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MINI-REVIEW

Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from *in vivo* work

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Luminescent vibrios, bacteria belonging to the species *Vibrio harveyi* and closely related species, are important pathogens in aquaculture that can affect almost all types of cultured animals. Due to large-scale use of antibiotics, many luminescent vibrios have acquired (multiple) resistance, which render antibiotic treatments ineffective. One of the alternative strategies that has recently been developed to control infections caused by antibiotic-resistant bacteria is the disruption of quorum sensing, bacterial cell-to-cell communication. The quorum sensing system of *V. harveyi* has been studied quite intensively *in vitro*. Recent studies have been directed towards understanding the impact of quorum sensing and quorum sensing disruption on the virulence of luminescent vibrios towards different host organisms *in vivo*. This mini-review aims at discussing the current knowledge of quorum sensing in luminescent vibrios *in vivo*. Subsequently, quorum quenching by halogenated furanones is discussed and finally, some directions for further research are presented.
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Introduction

Vibrio harveyi and closely related species such as *Vibrio campbellii* and *Vibrio parahaemolyticus* are amongst the most important bacterial pathogens in the intensive rearing of molluscs, finfish and especially shrimp (Austin and Zhang, 2006).

Vibrios are opportunists that only cause disease when the host organisms are immune-suppressed or otherwise physiologically stressed, with the frequency of infection often being attributable to adverse culture conditions (Alderman and Hastings, 1998). In our laboratories, adverse culture conditions are simulated in laboratory challenge tests by feeding the animals with a suboptimal diet (Defoirdt *et al.*, 2005; Marques *et al.*, 2005; Tinh *et al.*, 2007b). In order to overcome the negative consequences of adverse culture conditions, farmers traditionally rely on the use of antibiotics (Subasinghe *et al.*, 2001). Due to the indiscriminate misuse of antibiotics in aquaculture (Cabello, 2006), vibrios are now resistant to several antibiotics and consequently, antibiotics are no longer effective in treating luminescent vibriosis. Karunasagar *et al.* (1994), for instance, reported mass mortality in tiger shrimp (*Penaeus monodon*) larvae caused by *V harveyi* strains with multiple antibiotic resistance that was linked to the use of antibiotics in hatcheries. Hence, the quest for alternative methods to control infections caused by antibiotic-resistant bacteria is an important challenge for the sustainable development of aquaculture.

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Quorum sensing—bacterial cell-to-cell communication

One of the new strategies that has been proposed to control infections in aquaculture is disruption of quorum sensing, bacterial cell-to-cell communication by means of small signal molecules (Defoirdt *et al.*, 2004). Quorum sensing is a mechanism of gene regulation in which bacteria coordinate the expression of certain genes in response to the presence or absence of small signal molecules. This mechanism was first discovered in the marine bacterium *Vibrio fischeri* (Nealson *et al.*, 1970) and was thought to be restricted to only a limited number of species. Later on, similar systems were found to be present in many other bacteria.

The hypothesis that prevails in literature is that bacteria use quorum sensing to sense population density (Miller and Bassler, 2001). According to this hypothesis, quorum sensing-regulated genes are expressed (or repressed) depending on the bacterial cell density. However, this hypothesis has never been proven and is still under debate. Redfield (2002) argued for a more direct function of quorum sensing: the ability to determine whether excreted molecules rapidly diffuse away from the cell. This diffusion sensing would allow cells to regulate excretion of degradative enzymes and other gene products in such a way as to minimize losses owing to extracellular diffusion and mixing.

Quorum sensing-regulated gene expression is most often studied *in vitro* (that is, in bacterial cultures grown in liquid or on solid growth medium). However, microbiologists are becoming more and more aware of the fact that this gene regulation is linked to and influenced by environmental and host-derived signals (Newton and Fray, 2004). This mini-review aims at discussing the current knowledge of quorum sensing and quorum quenching in *V. harveyi* and closely related bacteria *in vivo* during *Vibrio*–animal interactions.

