Interfering with bacterial signaling mechanisms as a novel strategy to control diseases - aquaculture as a case study

Tom Defoirdt[1,2]

Abstract

Bacterial pathogens are quickly evolving resistance against all known antibiotics. As a result, antibiotic treatments are becoming ineffective, and diseases caused by antibiotic resistant bacteria are currently a major cause of death worldwide. Bacterial cell-to-cell signaling mechanisms (quorum sensing) control several phenotypes that are required by pathogens to infect their host. Therefore, interfering with these mechanisms currently is intensively studied as a novel strategy to control bacterial diseases.

Key words: host-pathogen interaction, *Vibrio,* quorum sensing interference, quorum sensing inhibitor, quorum quenching

Samenvatting: Het verstoren van bacteriële signaalmechanismen als een nieuwe strategie om ziekten te bestrijden – aquacultuur als case study

Bacteriële ziekteverwekkers ontwikkelen aan een hoog tempo resistentie tegen alle gekende antibiotica. Daardoor verliezen behandelingen met antibiotica hun genezende werking, en ziekten veroorzaakt door antibioticum-resistente bacteriën zijn momenteel één van de belangrijkste doodsoorzaken wereldwijd. Bacteriële signaalmechanismen (quorum sensing) reguleren verschillende fenotypes die ziekteverwekkers nodig hebben om hun gastheer te infecteren. Daarom wordt het blokkeren van deze mechanismen op dit moment intensief bestudeerd als nieuwe strategie om bacteriële ziekten te bestrijden.

Trefwoorden: gastheer-pathogeen interactie, *Vibrio*, quorum sensing interferentie, quorum sensing inhibitor, quorum quenching

[1] Presented on November 27th, 2018

[2] Research professor at the Center for Microbial Ecology and Technology (cmet), Ghent University. Address: Coupure Links 653, 9000 Gent, Belgium. e-mail: Tom.Defoirdt@UGent.be

1. Controlling bacterial diseases: a major challenge for sustainable aquaculture production

Given the still-growing human population and the depletion of wild fisheries stocks, the aquaculture sector will need to significantly increase its output in the near future in order to meet the increasing demand. A recent report of the World Bank stated *"Beyond 2030, aquaculture will likely dominate future global fish supply. Consequently, ensuring successful and sustainable development of global aquaculture is an imperative agenda for the global economy."* (World Bank, 2013). However, disease outbreaks are a major limitation to increasing aquaculture production. *Vibrio anguillarum* and vibrios belonging to the Harveyi clade (i.e. *V. harveyi* and closely related species such as *V. campbellii* and *V. parahaemolyticus*) are amongst the major pathogens of aquatic organisms (Frans et al., 2011; Ruwandeepika et al., 2012), causing severe diseases such as luminescent vibriosis (Defoirdt et al., 2007a) and acute hepatopancreatic necrosis disease (AHPND) (Lee et al., 2015a; Xiao et al., 2017). These diseases cause up to 100% mortality, and losses have been estimated to be more than $1 billion per year in the shrimp industry alone (FAO, 2013).

Antibiotics are still critically important as a first line therapy for the treatment of bacterial infections, both in humans and animals, and are currently often the only option farmers have to protect their animals from disease. However, bacteria showing clinically relevant resistance to antibiotics consistently appear within as little as a few years after first use and bacteria have now developed resistance against all known antibiotics (Hall, 2004; Hancock, 2014; Dickey et al., 2017). A major reason for this is that the modes of action of the currently available antibiotics are primarily variations on a single theme: bacterial eradication (Cegelski et al., 2008; Dickey et al., 2017). Such mode of action imposes strong selective pressure for resistance development, and as a result, resistance is spreading rapidly, rendering antibiotic treatments ineffective. According to the World Health Organisation, diseases caused by antibiotic resistant bacteria are currently the second leading cause of death worldwide and the situation is predicted to become even worse in the near future if no adequate measures are undertaken, with 10 million deaths per year by 2050, and a cumulative loss to the world's GDP of $ 100 trillion (WHO, 2014).

