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Review Article

REVIEW ARTICLE

Quorum quenching: role in nature and applied developments

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One sentence summary: Bacterial quorum sensing-mediated signalling can be disrupted by a wide variety of phenomena collectively known as quorum quenching: the mechanisms behind this inhibition, their biological and ecological impact in microbe-microbe and host-microbe interactions, as well as some of the most recent developments of their applications in human health, agriculture, aquaculture and environmentally-friendly technologies, are presented and discussed in this review.

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ABSTRACT

Quorum sensing (QS) refers to the capacity of bacteria to monitor their population density and regulate gene expression accordingly: the QS-regulated processes deal with multicellular behaviors (e.g. growth and development of biofilm), horizontal gene transfer and host–microbe (symbiosis and pathogenesis) and microbe–microbe interactions. QS signaling requires the synthesis, exchange and perception of bacterial compounds, called autoinducers or QS signals (e.g. *N*-acylhomoserine lactones). The disruption of QS signaling, also termed quorum quenching (QQ), encompasses very diverse phenomena and mechanisms which are presented and discussed in this review. First, we surveyed the QS-signal diversity and QS-associated responses for a better understanding of the targets of the QQ phenomena that organisms have naturally evolved and are currently actively investigated in applied perspectives. Next the mechanisms, targets and molecular actors associated with QS interference are presented, with a special emphasis on the description of natural QQ enzymes and chemicals acting as QS inhibitors. Selected QQ paradigms are detailed to exemplify the mechanisms and biological roles of QS inhibition in microbe–microbe and host–microbe interactions. Finally, some QQ strategies are presented as promising tools in different fields such as medicine, aquaculture, crop production and anti-biofouling area.

Keywords: anti-virulence; *Pseudomonas*; *Agrobacterium*; *Chromobacterium*; lactonase; paraoxonase; amidase; acylase; homoserine lactone; quorum-sensing inhibitors

INTRODUCTION (SCOPE)

Numerous bacterial populations are able to monitor their population density and regulate their gene expression accordingly through quorum sensing (QS) (Fuqua, Winans and Greenberg 1994). These bacteria use QS signals to coordinate and synchronize several behaviors under differing environments, including microbe–microbe and host–microbe interactions. Since the 1990s, the number of studies on QS has continuously increased, this has been translated into an increasing number of

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publications as well as a remarkable diversification of the explored areas.

Studies on QS permitted the discovery of a large diversity of QS signals and QS-regulated functions in a broad range of bacteria and archaea. They extended the number of biological functions assigned to the QS-signal molecules beyond their primary involvement in QS. Thus, QS signals may also exhibit antibiotic activities and may induce some responses in organisms which do not produce them, including bacteria and eukaryotes. Hence, QS signals appear as multifunctional signals driving gene regulation and organism behavior from the cell to the holobiont levels (Salvucci 2014; Vandenkoornhuyse *et al.* 2015). QS-related studies also contributed to the emergence and diffusion of novel concepts and terms such as quorum quenching, antivirulence, diffusion sensing, mass transfer and sociomicrobiology (Fuqua, Winans and Greenberg 1994; Dong *et al.* 2001; Redfield 2002; Parsek and Greenberg 2005; Hense *et al.* 2007).

Quorum quenching (QQ) refers to all processes involved in the disturbance of QS (Dong *et al.* 2001). QQ molecular actors are diverse in nature (enzymes, chemical compounds), mode of action (QS-signal cleavage, competitive inhibition, and so on) and targets, as all main steps of the QS pathway that are synthesis, diffusion, accumulation and perception of the QS signals may be affected. Usually, the enzymes that inactivate QS signals are named QQ enzymes, while the chemicals disrupting QS pathways are called QS inhibitors (QSIs). Physical parameters such as temperature and pH may also affect half-life of QS signals (Byers *et al.* 2002; Yates *et al.* 2002; Delalande *et al.* 2005).

QQ is considered as a natural mechanism evolved either by QS-emitting organisms for the recycling or clearing of their own QS signals or by QQ organisms in the context of a competitive relationship with QS-signal-emitting organisms. Investigations on QQ also extended to applied domains, to develop antibacterial and anti-disease strategies that target pathogens and invasive populations in medicine, agronomy and water engineering. The development of treatments based on QS interference is largely driven by the need of alternative or complementary approaches to phytochemicals and antibiotics.