The Quorum sensing system of *V. harveyi*

V. harveyi has been found to use a three-channel quorum sensing system (Figure 1). The first channel of this system is mediated by the harveyi autoinducer 1 (HAI-1), an acylated homoserine lactone (AHL) (Cao and Meighen, 1989). The second channel is mediated by the so-called autoinducer 2 (AI-2), which is a furanosyl borate diester (Chen *et al.*, 2002). The chemical structure of the third autoinducer, called cholerae autoinducer 1 (CAI-1), is still unknown. The autoinducers are detected at the cell surface by membrane-bound, two-component receptor proteins that feed a common phosphorylation/dephosphorylation signal transduction cascade (Taga and Bassler, 2003). Central in the signal transduction cascade is the LuxO protein. Phosphorylated LuxO indirectly inhibits production of the transcriptional regulator protein $LuxR_{Vh}$ through the action of five small regulatory RNAs (Tu and Bassler, 2007). $LuxR_{Vh}$ directly activates the *lux* operon (Swartzman *et al.*, 1992), whereas the majority of other quorum sensing-regulated genes appears to be indirectly controlled by $LuxR_{Vh}$ (Waters and Bassler, 2007). Tu and Bassler (2007) recently proposed that the multiple small regulatory RNAs function to translate increasing autoinducer concentrations into a precise gradient of $LuxR_{Vh}$, resulting in a gradient of expression of quorum sensing-regulated target genes. In other words, the concentration of $LuxR_{Vh}$ depends on the concentration of the five small regulatory RNAs, which is determined by the phosphorylation status of LuxO. The phosphorylation status of LuxO in its turn is determined by the net result of the kinase and phosphatase activities of the three receptors and thus dependent on the concentration of the three autoinducers.

Interestingly, Waters and Bassler (2007) recently reported that different quorum sensing-controlled

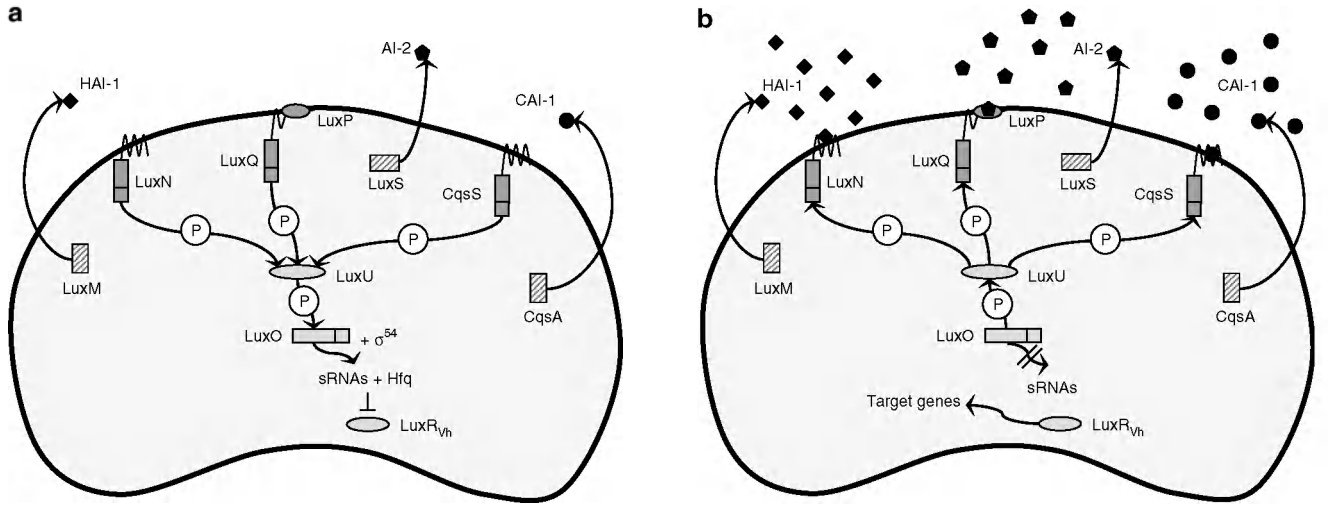

Figure 1 Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS and CqsA enzymes synthesize the autoinducers harveyi autoinducer 1 (HAI-1), autoinducer 2 (AI-2) and cholerae autoinducer 1 (CAI-1), respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. (**a**) In the absence of autoinducers, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ^{54} activates the production of five small regulatory RNAs (sRNAs). These sRNAs, together with the chaperone Hfq, destabilize the mRNA encoding the transcriptional regulator $LuxR_{Vh}$. Therefore, in the absence of autoinducers, the $LuxR_{Vh}$ protein is not produced. (**b**) In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, which result in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed and the transcriptional regulator $LuxR_{Vh}$ is produced. See text for more details. Ⓟ denotes phosphotransfer.