As a result of the frequent use of antibiotics in order to control bacterial diseases in aquaculture, aquaculture is a major source of antibiotic resistance genes, and this is an important problem with respect to public health (Cabello et al., 2016). Antibiotic resistance is common in aquaculture pathogens and in human pathogens that are associated with seafood (Heuer et al., 2009; Defoirdt et al., 2011a; Wang et al., 2011). This is only the tip of the iceberg since the pathogenic bacteria that are screened are only a fraction of the total microbial community that is associated with seafood, and the harmless bacteria also contain (transferable) antibiotic resistance genes (Liu & Pop, 2009). Hence, upon consumption of aquaculture products, consumers are exposed to bacteria containing antibiotic resistance genes that can be transmitted to human microbiota, ultimately leading to a loss of

protection of the currently known antibiotics against human infections. Although most of aquaculture production is taking place in Southeast Asia, it should be stressed that the health risks are of a global concern given the world-wide trade in aquaculture products.

From the above, it will be clear that on one hand, controlling bacterial diseases in aquaculture is essential to assure food security, whereas on the other hand, the current practice of using antibiotics for this purpose is causing major problems with respect to public health. Hence, there is an urgent need for novel methods to control bacterial diseases in aquaculture. This is reflected in the objectives of the Food and Agriculture Organization's Action Plan on antimicrobial resistance (FAO, 2016).

2. Antivirulence therapy, a new paradigm in the control of bacterial disease

Infection by bacterial pathogens is caused by the production of different virulence factors, i.e. molecules produced by pathogens that enable them to colonise or harm the host (Dickey et al., 2017). As virulence factors are required for infection, preventing pathogens from producing them constitutes an interesting alternative strategy for the control of disease, i.e. antivirulence therapy. Rather than killing, antivirulence therapy aims at "disarming" the pathogens, thereby preventing them from attacking their host (Clatworthy et al., 2007; Rasko & Sperandio, 2010; Maura et al., 2016; Dickey et al., 2017). Importantly, little to no negative impacts on the harmless and beneficial bacteria within the host are expected (Cegelski et al., 2008; Maura et al., 2016; Dickey et al., 2017; Certner & Vollmer, 2018). This is in sharp contrast to antibiotics, which kill harmless and beneficial bacteria as well as the pathogens, thus resulting in problems associated with the loss of the functions performed by the harmless and beneficial bacteria (e.g. their contribution to digestion). These problems can persist for some time after ending the treatment because the beneficial bacteria have to recolonise the host before their beneficial activity can restart (Willing et al., 2011).

3. Virulence mechanisms and virulence regulation in bacterial aquaculture pathogens

3.1. Virulence factors

The infectious cycle of pathogenic bacteria includes (1) entry of the pathogen, (2) establishment and multiplication (thereby causing damage to host tissues and cells), and (3) exit (Donnenberg, 2000). Each of these different steps involves the expression of specific virulence factors (**Figure 1**). Major virulence factors include flagella (rotating propulsion organelles enabling the cells to move and to colonize the host (Haiko & Westerlund-Wikström, 2013)), chemotaxis proteins (which enable bacteria to move either towards favourable or away from unfavourable environments by modulating the

direction or speed of flagellar rotation (Wadhams & Armitage, 2004)), pili (fiber-like structures involved in adhesion (Proft & Baker, 2009)), biofilm formation (the growth of complex communities of surface-associated cells enclosed in a polymer matrix (Hall-Stoodley et al., 2004)), production of exopolysaccharides (a major constituent of the intercellular matrix in biofilms (Costerton et al., 1981; Donlan & Costerton, 2002)), lytic enzymes (extracellular proteins causing damage to host tissues, thereby allowing the pathogen to obtain nutrients and to spread through tissues (Finlay & Falkow, 1997)), siderophores (secreted low molecular weight iron-binding compounds (Skaar, 2010)), toxins (Lee et al., 2015a), and secretion systems (which transport virulence factors out of the cells (Gerlach & Hensel, 2007)).

Virulence factors and the activities associated with them are often metabolically costly, and therefore, their expression is tightly controlled by various regulatory mechanisms (Rasko & Sperandio, 2010). Bacterial cell-to-cell signaling (quorum sensing) systems are amongst the best characterised virulence regulatory mechanisms (LaSarre & Federle, 2013).