In this review, we aimed at positioning QS interference in the fast moving area that is QS. In the first part, a presentation of the diversity of QS signals and cognate functions will be proposed. This should provide the reader with a better understanding of the targets of QQ. Next, a survey of the mechanisms and molecular actors associated with QS disruption is developed, with a special emphasis on the description of the natural QQ actors, including QQ enzymes and QSIs. QQ in some models will be described in detail to exemplify the mechanisms, biological and ecological roles of QS interference in microbe–microbe and host–microbe interactions. Finally, some applied developments on QS disruption will be presented in different fields such as medicine, aquaculture, crop production and anti-biofouling.

QS: MULTIPLE SIGNALS, MULTIPLE FUNCTIONS

In the QS gene regulatory pathway, bacteria produce, exchange, perceive and respond to biochemical signals termed autoinducers (Nealson 1977) or QS signals. In more detail, each individual cell of a given bacterial population produces a QS signal, the overall concentration of which therefore mimics the cell density of this population. For a given QS system, once a threshold concentration (i.e. a threshold population density) is reached, the signal can be sensed and the expression of the target genes regulated. However, the perceived concentrations vary according to the QS systems, the regulators (see below) responding to different concentrations of signals along a spectrum. Furthermore, QS signals are structurally diverse and used by bacteria to regulate a wide variety of genes and functions.

Cell–cell communication involving *N*-acylhomoserine lactones (AHLs)

AHLs are synthesized by LuxI synthases (Schaefer *et al.* 1996) from S-adenosyl-methionine (SAM) and an acyl chain carried by an acyl carrier protein (for review, see Pereira, Thompson and Xavier 2013). Some AHL structures are shown in Fig. 1. Common AHL names will be abbreviated based on the size of the acyl chain and substitution at carbon 3, e.g. *N*-(hexanoyl)-L-homoserine lactone will appear as C6-HSL, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone will appear as OHC8-HSL and *N*-(3-oxododecanoyl)-L-homoserine lactone will appear as OC12-HSL.

AHLs are commonly produced by α-, β- and γ-Proteobacteria (for reviews: Case, Labbate and Kjelleberg 2008; Uroz, Dessaux and Oger 2009; Churchill and Chen 2011). Beyond the Proteobacteria, the presence of short-chain AHL (C4-HSL) has been detected in culture media of several *Tenacibaculum maritimum* strains (Romero *et al.* 2010; Romero *et al.* 2011), a fish pathogen from the Cytophaga–Flavobacterium–Bacteroides group. QS was also discovered in cultures of the Cyanobacterium *Gloethece* sp. where it has been involved in the increase of the RuBisCo expression (Sharif *et al.* 2008). Some unusual AHL signals have also been reported, such as *N*-carboxylated AHLs in the methanogenic archeon *Methanosaeta harundinacea* (Zhang *et al.* 2012), cinnamoyl-homoserine lactone (Ahlgren *et al.* 2011) or the branched-chain isovaleryl-homoserine lactone in the nitrogen-fixing plant symbionts *Bradyrhizobium* sp. and *Bradyrhizobium japonicum* (Lindemann *et al.* 2011). An unusual AHL signal, *p*-coumaroyl-homoserine lactone, has also been identified in *Rhodopseudomonas palustris* that obtains the precursor *p*-coumarate from the degradation of plant lignin (Schaefer *et al.* 2008).