genes are differentially regulated by a given autoinducer input state, although they all depend on the $LuxR_{Vh}$ concentration. The authors explained this by the target promoters having different affinities for $LuxR_{Vh}$ and distinguished three classes of target genes. Genes that respond fully to either one of the autoinducers (resulting in relatively low $LuxR_{Vh}$ levels) were predicted to have promoters with high affinity for $LuxR_{Vh}$. Genes showing an additive response to the autoinducers (that is, significant expression in the presence of either one of the autoinducers, but a full response only in the presence of all autoinducers) were predicted to have intermediate affinity for $LuxR_{Vh}$. Genes only responding to the coincident presence of the autoinducers were suggested to have low affinity for $LuxR_{Vh}$ since they require the highest concentration of $LuxR_{Vh}$.

The impact of quorum sensing on the virulence of *V. harveyi in vivo*

Phenotypes that were found to be controlled by the *V. harveyi* quorum sensing system *in vitro* include bioluminescence (Bassler *et al.*, 1993) and the production of several virulence factors such as a type III secretion system (Henke and Bassler, 2004a), extracellular toxin (Manefield *et al.*, 2000), metalloprotease (Mok *et al.*, 2003) and a siderophore (Lilley and Bassler, 2000). Recently, using quorum sensing mutants, we found that the AI-2-mediated channel of the *V. harveyi* quorum sensing system regulates virulence of the bacterium towards the brine shrimp *Artemia franciscana in vivo* (Defoirdt *et al.*, 2005). Indeed, inactivation of the AI-2 synthase gene *luxS* or the AI-2 receptor gene *luxP* abolished mortality in brine shrimp larvae caused by a pathogenic *V. harveyi* strain. In contrast, inactivation of the HAI-1-mediated channel of the system had no effect on virulence of the strain towards the shrimp. The effect of the CAI-1-mediated channel was not studied since CAI-1 signalling was not yet described at the moment the experiments were performed. However, in later experiments, none of the signal molecule synthase double mutants MM77 ($HAI\text{-}1^-$, $AI\text{-}2^-$, $CAI\text{-}1^+$), JMH605 ($HAI\text{-}1^-$, $AI\text{-}2^+$, $CAI\text{-}1^-$) and JMH606 ($HAI\text{-}1^+$, $AI\text{-}2^-$, $CAI\text{-}1^-$) was found to cause mortality in brine shrimp (our unpublished results). Although it would be more illustrative to test the virulence of a single CAI-1 negative mutant, these observations suggest that disruption of the CAI-1-mediated channel probably will have the same effect as observed for the AI-2-mediated channel. It therefore seems that both AI-2 and CAI-1 are necessary for virulence towards brine shrimp, whereas HAI-1 is not.

The inactivity of the HAI-1-mediated channel of the *V. harveyi* quorum sensing system during infection of brine shrimp could be explained by the signal either not being produced or having a low stability *in vivo*. The first possibility would indicate that *V. harveyi* can alter the production of signals in accordance to the environment (and thus according to the type of animal during infection). Instability of HAI-1 could be either due to environmental conditions or host-derived enzyme activity. HAI-1 is an AHL, and this type of signal molecules has been shown to be unstable at relatively high pH, with short-acyl chain AHLs (such as *V. harveyi* HAI-1) being the most unstable ones (Byers *et al.*, 2002; Yates *et al.*, 2002). Although the pH of the brine shrimp gut is unknown, it might be sufficiently alkaline to hydrolize the HAI-1 signal and as a consequence, it would fail to activate the HAI-1 receptor. Given the fact that different higher organisms have been reported to produce AHL-inactivating enzyme activity (Chun *et al.*, 2004; Yang *et al.*, 2005), an alternative explanation could be that the shrimp produces enzymes that inactivate AHL-type of quorum sensing molecules.

