Disrupting bacterial quorum sensing as an alternative to antibiotics

Tremendous advances in our understanding of quorum sensing (QS) and its role in the pathogenesis of various disease conditions have occurred in recent years.

Deeper knowledge about how bacteria use QS signals to communicate with each other and coordinate their behaviours, including their assault on susceptible hosts, may provide new approaches to and options for disarming these potential pathogens and preventing and/or treating bacterial diseases.

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This article highlights why advances in QS – particularly as they relate to Clostridium perfringens virulence and necrotic enteritis (NE) pathogenesis – matter to the health of production animals (and humans), and how we can leverage this unique niche to develop novel alternatives to conventional antibiotics.

In-depth understanding

An in-depth understanding of the host-pathogen interaction has become important to identifying new approaches to infectious disease control in an era of increasing antimicrobial resistance.

The ability of bacteria to cause disease is composed of the following two factors:

- Infectivity, or the ability to infect and colonise a host.
- Virulence, or the capability to damage host cells and evade host defences.

While infectivity results from an imbalance between bacterial virulence and host resistance, bacterial virulence is derived from physical characteristics that bacteria have or chemical substances that they can produce.

Key bacterial virulence factors include toxins and gene products involved in adhesion, host tissue degradation, iron acquisition, motility, toxin secretion and protection from host defences.

Many of these virulence factors are regulated by genes controlled by quorum sensing. In fact, successful bacterial infection is now recognised as a collective process based on information sharing and active collaboration among bacterial community members – in other words, one that employs QS.

A brief historical overview of bacterial quorum sensing

Quorum sensing is a cell-to-cell communication process used by bacteria that involves production, detection and response to extracellular, small-molecule signals called autoinducers (AIs) to coordinate group behaviours based on cell population density.

Bacteria themselves make and secrete AIs into their local environments, which they then monitor to track changes in the cell numbers. Autoinducer concentrations accumulate in the local environment as the bacterial population density increases, and when AI levels reach a predetermined threshold, specific gene transcription is activated and gene expression, along with the behaviour of the total population, are collectively altered.

Elucidation of the QS concept required a series of discoveries made by many microbiologists over the past 50 years. Hints about bacterial QS were first seen in the mid-1960s when scientists showed the ability of Streptococcus pneumoniae to take up free, extracellular DNA and the luminescence of two marine bacteria species, Vibrio fischeri and V. harveyi, necessitated production of extracellular molecules.

At the time, researchers hypothesised the cell-to-cell signalling conducted through these extracellular molecules was a type of bacterial chemical communication. Unfortunately, these early reports were viewed sceptically and generally ignored.

Two milestone discoveries made during the 1980s would provide still more clues about QS. The first significant discovery was the findings of V. fischeri luminescence (lux) genes and the genes required for luminescence control, luxI and luxR, which were shown to control lux transcription.

The second landmark breakthrough was identification of the V. fischeri QS signal, N-3-oxohexanoyl-l-homoserine lactone.

Still, general interest in the potential bacterial communication system now known as QS remained quiet. But once DNA sequencing and comparative sequence analysis became commonplace in many laboratories, gene pairs with homology to luxR and luxI were found, and subsequently aroused some researchers' curiosity.

The increased attention resulted in numerous findings: other species of bacteria also controlled genes for activities such as conjugation, exoenzyme production and antibiotic synthesis with luxI–luxR-like systems.

Another common theme emerged as well: scientists found the LuxI homologs stimulated production of an acylated homoserine lactone (AHL) and the LuxR homologs all showed specificity for their related AHL. The merging of these discoveries resulted in the concept of QS.

Three other significant findings also occurred during the mid-1990s.

First, the QS signal from Streptococcus pneumoniae, which had often been referred to as a pheromone, was found to be a small peptide.

Second, researchers demonstrated that the virulence factors of Staphylococcus aureus, a Gram-positive bacterium, were controlled by a cell density-sensing system that used a small cyclic peptide made by the organism itself and that peptide activated the expression of the agr locus.

The third important discovery was a second QS system was identified in V. harveyi that functioned in parallel with the first system to control density-dependent expression of bioluminescence. Thus, QS was shown to occur in both Gram-positive and Gram-negative bacteria via diverse chemical signals.