Cell–cell communication involving other signals

AI-2 (Fig. 1) is the signal involved in the induction of bioluminescence in *Vibrio harveyi*, a bacterium closely related to *Photobacterium fischeri*. The AI-2 signal was originally identified as a borated tetrahydrofurane (Chen *et al.* 2002). The designation AI-2 now describes an interconverting equilibrium mixture of compounds, borated or not (for review, see Guo *et al.* 2013). These compounds originate from the spontaneous cyclization of the molecule 4,5-dihydroxy-2,3-pentanedione (DPD) synthesized via the LuxS synthase (Surette, Miller and Bassler 1999). Production of AI-2 was detected in a number of Gram-positive and Gram-negative bacteria, including pathogens such as *Salmonella typhimurium* or *V. cholerae* (for review, see Federle and Bassler 2003) and in the hyperthermophile archeon *Pyrococcus furiosus* (Nichols *et al.* 2009) with, in most cases, no yet-identified QS functions. AI-2 was also detected in bacterial cultures lacking either a QS-like response to the signal (e.g. Dove *et al.* 2003; Doherty *et al.* 2006) or the LuxS gene (Nichols *et al.* 2009). Some authors have therefore proposed that it could be a universal metabolic by-product rather than a QS signal (Winzer *et al.* 2002; Winzer, Hardie, Williams 2002; Turovskiy *et al.* 2007). A review of