The *V. harveyi* quorum sensing system has been described as a three-way detector, with the expression of quorum sensing-regulated genes being proportional to the levels of the three signal molecules (Henke and Bassler, 2004b). Apparently, the detection of AI-2 and CAI-1 results in sufficiently high levels of the $LuxR_{Vh}$ transcriptional regulator to allow expression of the virulence factors that are essential to kill brine shrimp, whereas the $LuxR_{Vh}$ concentration produced in the presence of only one of these two signal molecules is not. Interestingly, Tinh *et al.* (2007b) found that both HAI-1 and AI-2 signalling needed to be inactivated in order to neutralize the negative effects of *V. harveyi* BB120 towards gnotobiotic rotifers (*Brachionus plicatilis*). This indicates that, in contrast to what was found in brine shrimp, HAI-1 signalling is active in the rotifers and suggests that higher levels of the $LuxR_{Vh}$ transcriptional regulator are needed to infect these organisms when compared to brine shrimp. Alternatively, although all currently known quorum sensing-regulated genes are controlled by the model as described in Figure 1, we cannot exclude the possibility that pathogenicity to rotifers is mediated by a specific virulence factor that is regulated by HAI-1 through a yet unknown signal transduction loop.

On the value of a three-channel quorum sensing system

The above-mentioned *in vivo* work seems to confirm the hypothesis of Taga and Bassler (2003) that *V. harveyi* uses a three-channel quorum sensing system in order to counteract bias. Indeed, if quorum sensing-regulated gene expression would be based on the detection of only one signal molecule, then bias could be caused by inactivation of the signal, either chemically (for example, AHLs such as HAI-1 are unstable at high pH; Byers *et al.* (2002)) or

biologically (for example, HAI-1 could be degraded by *Bacillus* spp.; Dong *et al.* (2002)). Indeed, the HAI-1-mediated channel of the *V. harveyi* quorum sensing system seems to be inactive during infection of brine shrimp (see above). Consequently, the vibrios would fail to infect brine shrimp if they would only rely on HAI-1 signalling. On the other hand, interference due to signal production by bacteria belonging to other species (for example, in case of AI-2) will not drastically bias the three-channel system since the two other signals are needed as well for maximal activation (Henke and Bassler, 2004b). Moreover, this kind of bias would not be so dramatic since activation of the quorum sensing cascade by a signal produced by other bacteria would indicate that mixing and diffusion in the micro-environment is low and hence, the vibrios would have a high chance to benefit from their own quorum sensing-activated gene products (according to the diffusion sensing hypothesis of Redfield (2002)).

Quorum sensing disruption by halogenated furanones

Halogenated furanones have been shown before to disrupt AHL- as well as AI-2-mediated signalling in Gram-negative bacteria, without affecting growth (Ren *et al.*, 2001; Hentzer and Givskov, 2003). Consistent with this, the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone, added to the culture water at 20 mg l^{-1}, was shown to increase the survival of brine shrimp larvae challenged to different pathogenic isolates belonging to the species *V. campbellii*, *V. harveyi* and *V. parahaemolyticus* (Defoirdt *et al.*, 2006). This suggests that virulence attenuation caused by quorum sensing disruption is a general feature for luminescent vibrios and not specific for one strain. For some of the strains, the protection offered by the furanone treatment was complete, whereas other strains still caused significant mortality in furanone-treated larvae. Hence, apparently there is a difference between different strains in the degree of quorum sensing disruption by the compound. Unfortunately, it was not possible to investigate whether higher furanone concentrations could protect the larvae from the isolates that still caused mortality at 20 mg l^{-1} of furanone because the compound was highly toxic to the larvae at 50 mg l^{-1} (Defoirdt *et al.*, 2006). This implies that the therapeutic index for the furanone is too low to be applied in practice. Hentzer and Givskov (2003) also mentioned that the currently known halogenated furanones are too reactive for the treatment of infections in higher organisms.