Three common principles define QS

Rutherford and Bassler (2012) have identified three basic tenets common to all known QS systems despite differences in regulatory components and molecular mechanisms.

- Members of the bacterial community produce AIs, which are the signalling molecules. When cell density is low, AIs diffuse away and consequently are present at concentrations below the detection threshold. At high cell density, the cumulative production of signalling molecules leads to high concentrations in the local environment, which enables detection and response.
- AIs are detected by either cytoplasmic or membrane-bound receptors.
- Detection of AIs results in activation of AI production, in addition to activating gene expression for cooperative behaviours. Scientists believe this feed-forward autoinduction loop promotes population synchrony.

Mechanisms of quorum sensing

Cell-to-cell communication is now recognised as typical in the bacterial world, occurring not only within a species, but between bacterial genera and even across kingdom boundaries.

- **Intraspecies communication.**

Most QS autoinducers promote signalling within a given bacterial species in order to collectively modify their behaviour in response to changes in cell density and species composition of the bacterial community.

Both Gram-negative and Gram-positive species use QS, although they tend to use different QS systems for intraspecies communication.

- **Canonical QS in Gram-negative bacteria.**

Gram-negative bacteria use small

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molecules as AIs to communicate. These AIs are either acyl-homoserine lactones (AHLs) or other molecules produced from S-adenosylmethionine.

Generally speaking, the signal molecules are produced in the cell and, depending on the length of the acyl side chain, they either freely diffuse or are actively transported across inner and outer bacterial cell membranes.

When the extracellular AI concentration reaches a minimum threshold, which happens when the bacterial cell population density increases, AIs bind to cytoplasmic receptors that are transcription factors.

The AI-receptor complex then regulates gene expression in the QS regulon. Instead of transcription factor receptors, AIs may be detected by two-component histidine kinase receptors in some Gram-negative bacterial QS systems.

● **Canonical QS in Gram-positive bacteria.**
Gram-positive bacteria use peptides, called autoinducing peptides (AIPs), as signalling molecules. Once produced in the cell as a precursor molecule, AIPs are processed and secreted outside of the cell by specialised transporters because the cell membrane is impermeable to peptides.

When the extracellular AIP concentration reaches a predetermined threshold, which occurs at high bacterial cell density, the AIP binds to its associated membrane-bound receptor, which is typically a two-component histidine kinase receptor. Binding usually activates receptor kinase activity, causing the sensor kinase to autophosphorylate and pass the phosphate group to a related cytoplasmic response-regulator protein.

The phosphorylated response regulator then triggers gene expression in the QS regulon. In some Gram-positive bacteria, AIPs are returned to the cell cytoplasm where they interact with and alter activity of transcription factors that, in turn, modify gene expression changes.

The same QS signals used by Gram-positive and Gram-negative bacteria to induce and orchestrate gene transcription within a given species can sometimes activate the transcriptional programs of related bacterial strains.

Those QS signalling molecules may also simultaneously inhibit transcriptional functions of competing bacteria and fungi within the same microenvironment.

● **Interspecies communication.**
Most QS signalling molecules promote intraspecies communication. However, the discovery of a second QS system in V. harveyi resulted in the identification of a family of molecules that are commonly called autoinducer-2 (AI-2).

AI-2 is created by the LuxS enzyme, which is broadly conserved in a wide variety of bacteria.

Components of the AI-2 system have been detected in almost half all sequenced bacterial genomes, making the system the most pervasive signalling system used by Gram-negative and Gram-positive bacteria.

While AI-2 has been proposed as a universal signal for interspecies communication, it has also been suggested that the role of AI-2 in enteric bacteria is mainly metabolic.

● **Interkingdom signalling.**
More recently, bacterial communication has been shown to cross kingdom boundaries. Microbiologists now recognise the ability of bacteria to sense host signalling chemicals, including hormones such as adrenaline and noradrenaline.

Known as interkingdom signalling, bacterial pathogens use host signalling molecules as cues of the local environment and the host's physiological status, then respond by modulating expression of genes important for pathogenesis. The communication is a two-way street: QS signal molecules can alter transcriptional programs of mammalian epithelial cells and immune system cells.