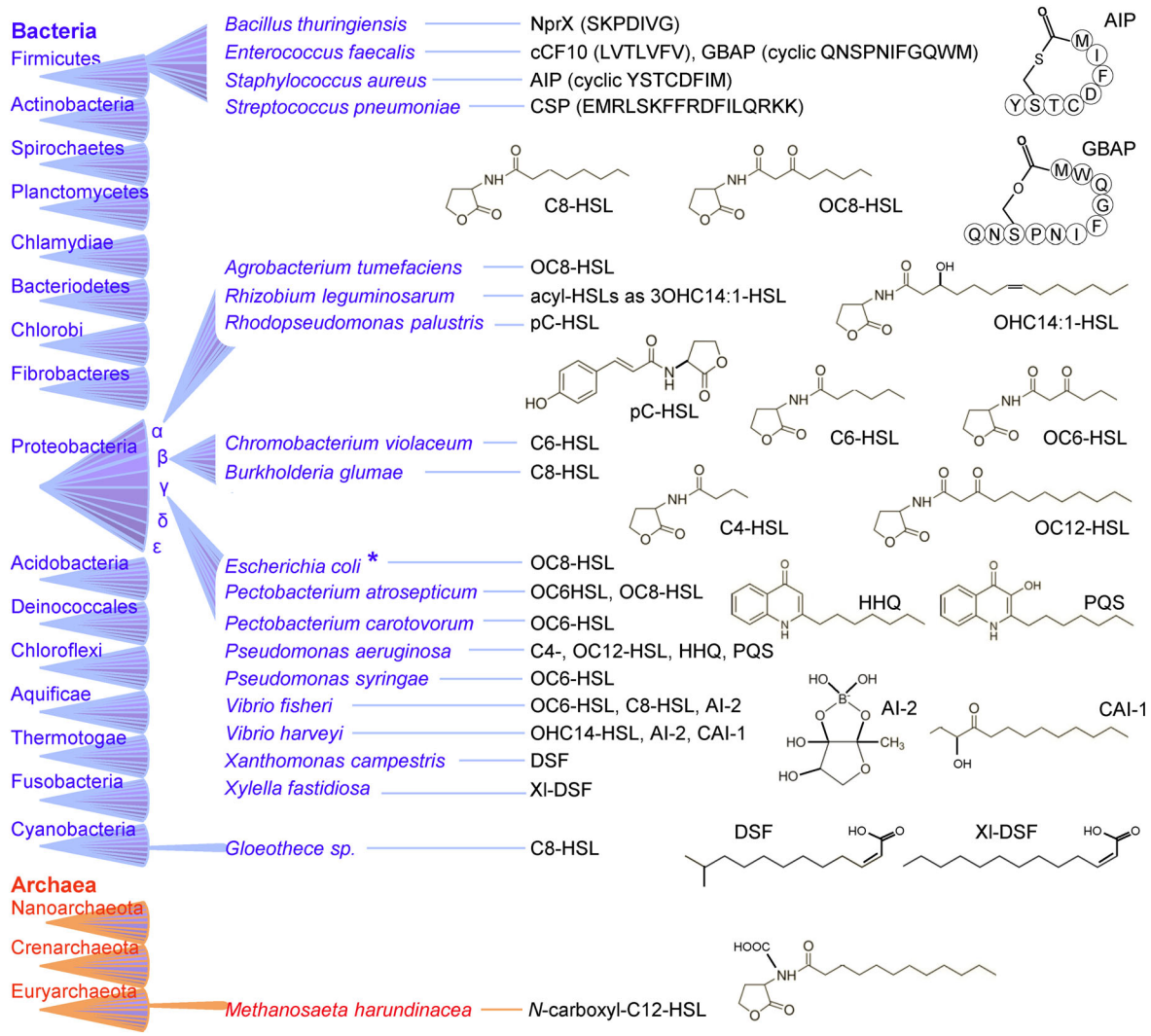

Figure 1. Diversity of QS signals. The diversity of QS signals is exemplified in different clades of bacteria and archaea. The asterisk indicates that *Es. coli* responds to, but does not produce, an OC8-HSL signal.

the literature suggests that the function of the AI-2 signal depends on the bacterial species (for review, see Pereira, Thompson and Xavier 2013). In this respect, it is tempting to speculate that the specific QS signaling function may have evolved from the wide metabolic signaling functions of AI-2.

Some other QS signals (Fig. 1) are derived solely from fatty acids such as the *cis*-11-methyl-2-dodecenoic acid (or diffusible signal factor, DSF) from *Xanthomonas* (for a review, see He and Zhang 2008) or the 3-hydroxypalmitate methyl ester (3-OH-PAME) from *Ralstonia* (Flavier *et al.* 1997). Others derive from amino acids, such as the diketopiperazines (e.g. cyclo(L-Pro-L-Tyr)) in Proteobacteria such as *Pseudomonas*, *Citrobacter*, *Enterobacter* (Holden *et al.* 1999) or the more complex autoinducer (cyclo)peptides in several Gram-positive bacteria (for a review, see Monnet, Juillard and Gardan 2014). Several archaea produce extracellular compounds that can activate an *Agrobacterium* AHL biosensor (Paggi *et al.* 2003; Tommonaro *et al.* 2012). One of these was identified as the diketopiperazine cyclo-(L-prolyl-L-Val) (Tommonaro *et al.* 2012). Other known signal molecules are butyrolactones in *Streptomyces* spp. and quinolones such as 2-heptyl-3-hydroxy-4-quinolone (PQS) in *Pseudomonas* (for review, see Jimenez *et al.* 2012).

QS signals: not only a perception of a population cellular density

The QS-controlled genes and functions are diverse and may be classified into four functional categories: cell maintenance and proliferation (exoenzymes production, sidérophores synthesis, sporulation, acid resistance, and so on); cell behaviors (biofilm formation and dispersal, motility, adhesion, and so on); horizontal gene transfer (plasmid conjugation, competence); and interactions with host and other microbes (virulence factors, exopolysaccharide production, bioluminescence, antibiotics, host colonization factors). Several reviews have described QS functions in diverse bacteria (Fuqua and Winans 1994; Whitehead *et al.* 2001; Jimenez *et al.* 2012; Monnet, Juillard and Gardan 2014).

Aside from being a density-dependent regulatory process that permits a synchronous expression of gene(s) in a bacterial population, QS can also be viewed as a way to sense how 'open' the bacterial environment is (Redfield 2002). This theory, known as diffusion sensing, has been reunited with the QS principle to generate the efficiency sensing concept. Efficiency sensing proposes that bacteria—via production of autoinducers—'can only measure the combination of cell density, spatial distribution and

limitations to autoinducer mass transfer' (Hense *et al.* 2007). According to this concept, signal molecules would therefore be a way for bacteria to probe the 'worthiness' of the production of costlier molecules, such as extracellular enzymes. Based on ecological models, it is clear that diffusion sensing and QS should not be viewed as opposed theories (West *et al.* 2012) and that additional roles for QS signals can be suggested (Platt and Fuqua 2010), such as cluster sensing (sensing the existence of a microcolony in an open environment) or position sensing (at the single cell level, possibility to sense its position with respect to the group).

