative bacteria consist of signalling molecules (autoinducers), autoinducer synthase (LuxI), Lux-R type regulators and target genes[58]. The signaling circuits composed of LuxI/LuxR appear to be the standard communication mechanism in Gram-negative bacteria, where QS system resembling the canonical. *V. fischeri* circuit have been shown to be the model system in controlling gene expression in over 100 species of Gram-negative bacteria[59]. Commonly, the acylated HSL synthesized by the responsible enzyme LuxI-like protein; a cognate LuxR-like protein that will recognize the HSL autoinducer and activation of transcription downstream target genes occurred subsequently[60]. The mode of action of the LuxI/LuxR pairs is highly conserved across all cases. The coupling of acyl-side chain of a specific acyl-acyl carrier protein (acyl-ACP) from the fatty acid biosynthetic machinery with the homocysteine moiety of *S*-adenosylmethionine (SAM) is produced by LuxI homologue. The coupling process forms a ligated intermediate which then convert to form acyl-HSL and methylthioadenosine (MTA). The LuxR homologue, on the other hand function by binding their substrate, autoinducer and activating the transcription of targeted DNA. The LuxR homologue consist of an amino-terminal region that binds to autoinducers and the C-terminal domains responsible for oligomerization and promoter DNA binding[60–62].

Rather delicate signalling specificity exists in LuxI/LuxR type circuits and specificity inherent stems from a high selectivity of the LuxR proteins to its signalling molecules. As evolutionary goes by, more regulatory complexity has been added to the basic backbone of QS circuit, such as the use of multiple AHL autoinducers and LuxR proteins that can act either parallel or in series[63]. This can be seen in the plant phytopathogen, the gene regulation of *Ralstonia solaacearum* LuxI/LuxR like autoinduction system (SolI/SolR) are regulated by PhcA and also RpoS[64]. Next, the opportunistic pathogen *P. aeruginosa* employ two pairs of LuxI/LuxR homologues (LasI/LasR, RhlI/RhlR) and function in tandem to control the virulence factors production[65]. Recently QscR, identified as third LuxR homologue was found from the complete genome sequence of *P. aeruginosa*. However, there are yet indication of cognate LuxI homologue that could be responsible in producing the autoinducer in which QscR can respond[66]. In fact, level of communication complexity layered to the LuxI/LuxR backbone circuit highly dependent on the nature of bacteria. Apparently the complex interconnected network could serve for precise timing of the expression of various QS controlled phenotypes[60]. QS has been potentially responsible in managing various bacterial physiological activities such as expression of virulence factors, biofilm formation and swarming. Hence, by aiming bacterial communication circuit would be a novel way either in interfering its virulence or to enhance them for biotechnological purposes.

Employment of AHL Biosensors

The discovery of vast diverse AHL QS system has been rendered possible by adopting bacterial biosensors capable in sensing the AHLs production in a rapid manner. The bacterial biosensors contain defective LuxI protein which led them in disability in producing their own AHLs[67]. However, these biosensors carry a functional LuxR-family protein cloned with a cognate target promoter that up-regulates the transcription of reporter genes that exhibits phenotypes such as green-fluorescent protein, bioluminescence and purple violacein pigmentation. Specificity in sensing exogenous AHL by bacterial biosensors strongly relies on the LuxR family protein and hence it is essential to carry out the detection with several biosensors.

There are several biosensors available in detecting short and medium acyl chain AHLs (acyl chain range within C4 to C8 in length). *Chromobacterium violaceum* (CV026), a Gram-negative water and soil bacterium is commonly employed to serve this type of detection[38]. CV026 was developed after mini-Tn5 transposons insertion into *cviI* AHL synthase gene while the ability to induce purple pigmentation via *cviR* was retained. This mini-Tn5 mutant forms white colony and will only be able to turn purple in the presence of exogenous AHLs. CV026 is incapable to detect any AHLs with acyl chains of C10 or longer and all 3-hydroxy-AHLs. Several biosensors available in detecting short and medium acyl chain AHLs rely on a plasmid construct harboring *luxCDABE* operon and the host *E. coli* was commonly used for the cloning of plasmids because *E. coli* is not able to produce any AHLs. Genetically modified *E. coli* carrying AHL sensors plasmids; pSB401, pSB536 and pAL101 containing fusion of *luxRI'::luxCDABE, ahyRI'::luxCDABE, rhlRI'::luxCDABE* respectively are able to exert bioluminescence in presence of AHL molecules[68].

Other than short and medium length acyl chain AHLs, there are also some biosensors available in detecting long acyl chain AHLs (length of C10 and above). One of the biosensors is genetically modified *E. coli* harboring pSB1075 and pKDT17 both containing same fusion of *lasRI'::luxCDABE* but pSB1075 luminesce[68] while pKDT17 responds through standard β-galactosidase ac-

tivity upon exposure of exogenous AHLs[69]James. On the other hand, the *P. aeruginosa* PAO1 M71LZ could be utilized in detecting particularly C12 3-oxo-HSL and this biosensor is a *lasI* genomic knock-out mutant under control of *rsaL* promoter with transcriptional fusion of *lasR::lacZ* [70].