3.2. Virulence regulation by three-channel quorum sensing systems in vibrios

Quorum sensing refers to gene regulation mechanisms in which bacteria coordinate the expression of certain genes in response to the presence of small signal molecules. The most well-known signal molecules are acylated homoserine lactones (AHLs), which are produced by many Gram-negative bacteria (Jayaraman & Wood, 2008). AHLs of different species differ in the acyl side chain, which usually contains between 4 and 18 carbons and which can have an oxo or a hydroxyl substitution at the third position (**Figure 2**). Quorum sensing systems control the virulence of various bacterial aquaculture pathogens (**Table 1**).

The major aquaculture pathogen *V. campbellii* is one of the model species in quorum sensing research (Ng & Bassler, 2009). This bacterium uses three different signals, called Harveyi Autoinducer 1 (HAI-1), Autoinducer 2 (AI-2) and Cholerae Autoinducer 1 (CAI-1) (**Figure 3**). The signal molecules are detected at the cell surface by membrane-bound, two-component receptor proteins that feed a shared signal transduction cascade controlling the psroduction of the quorum sensing master regulators LuxR and AphA. In total, hundreds of genes are controlled by these regulators (van Kessel et al., 2013). In addition to bioluminescence, *V. campbellii* quorum sensing has been found to control the expression of different virulence genes (Mok et al., 2003; Henke & Bassler, 2004; Defoirdt et al., 2010; Natrah et al., 2011b; Yang & Defoirdt, 2015) and to be required for full virulence of the pathogen (Defoirdt & Sorgeloos, 2012; Pande et al., 2013; Noor et al., 2019). A similar quorum sensing system is found in several other vibrios, including the major fish pathogen *V. anguillarum* (Milton, 2006). However, in contrast to *V. campbellii*, the three-channel quorum sensing system of *V. anguillarum* has no effect on its virulence (Li et al., 2018).

3.3. Indole signaling

Indole has been known for quite some time to be synthesised from tryptophan by tryptophanase (TnaA) in many different bacteria, both Gram-negative and Gram-positive (Lee & Lee, 2010), and enteric bacteria can produce copious amounts of indole (up to mM levels) in the mammalian gut (Lee et al., 2015b). However, the appreciation of its role as a signal molecule is of relatively recent origin. Indole has been reported to control various virulence-related phenotypes (most notably biofilm formation and motility) and virulence in human, animal as well as plant pathogens (Lee et al., 2015b).

Major virulence factors that are affected by indole in vibrios include biofilm formation, exopolysaccharide production, and motility (the latter only in *V. campbellii*). Furthermore, indole signaling controls the virulence of *V. anguillarum* towards sea bass larvae and the virulence of *V. campbellii* towards brine shrimp and giant river prawn larvae (Li et al., 2014; Yang et al., 2017).

4. Interfering with signaling mechanisms in bacterial aquaculture pathogens

4.1. Application of quorum sensing inhibitors

Many different agents that are able to interfere with signaling mechanisms in bacteria have been documented (**Table 2**). Halogenated furanones constitute one of the most intensively studied class of quorum sensing inhibitory compounds, including both natural compounds produced by marine algae and synthetic derivatives (**Figure 4A and 4B**) (Janssens et al., 2008). Halogenated furanones were also shown to block the multichannel quorum sensing systems of vibrios by decreasing the DNA-binding activity of the quorum sensing master regulator LuxR (Defoirdt et al., 2007b), and protect both fish and crustaceans against vibriosis (Defoirdt et al., 2006; Rasch et al., 2004). Unfortunately, these compounds are too toxic to higher organisms to be applied in practice, with toxic concentrations being only slightly higher than quorum sensing-disrupting concentrations.

In search for compounds with a higher therapeutic potential, the synthesis of brominated thiophenones, sulphur analogues of brominated furanones, has recently been reported (Benneche et al., 2011). These compounds have the same effect on the quorum sensing system of vibrios as brominated furanones, i.e. they decrease the DNA-binding activity of LuxR. It has been proposed that both types of compounds covalently bind to proteins through an addition-elimination mechanism. Thiophenones are more active than the corresponding brominated furanones, with a concentration of 2.5 µM having a similar effect as ~100 µM of the furanones. One thiophenone compound, (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxo-butanoic acid (**Figure 4C**), was shown to have an interesting therapeutic potential to treat luminescent vibriosis, with a therapeutic index of approximately 100 (Defoirdt et al., 2012).