More than a signal of cell density. Quorum sensing is now known to be much more than a simple estimate of bacterial cell density; it is used to govern a wide variety of functions. On the one hand, bacteria use QS to coordinate gene expression within their own species to direct activities that are most beneficial when performed by groups of bacteria acting simultaneously.

Among the processes now known to be controlled through QS are bioluminescence, sporulation, competence, antibiotic production, antibiotic resistance expression, biofilm formation, virulence factor secretion and several others.

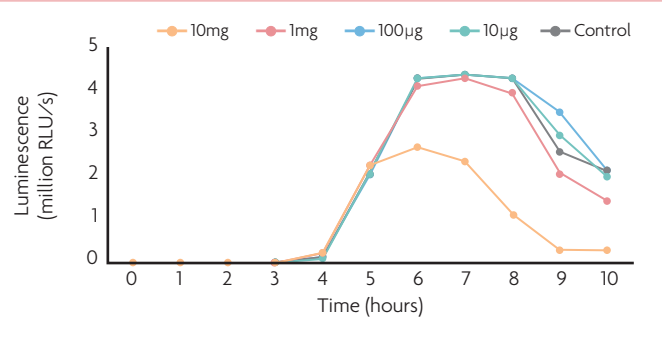


Fig. 1. Bacterial luminescence from Vibrio harveyi cultures treated with different concentrations of Calibrin-Z, medium control and V. parahaemolyticus during method one.

Role of QS in Clostridium perfringens infection in chickens

Quorum sensing is used by various bacterial pathogens of veterinary importance, including Clostridium perfringens. A ubiquitous inhabitant of normal human and animal intestinal microbiota, C. perfringens is a Gram-positive, spore-forming, aerotolerant anaerobic bacterium.

It is recognised as one of the fastest-growing bacterial pathogens, capable of using living or dead tissues as its nutrient source. When conditions are not favourable for growth, C. perfringens forms spores that enable its survival for long periods of time.

Certain C. perfringens type A strains can cause necrotic enteritis (NE), an enteric disease of poultry that has been estimated to cause nearly US$6 billion in losses to the global poultry industry. While considerable advances have been made in recent years in understanding the pathogenesis of C. perfringens-induced NE, there is much more to learn.

The discovery of NetB toxin and its essential role in the ability of C. perfringens isolates to cause NE in poultry was a paradigm shift in our understanding of NE pathogenesis.

Onset of NE is a complex process that involves several C. perfringens virulence factors linked to colonisation, adhesion and acquisition of nutrients.

Development of necrotic enteritis

Development of NE in poultry is generally attributed to rapid overgrowth of C. perfringens in the intestinal tract and subsequent production of virulence factors that damage host tissues. This progression is consistent with a QS regulatory system.

By co-ordinating the production of extracellular toxins and enzymes to occur only after a sufficient population density is reached, QS enables a pathogen to inflict the greatest damage to the host while minimising its expenditure of metabolic resources.

Limiting use of metabolic resources may be especially important to C. perfringens because its ability to cause disease and its metabolism are closely connected – even more than many other bacterial pathogens.

The tight linkage of virulence and metabolic functions is governed and directed by the two-component regulatory system, VirR/VirS.

C. perfringens virulence regulation is complicated and can vary from one strain to the next. However, some of the key regulators are now known. Production of multiple C. perfringens toxins is controlled by the VirR/VirS two-component regulatory system, including NetB toxin, α-toxin, perfringolysis-O and β-toxin.

However, QS has recently been shown to play a key role in regulating expression of C. perfringens virulence-related proteins, most notably NetB toxin, α-toxin and perfringolysin-O.

Clostridium perfringens uses at least two different QS systems: the Agr-like (accessory gene regulator) and the LuxS/AI-2 systems. In C. perfringens, the Agr-like system switches on the VirR/VirS two-component regulatory system, and initiates the disease process. The Agr-like QS system is found only in Gram-positive bacterial species. Small autoinducing peptides (AIPs) are produced and used as signalling molecules, which are subsequently recognised by a two-component system to ultimately modulate gene expression.

This system is represented by the Agr locus in Staphylococcus aureus, which entails four co-transcribed genes: agrB, agrD, agrC and agrA. The autoinducer propeptide, encoded by agrD, is first processed to the active AIP by the AgrB transporter and then released into the extracellular environment.