In order to detect AHLs with 3-hydroxyl group attached, a bacterial biosensor known as *P. fluorescens* 2-79 could be employed. Strain 2-79 employed genetically linked PhzI/R QS system that regulates expression of *phzABCDEFG* operon[71]. The strain 2-79 biosensor basically was developed from wildtype *P. fluorescens* 1855 that harbors two plasmid system; (i) pSF105 carrying *phzR* gene regulates by *trc* promoter (ii) pSF107 harbors *phzR-phzA* divergent that regulates by dual promoter region and fuse with two different reporters, *uidA* and *lacZ*. The sensing of exogenous AHLs could be easily detected via β-glucuronidase and β-galactosidase activity.

There are some other biosensors that have been eveloped to detect broad range of AHLs such as *Agrobacterium tumefaciens* WCF47, biosensors to detect uncommon AHLs, such as SinI/R-based biosensors to detect any AHLs with longer than 12 carbon length acyl chain, and there is also biosensors with gene encoding for the green fluorescent protein allowing detection of AHLs at single-cell level[67]. Although a negative results usually indicates that the no AHLs are produced by the tested bacterial strain, but this could be due to the biosensors used could not detect novel AHLs or the AHLs produced are in low concentration and below a threshold that biosensors could barely detect[67]. Hence, other methods such as thin layer chromatography (TLC) or high-resolution tandem-mass spectrophotometry could be used in detecting the AHLs.

Other Signalling Molecules

Besides AHLs as QS signalling molecules, there are presence of other signalling molecules being reported. Some of the well-documented intercellular signalling molecules are the members of a family of quinolone compounds termed 4-hydroxy-2-alkylquinolines (HAQs)[72]. The transcriptional regulator MvfR controls the synthesis of HAQs, which leads to modulation of several genes expression in the production of anthranilic acid and its conversion to 4-hydroxy-2-heptylquinoline (HHQ). The molecule 3,4-dihydroxy-2-heptylquinoline or known as *Pseudomonas* quinolone signal (PQS) is produced from the conversion of HHQ via PqsH action. However, the production of MvfR and PqsH are tightly control by LasR to intertwine with AHL-based pathway. The signalling of PQS is incorporated in the AHL QS pathway that is governed by Las and Rhl systems and known to be upregulated in cystic fibrosis patients during lung infections[73].

Plant pathogen *Xanthomonas campestris* and *X. fastidiosa* utilize a new type of communication language in regulating their virulence factors[74]. This signalling molecule is known as diffusible signalling factor (DSF) which was later identified as unsaturated fatty acid, cis-11-methyl-2-dodecenoic acid. Three major QS components are needed in this QS pathway: RpfF, RpfC and RpfG where they are involve in catalyzes, perception and transduction of the signalling molecules. The DSF-based QS mechanisms have been expanding and found to be utilized in other microorganisms such as *Xylella fastidiosa*[75], *Stenotrophomonas maltophila*[76] and *Burkholderia cepacia*[76]. Gram-negative bacteria are also capable in producing other types of signaling molecules; the iron mediated oxetane ring containing bradyoxetin namely 2-4-[[4-(3-amino-oxetan2-yl)phenyl](imino)methyl]phenyl oxetane-3yl-amine and 3-hydroxypalmitic acid methyl ester (3-OH PAME) by *Bradyrhizobium japonicum*[77] and *Ralstonia solanacearum*[78]. Both these signalling molecules are involved in protruding symbiotic relationship with higher organism, the plant.

While majority Gram-negative bacteria uses AHLs as autoinducers, Gram-positive bacteria uses post-translationally modified autoinducing peptide (AIP) molecules for QS[63]. The AIP is secreted through an ATP Binding cassette (ABC) transporter protein[63]. Gram-positive bacteria employ the two-component QS systems for AIP detection where it involved a membrane-bound histidine kinase receptor and a cognate cytoplasmic response regulator. The two component system is regulated by a series of auto-phosphorylation cascade. Similar with the AHL-based QS, the concentration of secreted AIP increases parallel with increasing cell density. Several peptide-based QS systems include the AgrC/AgrA system of *Staphylococcus aureus* in regulating virulence[79], the ComD/ComE system of *Streptococcus pneumoniae* in controlling bacterial competence[80], the ComP/ComA system of *Bacillus subtilis* in regulating DNA uptake and sporulation[81] and FsrB/FsrD system of *Enterococcus faecilis* for conjugation [82].

CONCLUSION

In summary, bacteria have adapted quorum sensing systems to enable them for regulating various activities. This bacteria communication system is unique and important for researchers in order to gain fundamental insight on how bacteria connect in their community. By understanding the various signaling molecules of bacteria, it will provide us with information that could be utilized in the management of biofilm, virulence and pathogenesis traits of various foodborne pathogens such as Listeria sp. [5, 83, 84], Vibrio sp. [85-91], and Salmonella sp. [92-96] that commonly affect human and animal health.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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