Another compound that has recently been reported to have quorum sensing-disrupting activity is the non-toxic flavouring substance cinnamaldehyde (**Figure 4D**). This compound has long been known for its antibacterial properties. However, at subinhibitory concentrations (~100 µM), it has the same activity as brominated furanones and thiophenones (Brackman et al., 2008). Cinnamaldehyde has been shown effective in different aquatic host-microbe systems (Brackman et al., 2008; Natrah et al., 2012). A major advantage of this compound with respect to practical application is that it is Generally Recognised As Safe (GRAS).

As mentioned earlier, marine algae produce different quorum sensing-disrupting halogenated furanones. In addition to this, extracts from different macro-algae belonging to the families of Caulerpaceae, Rhodomelaceae and Galaxauraceae have been reported to contain quorum sensing-inhibitory activity (Skindersoe et al., 2008) and the production of three new AHL antagonists by the red alga *Ahnfeltiopsis flabelliformis* has been reported (Kim et al., 2007). The micro-algae *Chlamydomonas reinhardtii*, *Chlamydomonas mutablis*, *Chlorella vulgaris* and *Chlorella fusca* were also found to produce quorum sensing mimic compounds (Teplitski et al., 2004). More recently, different micro-algae commonly used in aquaculture have been reported to interfere with AHL quorum sensing (Natrah et al., 2011a). Some of the algae stimulated the activity of AHL reporter strains, whereas others inhibited their activity. The chemical nature of the quorum sensing mimic compounds secreted by these micro-algae still has to be elucidated. Furthermore, we recently found that natural indole analogues that are produced by plants and seaweeds, i.e. the auxin hormones indole-3-acetic acid and indole-3-acetamide (**Figure 5**), have a similar antivirulence effect as indole (Yang et al., 2017).

Finally, quorum sensing inhibitors have also been identified in marine bacteria. Extracts from different cyanobacterial species were reported to disrupt AHL quorum sensing, with the highest activity being observed in extracts from a *Symploca hydnoides* and two *Lyngbya majuscula* isolates (Dobretsov et al., 2010). The active compound produced by *Lyngbya majuscula* was identified to be malyngolide. Further, two phenetylamide metabolites could be identified as active quorum sensing-disrupting compounds produced by *Halobacillus salinus* (Teasdale et al., 2009). Further research revealed that quorum sensing inhibitory activity is rather widespread among marine bacteria belonging to the genera *Bacillus* and *Halobacillus* that were isolated from algae and aquatic biofilms and sediments (Teasdale et al., 2010).

4.2. Enzymatic inactivation and biodegradation of quorum sensing molecules

The ability to degrade AHLs is widely distributed in the bacterial kingdom (Dong et al., 2007). The actual inactivation of the signal compound can be mediated by two major types of enzymes: AHL lactonases and AHL acylases (**Figure 6**).

The lactonase enzyme responsible for the AHL-inactivating activity (AiiA) from *Bacillus* spp. opens the lactone ring of AHLs to produce the corresponding acylhomoserines. Genes encoding AHL-degrading lactonases are widespread in *Bacillus* species (Dong et al., 2002). AHL lactonases hydrolise both short- and long-chain AHLs with similar efficiency, but show no or little activity to other chemicals, including non-acyl lactones and aromatic carboxylic acid esters (Dong et al., 2007). Cleavage of AHLs by an AHL acylase enzyme results in the release of homoserine lactone and a fatty acid (Fast & Tipton, 2012). Subsequently, the fatty acid is used as carbon source via the β-oxidation pathway. Some substrate specificity of these AHL-inactivating enzymes can exist. For example, the AHL acylase enzyme PvdQ, produced by *Pseudomonas aeruginosa*, could only utilise AHLs with acyl side chains longer than 8 carbons (Fast & Tipton, 2012).