When the necessary concentration of AIP has been reached, it activates the agrC-agrA two-component system to subsequently modify gene expression. Orthologs – genes related by vertical descent from a common ancestor that encode proteins with the same function in different species – of agrB and agrD were identified in the C. perfringens genome within the last decade.

These genes were found to regulate production of several toxins: α-toxin, perfringolysin-O, β-toxin, ε-toxin, β2-toxin and enterotoxin.

In terms of NE pathogenesis, Prescott et al. (2016) acknowledged the framework they described is speculative but is considered reasonable given our current knowledge.

Colonisation and degradation of

the mucus layer in the small intestine are most likely to be essential prerequisites to NE development.

Although mucus contains a wide variety of antimicrobial molecules to discourage bacterial colonisation of intestinal epithelial cells, mucus is rich in O-glycosylated mucin glycoproteins.

The oligosaccharides present in areas of mucin glycoproteins provide many potential binding sites for bacterial adhesins and an energy source for bacteria that can metabolise them.

C. perfringens typically have glycoside hydrolases that can break down specific O-glycans in mucin. NE-inducing C. perfringens isolates also possess additional mucin-degrading enzymes.

Since glycoproteins are also found in epithelial cell surface membranes, clostridial glycoside hydrolases may have broader degradative functions.

Mucus degradation in the small intestine likely provides the critical source of nutrients that C. perfringens needs to enable it to form microcolonies on the mucosal surface.

It is during this phase of colonisation that QS likely plays a role that results in the upregulation of VirR/VirS regulon expression and of virulence and metabolism genes.

In C. perfringens, the Agr-like QS system activates the VirR/VirS two-component regulatory system and initiates the disease process. The autoinducing peptide of the Agr-like QS system in C. perfringens has been recently identified, as has an artificial 6-mer peptide that can inhibit toxin production.

A role for disrupting quorum sensing in strategic bacterial control

Increasing antimicrobial resistance among pathogenic bacteria has created a need for new, novel therapeutic options for preventing and/or treating bacterial diseases. The early connection between QS and pathogen virulence generated excitement for a potentially new approach to fighting bacterial infections – targeting QS.

As more has been learned about the important role that QS has in the pathogenesis of many bacterial infections, many researchers have investigated avenues for interfering with QS systems.

Numerous potential agents for use in human medicine have been put forward, although very few have been approved for use in current medical practice in the United States.

Many bacterial species with clinical relevance to veterinary medicine – E. coli, C. perfringens, S. aureus and Pseudomonas aeruginosa, to name a few – use QS to regulate virulence factor production. Since virulence factors are required for bacterial infection, preventing pathogens from producing those virulence factors could be an important alternative strategy for controlling bacterial diseases.

The modes of action of currently available antibiotics are variations on a single theme: bacterial eradication. In contrast, the antivirulence paradigm focuses on disarming bacteria by preventing virulence factor production or by neutralising those factors.

Three routes by which to interfere with QS as part of an antivirulence strategy have been identified:

- Inhibit synthesis of QS signal molecules.
- Inhibit interaction between a QS signal molecule and its related receptor.
- Quench extracellular QS signal molecules by neutralisation or degradation.

Interfering with or blocking QS do not kill pathogenic bacteria but instead inhibit virulence factor production and have been suggested to apply less selection pressure that could result in resistance development than conventional antibiotics.

The ideal virulence factor disruptor (or inhibitor) would not affect the natural survival or replication of the bacterial target and, consequently, would not alter the overall selective pressures that guide bacterial evolution.

An antivirulence approach to controlling bacterial diseases can also avoid an underappreciated, yet still harmful, side effect associated with today's antibiotics: damage to beneficial, commensal bacteria.

Even narrow-spectrum antibiotics can alter the gastrointestinal microbiota and broad-spectrum medications may dramatically deplete it, which can lead to complications such as Clostridium difficile colitis.

Because commensal bacteria lack many virulence factors, particularly toxin production, they should be unaffected by antivirulence therapy.

Efforts are underway to develop therapies against S. aureus infections using AIP analogs to interfere with the Agr QS system.