Initial *in vitro* bioluminescence experiments showed that halogenated furanones block HAI-1- and AI-2-mediated signalling in *V. harveyi* (Ren *et al.*, 2001; Defoirdt *et al.*, 2006). It was originally hypothesized that the compounds disrupt quorum sensing in this bacterium by displacing the signal molecules from their receptors (Manefield *et al.*, 2000; Ren *et al.*, 2001). However, recent research at our laboratories showed that the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone blocks quorum sensing-regulated gene expression in *V. harveyi* by decreasing the DNA-binding activity of the quorum sensing transcriptional regulator $LuxR_{Vh}$ and not by interacting with the signal molecule receptors (Defoirdt *et al.*, 2007). The fact that the furanone affects the master regulator rather than selectively blocking one of the channels of the *V. harveyi* quorum sensing system is quite important with respect to possible practical applications. As mentioned earlier, there seems to be a difference in the relative importance of the three channels for a successful infection of different hosts. Hence, since the furanone blocks all three channels of the system at once by acting at the end of the quorum sensing signal transduction cascade, it will not be necessary to develop different furanone compounds to protect different hosts. Indeed, the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone has been shown to protect both brine shrimp and rotifers against luminescent vibrios (Defoirdt *et al.*, 2006; Tinh *et al.*, 2007b).

Interestingly, the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone had no effect on growth of the vibrios (Manefield *et al.*, 2000; Defoirdt *et al.*, 2006), indicating that it poses no (or very small) selective pressure on the bacteria. Consequently, the chance of resistance development will probably be smaller than for conventional antibiotics. Because quorum sensing disrupting compounds attenuate virulence of pathogenic bacteria without affecting growth, they have been termed antipathogenic drugs, as opposed to antibacterial drugs (Hentzer and Givskov, 2003). Antipathogenic drugs target key regulatory systems in bacterial pathogens that regulate the expression of virulence factors. The fact that antipathogenic compounds are unlikely to pose a selective pressure for the development of resistance, makes this concept highly attractive as a sustainable biocontrol strategy. Hence, it is certainly worthwhile to try synthesizing analogous compounds with lower toxicity, which could then be applied in practice.

Directions for further research

Biodegradation of signal molecules

AI-2 is produced from *S*-adenosylmethionine (SAM) in three enzymatic steps (Figure 2). When SAM is used as a methyl donor, *S*-adenosylhomocysteine (SAH) is produced. The enzyme Pfs converts SAH to *S*-ribosylhomocysteine (SRH), and subsequently, LuxS acts on SRH to make homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (Schauder *et al.*, 2001).