AHL-degrading enrichment cultures can be obtained by using media containing AHLs as the sole carbon and/or nitrogen source (Tinh et al., 2007). Such kind of enrichment cultures have been isolated from micro-algal cultures and from the digestive tract of healthy shrimp and fish, and pure strains of AHL-degrading *Bacillus* sp. have been isolated from these enrichment cultures (Defoirdt et al., 2011b; Pande et al., 2015). Interestingly, the addition of AHL-degrading bacteria to larval cultures significantly increases their survival (Tinh et al., 2008; Nhan et al., 2010; Pande et al., 2015). Hence, bacteria that are able to degrade quorum sensing signal molecules might be useful as a new kind of probionts for aquaculture. Alternatively, purified signal molecule-degrading enzymes can be used as biocontrol agents. Cao et al. (2012), for instance, reported that oral administration of a thermostable lactonase enzyme from a *Bacillus* sp. significantly attenuated *Aeromonas hydrophila* infection in zebrafish.

5. Conclusions and further perspectives

Most research efforts with respect to exploring the possibilities to control bacterial infections in aquaculture by means of antivirulence therapy have focused on the disruption of quorum sensing, bacterial cell-to-cell communication. These studies revealed that interefering with pathogenicity mechanisms of aquaculture pathogens indeed is an effective way to control bacterial disease in aquatic hosts. Moreover, they revealed that quorum sensing-disrupting agents can be recruited from the aquatic environment itself (i.e. bacterial or algal secondary metabolites or enzymes produced by signal-degrading bacteria) and that these agents can protect different aquatic hosts from disease. In addition to quorum sensing, scientific progress during the past years has unravelled more pathogenicity mechanisms in bacterial pathogens that might be suitable targets for antivirulence therapy for aquaculture (e.g. sensing of host cues, or specific virulence factors such as secretion systems and adhesion structures) (Defoirdt, 2014), and it will be interesting to explore the possibility to interfere with these mechanisms as a novel therapeutic strategy. Finally, before this kind of novel

therapeutics can be used in practice, large-scale (e.g. hatchery) trials will be needed to prove their efficacy in a real aquaculture environment and to exclude possible negative side effects. Finally, there still is a debate going on with respect to the possibility that bacteria will evolve resistance to quorum sensing inhibition (Liu et al., 2018). In order to solve this question, further research is needed in order to address the impact of quorum sensing inhibition on the fitness of bacterial pathogens in association with a host.

6. References

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Tables

Table 1. Examples of quorum sensing systems in aquaculture pathogens and the link between quorum sensing and virulence.

Species	Signal molecules	Quorum sensing-regulated virulence and virulence factors	References
Aeromonas hydrophila	BHL, HHL	Biofilm formation, exoprotease, haemolysin and siderophore production, secretion, lethality to burbot	Natrah et al. (2012); Swift et al. (1999)
Edwardsiella tarda	BHL, HHL, OHHL, HeHL, AI-2	Motility, biofilm formation, type III secretion	Han et al. (2009); Leung et al. (2012)
Vibrio alginolyticus	AI-2	Protease production, haemolytic activity, extracellular polysaccharide production and siderophore production, lethality to red seabream	Rui et al. (2008); Wang et al. (2007); Ye et al. (2008)
Vibrio anguillarum	OH-HHL, ODHL, AI-2, CAI-1, indole	Extracellular ptotease activity, pigment production, biofilm formation	Milton (2006); Li et al. (2014)
Vibrio campbellii	HAI-1, AI-2 and CAI-1, indole	Siderophore, chitinase, metalloprotease, phospholipase and extracellular polysaccharide production, type III secretion, lethality to crustaceans and fish	Ruwandeepika et al. (2012); Yang & Defoirdt (2015); Yang et al. (2017)
Vibrio mimicus	AI-2	Protease activity	Sultan et al. (2006)
Vibrio salmonicida	OHHL, HHL	Motility, adhesion, cell-to-cell aggregation, and biofilm formation, virulence towards Atlantic salmon	Bjelland et al. (2012)

BHL: N-butanoyl-L-homoserine lactone, HAI-1: N-(3-hydroxybutanoyl)- L-homoserine lactone, HHL: N-hexanoyl-L-homoserine lactone, OHHL: N-(3-oxohexanoyl)-L-homoserine lactone, OH-HHL: N-(3-hydroxyhexanoyl)-L-homoserine lactone, HeHL: N-heptanoyl-L-homoserine lactone, ODHL: N-(3-oxodecanoyl)-L-homoserine lactone, AI-2: Autoinducer 2, CAI-1: (S)-3-hydroxytridecan-4-one

Table 2. Examples of quorum sensing-interfering agents.