In addition, some progress has been made in targeting the C. perfringens AIP.

Targeting the Agr-like QS system of C. perfringens, which is essential for NE pathogenesis in vivo, may help prevent NE in chickens and provide an alternative to the antibiotic therapies currently used.

Specific advanced minerals show promise

Scientists at Amlan International recently demonstrated that specific advanced minerals show promise as potential inhibitors of QS via neutralisation or degradation of extracellular QS signal molecules in an aqueous system in vitro.

An AHL-type quorum sensing signal molecule, N-(3-oxooctanoyl)-dl-homoserine lactone, was used for the first of two in vitro studies.

This study was followed by an in vitro study in which researchers directly monitored quorum quenching in V. harveyi bacteria in the presence of specific minerals under simulated growth conditions.

The advanced minerals used to disrupt quorum sensing functioned as adsorption/catalytic antagonists. The AHLs involved in QS activity in some bacteria were either removed by adsorption or were catalytically broken down to smaller molecules on the active sites of the mineral material's surface.

In the second study, a thermally processed calcium montmorillonite (Calibrin-Z) was evaluated for its ability to disrupt QS in live/growing V. harveyi cultures.

V. harveyi is a common pathogen that causes vibriosis, a major disease of shrimp, fish and shellfish, and results in significant losses for the global aquaculture industry.

In addition, V. harveyi has been reported to channel information from three AI signals into one QS cascade.

Two different methods were used to directly monitor the luminescence emitted by bacteria as a measure of catalytic quorum quenching by Calibrin-Z.

In method 1, Calibrin-Z added to bacterial broth at an inclusion level at and above 1mg/mL starts to reduce overall luminescence in comparison to the control (no added Calibrin-Z).

At the 10mg/mL Calibrin-Z concentration, bacterial luminescence was reduced by 55% (determined from the area under the curves), which indicated a significant catalytic quorum quenching or interference in the overall QS of V. harveyi (see Fig. 1).

Just as important was the effect – or lack of effect – the addition of Calibrin-Z had on bacterial counts during the measurement period (see Fig. 2).

This confirms that catalytic quorum quenching occurred by transformation or separation of QS signal molecules or by interference of the QS process and not by killing the bacteria in the system.

In method two, just-formed QS molecules or their precursors from the V. harveyi cultures were separated into a supernatant by centrifugation.

This supernatant was treated with different concentrations of Calibrin-Z and luminescence was monitored over time.

Those systems that originated from calcium montmorillonite-treated filtrates showed reduced luminescence after one hour of growth, after which the intensity of luminescence began to increase.

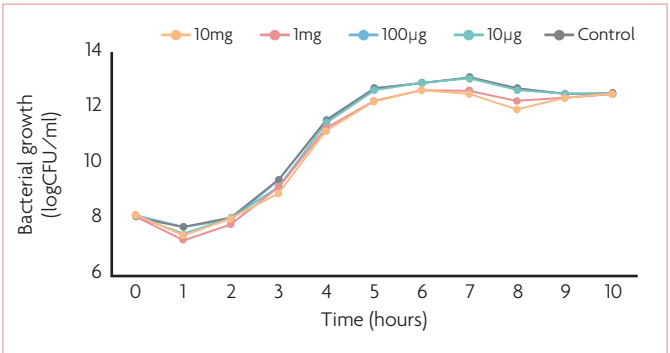
By the third hour of culture, luminescence had recovered to the original level of the medium control.

The amount of Calibrin-Z significantly affected bacterial luminescence with higher levels of Calibrin-Z resulting in lower luminescence after one and two hours of culture.

These results demonstrate the Calibrin-Z caused a delay in luminescence by selectively interfering with the QS system without causing any change in the bacterial population.

Although the exact mechanisms underlying the ability of Calibrin-Z to neutralise or degrade QS signal molecules needs to be further explored, its use as an extracellular QS molecule quencher could be a very promising approach to controlling bacterial diseases caused by toxin production or other bacterial secretions controlled by a QS system. ■

Fig. 2. Bacterial count measured as the concentration (CFU/mL) of viable bacterial cells at each time point from V. harveyi culture at different concentrations of Calibrin-Z, medium control and V. parahaemolyticus during method one.



References are available from the author on request