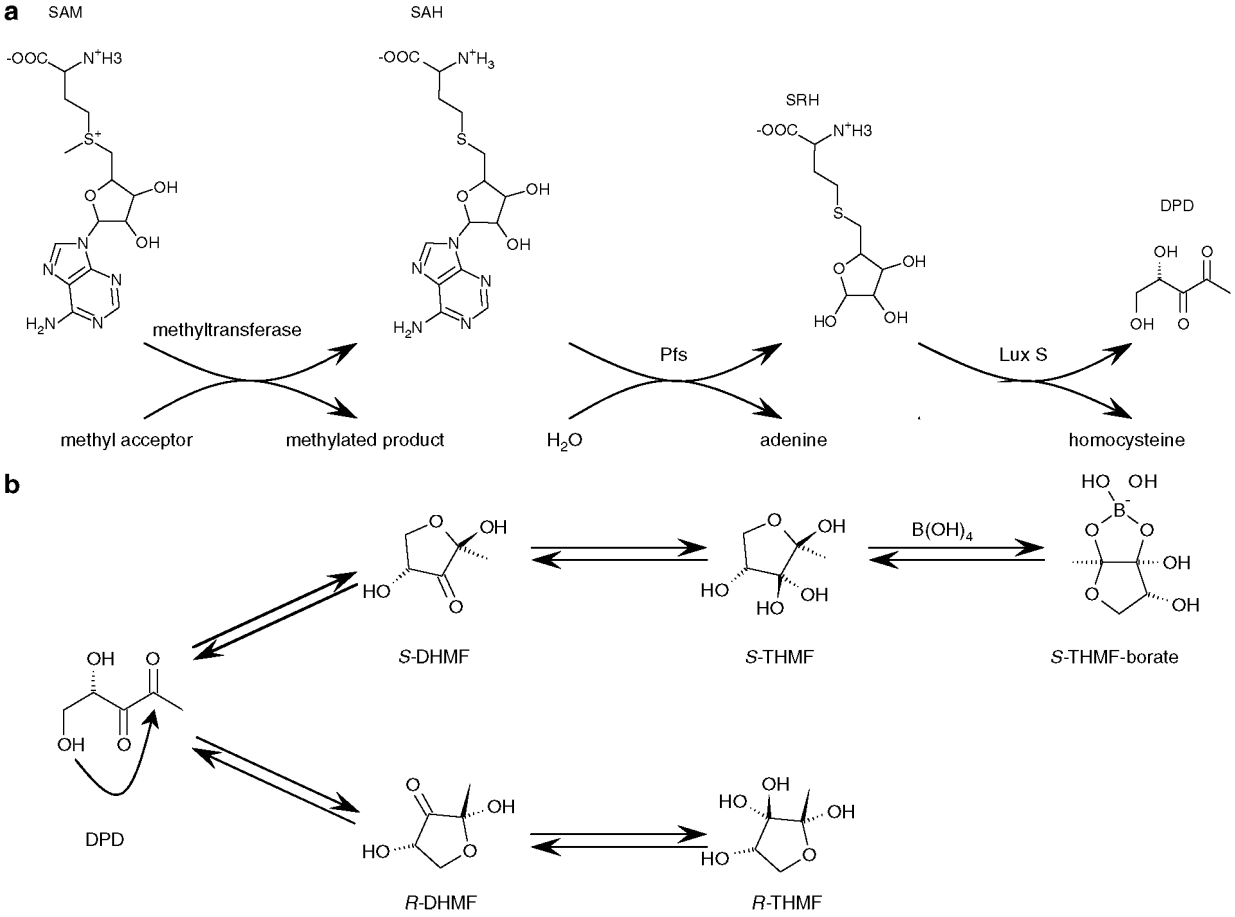

Figure 2 Biosynthesis of AI-2. (**a**) 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor to all AI-2, is synthesized from *S*-adenosylmethionine (SAM) in three enzymatic steps. SAH, *S*-adenosylhomocysteine; SRH, *S*-ribosylhomocysteine. (**b**) DPD rearranges and undergoes further reactions (all equilibria) to form distinct biologically active signal molecules that are generically termed AI-2. *Vibrio harveyi* AI-2 is produced by the upper pathway; *Salmonella typhimurium* AI-2 by the lower one. *S*-DHMF: (2*S*,4*S*)-dihydroxy-2-methyldihydro-3-furanone; *R*-DHMF, (2*R*,4*S*)-dihydroxy-2methyldihydro-3-furanone; *S*-THMF, (2*S*,4*S*)-2-methyl-2,3,3,4-tetra-hydroxy-tetrahydrofuran; *R*-THMF, (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydro-furan (based on Vendeville *et al.* (2005); De Keersmaecker *et al.* (2006)).

In *V. harveyi*, DPD cyclizes, is hydrated and is converted into the active AI-2 signal molecule (Chen *et al.*, 2002).