Agent	Target bacterium	Molecular target	References
Natural compounds			
Ajoene	*P. aeruginosa*	Signal transduction	Jakobsen et al. (2012)
Citrus limonoids	*V. harveyi*	Signal transduction	Vikram et al. (2011)
Flavonoids	*P. aeruginosa*	AHL receptor	Paczkowski et al. (2017)
Indole-3-acetic acid	*V. harveyi*	Indole signaling	Yang et al. (2017)
Naringenin	*P. aeruginosa*	AHL production and detection	Vandeputte et al. (2011)
Synthetic compounds			
Brominated thiophenones	*V. harveyi*	Signal transduction	Yang et al. (2015)
Thiazolidinediones and dioxazaborocanes	*V. harveyi*	AI-2 receptor	Brackman et al. (2013)
3-acylpyrroles	*V. cholerae*	CAI-1 receptor	Perez et al. (2014)
Signal molecule-degrading bacteria and enzymes			
Bacillus sp. strain NFMI-C	*V. harveyi*	AHL signal molecules	Pande et al. (2015)
Lactonase from *Bacillus* sp. strain QSI-1	*A. hydrophila*	AHL signal molecules	Chu et al. (2014)
Modified acylase PvdQ	*B. cenocepacia*	AHL signal molecules	Koch et al. (2014)

Figures

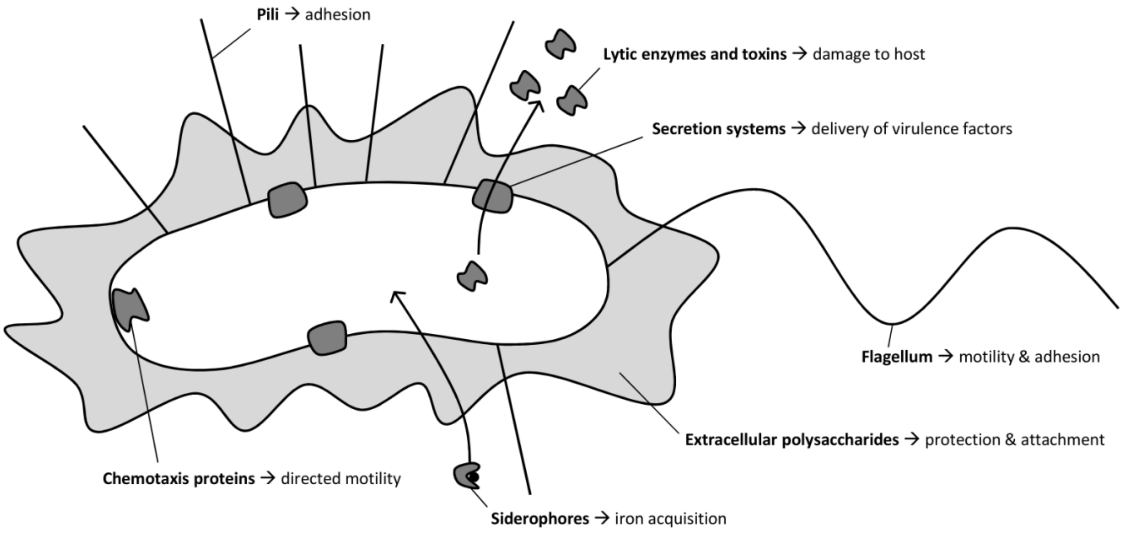


Figure 1. Schematic overview of different virulence factors produced by a pathogenic bacterium.