More recent research showed that the active AI-2 signal in *Salmonella typhimurium* has a different chemical structure when compared to *V. harveyi* AI-2 (Miller *et al.*, 2004). In *S. typhimurium*, AI-2 induces transcription of the *lsrACDBFGE* operon. The first four genes in this operon encode an ABC transporter, through which AI-2 is imported into the cells (Taga *et al.*, 2003). The other genes of the operon, together with the *lsrK* en *lsrR* genes, encode proteins that phosphorylate AI-2 and further degrade the signal (Taga and Bassler, 2003). A similar system has been described in *Escherichia coli* (Xavier and Bassler, 2005b). It is still unclear why these two species produce a signal, which then activates its own degradation. The signal might be used as a nutrient, although the bacteria cannot grow in minimal media containing AI-2 as the sole carbon source (Taga and Bassler, 2003). Another hypothesis is that by degrading AI-2, these bacteria trick their competitors into behaving as if there were no AI-2 (Federle and Bassler, 2003). In an exciting report, Xavier and Bassler (2005a) studied AI-2 cross talk between *V. harveyi* and *E. coli* and found that when co-cultured, *V. harveyi* produced only 18% of the bioluminescence it produced in pure culture. The effect was shown to be due to internalization and degradation of AI-2 by *E. coli* since no reduction occurred in co-cultures with mutants that are defective in AI-2 internalization.

The degradation of AI-2 by *E. coli* is not constitutive but is under control of several regulatory mechanisms, such as cAMP-CRP, the repressor LsrR and RpoS (De Keersmaecker *et al.*, 2006), which makes it inappropriate for practical applications. Since the different active AI-2 signal molecules are all in equilibrium with each other and with DPD (see Figure 2), inactivation of one of them will result in a decrease in the concentrations of all the other forms. However, no other bacteria than *E. coli* and

S. typhimurium have been reported that are able to degrade AI-2 or its precursor DPD. The enrichment, isolation and identification of AI-2- or DPD-degrading bacteria constitutes an intriguing area for further research.

Several reports have been published describing the degradation of AHLs by different bacteria. Enzymes that are able to inactivate AHLs have been discovered in different Gram-negative species belonging to the α-proteobacteria (Zhang *et al.*, 2002), the β-proteobacteria (Leadbetter and Greenberg, 2000; Lin *et al.*, 2003; Uroz *et al.*, 2003) and the γ-proteobacteria (Uroz *et al.*, 2003) as well as in some Gram-positive species (Dong *et al.*, 2002; Lee *et al.*, 2002; Uroz *et al.*, 2003). Interestingly, Tinh *et al.* (2007a) very recently isolated AHL-degrading enrichment cultures from the digestive tract of healthy Pacific white shrimp (*Penaeus vannamei*). The enrichment cultures protected rotifers from the negative effects of an AI-2 non-producing *V. harveyi* mutant, suggesting that the bacteria interfered with HAI-1-regulated gene expression *in vivo*. Also *Bacillus* strains that are currently used as probiotics in aquaculture (for example, Moriarty, 1998) would be worthwhile testing in this respect since different *Bacillus* spp. have been reported to degrade AHLs (Dong *et al.*, 2002; Lee *et al.*, 2002). It would be highly interesting to further investigate whether signal molecule-degrading bacteria (either AI-2 degraders alone or both AHL and AI-2 degraders together, depending on the host system) would be capable to disrupt *V. harveyi* quorum sensing and whether this could protect cultured animals from luminescent vibriosis. If they would be effective in controlling luminescent vibriosis, these bacteria could be used as a new kind of probiotics.

Inhibition of signal molecule biosynthesis

Another possibility to disrupt quorum sensing is the inhibition of signal molecule biosynthesis. This strategy has already been tested in the AHL-mediated quorum sensing system of *Pseudomonas aeruginosa* by applying substrate analogues for the AHL synthase enzyme RhlI (Parsek *et al.*, 1999). Interestingly, Alfaro *et al.* (2004) synthesized two inhibitors of the AI-2 synthase enzyme LuxS, *S*-anhydroribosyl-L-homocysteine and *S*-homoribosyl-L-cysteine. Both compounds are analogues of the LuxS substrate *S*-ribosyl-L-homocysteine. The compounds were tested *in vitro* using purified LuxS and were found to inhibit AI-2 production by the enzyme. Very recently, Shen *et al.* (2006) synthesized the first LuxS inhibitors with activity in the submicromolar range, with K_I values as low as 0.43 μM (≈115 μg l^{-1}). It would be interesting to test whether signal molecule synthase inhibitors could disrupt quorum sensing in *V. harveyi* and if they do, whether they have any impact on the virulence of luminescent vibrios towards aquatic organisms.

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