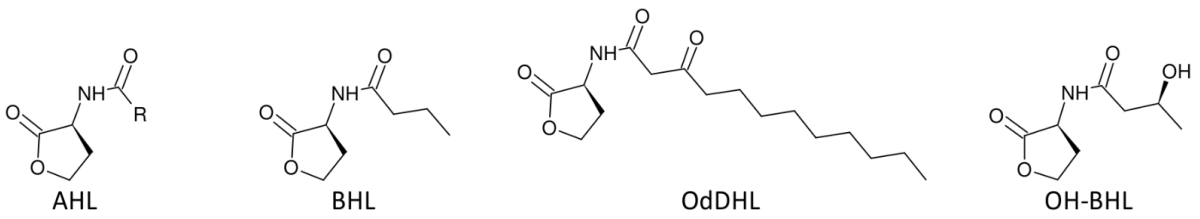


Figure 2. Chemical structures of selected acylhomoserine lactones (AHLs). From left to right: general structure of an AHL; N-butanoyl-L-homoserine lactone (BHL) and N-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL), the AHLs produced by *Pseudomonas aeruginosa*; N-(3-oxo-butanoyl)-L-homoserine lactone (OH-BHL), the AHL produced by *Vibrio ahrveyi.*

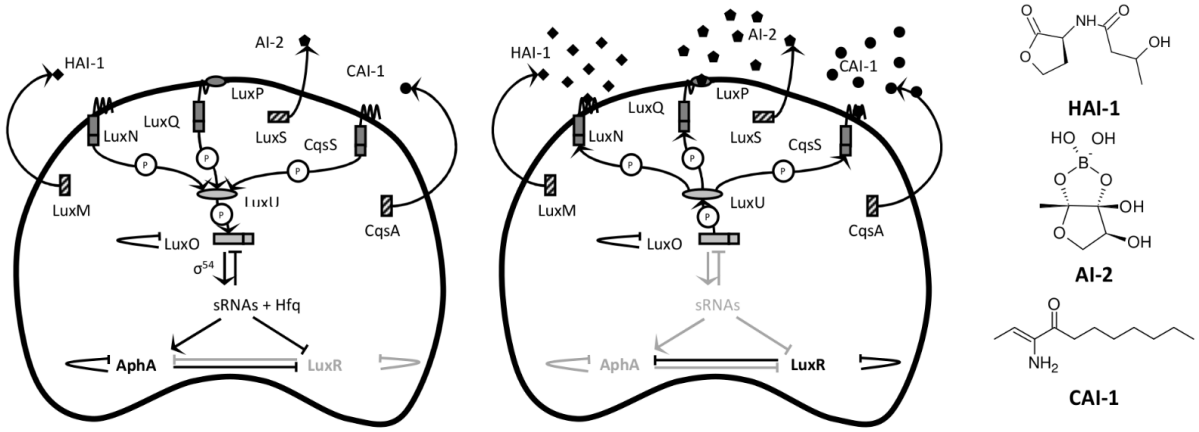


Figure 3. Quorum sensing in *V. campbellii*. The LuxM, LuxS and CqsA enzymes synthesise the signal molecules HAI-1, AI-2 and CAI-1, respectively. These signal molecules are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. In the absence of signal molecules (left), the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ54 activates the production of five small regulatory RNAs (sRNAs). The sRNAs promote translation of the master regulator AphA and inhibit translation of the master regulator LuxR. In the presence of high concentrations of signal molecules (right), the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed, AphA is not translated and LuxR is translated. AphA and LuxR are transcriptional regulators that (either individually or together) affect the transcription of many target genes.

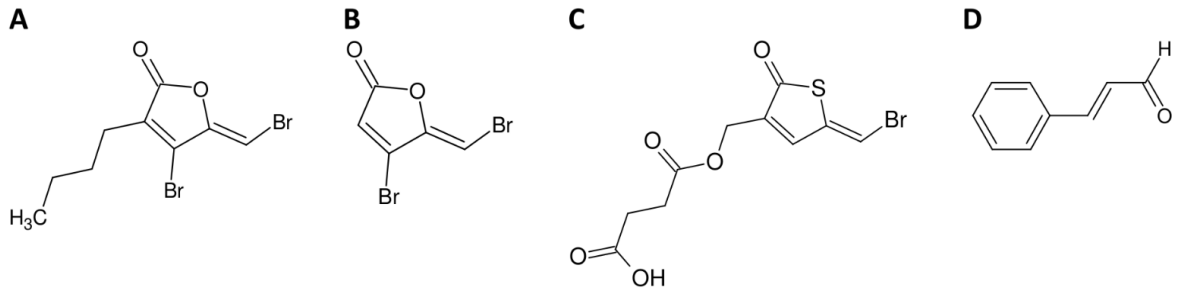
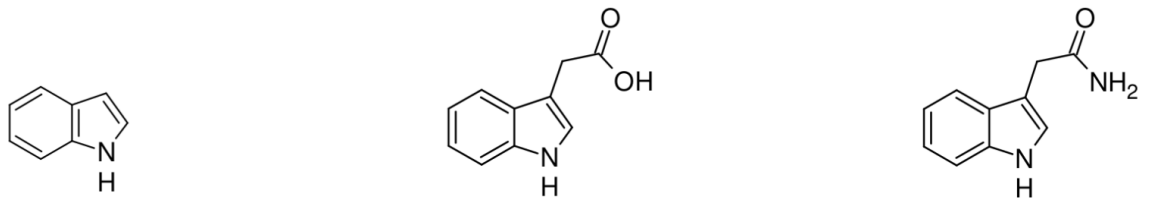


Figure 4. Chemical structure of quorum sensing inhibitors. (A) The natural furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone, produced by the red marine alga Delisea pulchra. (B) The synthetic derivative (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone. (C) The brominated thiophenone (Z)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxo-butanoic acid. (D) Cinnamaldehyde.



indole ***indole-3-acetic acid*** ***indole-3-acetamide***

Figure 5. Structures of indole, and of indole-3-acetic acid and indole-3-acetamide, two natural indole analogues produced by plants and algae with a similar antivirulence effect as indole

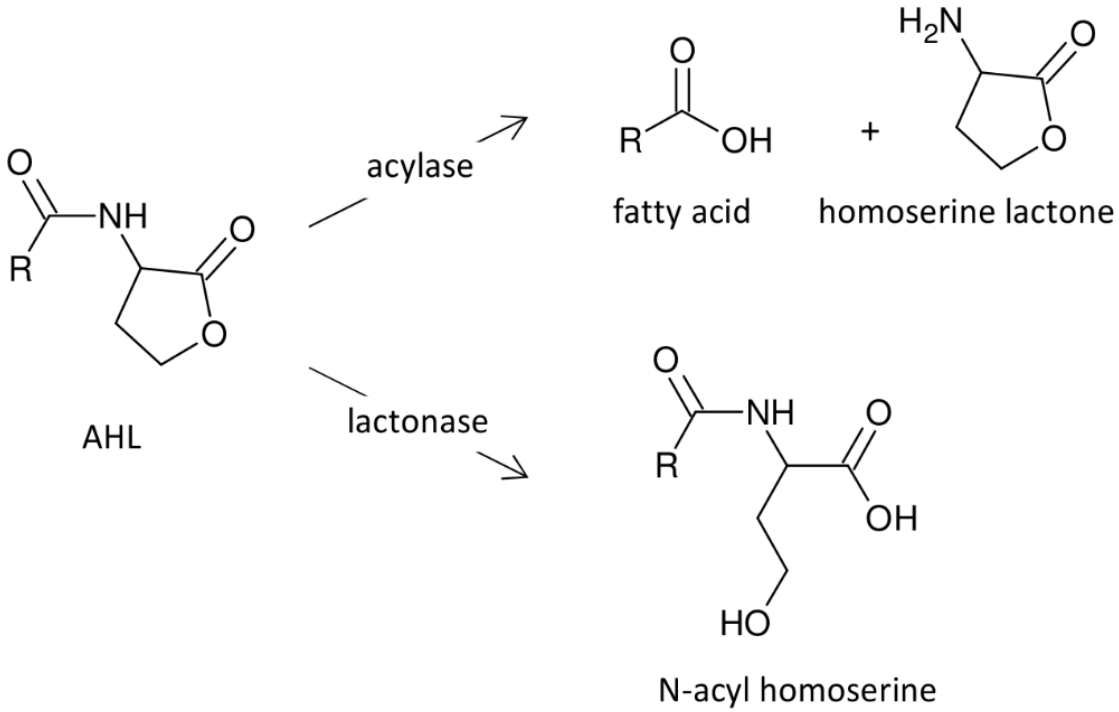


Figure 6. Enzymatic inactivation of acylhomoserine lactone (AHL) signal molecules. Cleavage of the amide bond by an AHL acylase enzyme yields a fatty acid and homoserine lactone. Cleavage of the lactone ring by an AHL lactonase enzyme yields the corresponding acylated homoserine.