In addition to their signaling roles, AHLs exhibit other biological properties which can modify interactions of the AHL-producing cells with their environment. For instance, long-chain AHLs may act as biosurfactants and antibiotics (Schripsema *et al.* 1996; Kaufmann *et al.* 2005; Daniels *et al.* 2006). The antibiotic activity of AHLs is exemplified by the compound *N*-(3-hydroxy-7-cis tetradecenoyl)-L-homoserine lactone (OHC14:1-HSL), also named 'small bacteriocin' (Wilkinson *et al.* 2002). This molecule is produced by a chromosomally encoded *luxI*-like gene and inhibits the growth of *Rhizobium leguminosarum* strains that host the symbiotic plasmid pRL1JI. The sensitivity to OHC14:1-HSL is conferred by two *luxR*-like genes located on pRL1JI. A third *luxR*-like gene located on the same plasmid mediates the repression of OH-C14:1-HSL synthesis, the presence of which is, however, necessary to induce the transfer of pRL1JI. This complex system therefore regulates the transfer of the symbiotic plasmid and the bacterial growth rate. The target of the small bacteriocin could be translation, but this remains to be formally demonstrated (Wilkinson *et al.* 2002). Another instance of antibiotic activity is OC12-HSL and its tetramic acid degradation product, 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione (Lowery *et al.* 2009b). This degradation compound is active on numerous Gram-positive bacteria unless they have the capacity to open the lactone ring of the AHL (Kaufmann *et al.* 2005—see section quorum quenching enzymes: biological roles below).

QS signals perception by eukaryotes

Several remarkable results changed our views on QS by demonstrating that bacterial QS signals can be sensed by eukaryotic organisms, including microorganisms. For instance, the growth of the filamentous form of the Saccharomycetaceae fungi *Candida albicans* is affected by OC12-HSL produced by *Pseudomonas aeruginosa* (Hogan, Vik and Kolter 2004). In relation, in dense culture, *C. albicans* produces the sesquiterpene alcohol farnesol that interferes with the PQS-mediated QS regulation in *P. aeruginosa* (Cugini *et al.* 2007). This interaction therefore appears as a good example of the chemical warfare that involves two organisms competing for the same ecological niche.

Another report of an interkingdom sensing of QS signals involves the green alga of the *Enteromorpha* genus, the zoospores of which attach themselves to bacterial biofilms. Using AHL-producing and AHL-nonproducing *V. anguillarum* strains, Joint *et al.* (2002) demonstrated that the zoospores only adhere to AHL-producing strains. A similar result has been reported for the zoospores of the macroalga of the genus *Ulva* that are attracted to bacterial biofilms and also preferentially settle on biofilms of AHL-producing bacteria (Tait *et al.* 2005). Similarly, cypris larvae of the barnacle *Balanus improvisus* explore bacterial biofilms and preferentially settle on those producing AHL (Tait and Havenhand 2013).

Plants also respond to QS signals. *Medicago truncatula* cells exposed to the QS signals OC16:1-HSL and OC12-HSL induce an increased production of 154 proteins (Mathesius *et al.* 2003). A comparable result was obtained in a transcriptomic study of *Arabidopsis* seedlings submitted to 3-oxo-octanoylhomoserine lactone (OC8-HSL) (Miao *et al.* 2012). Interestingly, the authors indicated that the chloroplasts (that originate from a microbial ancestor) are the intracellular organelles most influenced by the exposure to OC8-HSL. AHL may also modulate plant immunity. This was experimentally demonstrated using the AHL-producing bacterium *Serratia liquefaciens* which colonized roots of tomato plants (*Solanum lycopersicum*) and enhanced the systemic resistance of the plant challenged with the leaf pathogen *Alternaria alternata* (Schuhegger *et al.* 2006). Similarly, barley plants treated with OC14-HSL exhibit an elevated resistance to the fungal pathogen *Blumeria graminis*, and *Arabidopsis* roots treated with the same AHL generate a systemic resistance to the biotrophic fungus *Golovinomyces*. This systemic protection may be related to the priming effect that this AHL exerts on *Arabidopsis* plants (Schenk *et al.* 2014).

Bacterial QS signals can also be sensed by animals. Thus, OC12-HSL but not OC8-HSL inhibits lymphocyte proliferation and the production of the tumor necrosis factor alpha (TNF-α) by lipopolysaccharide-stimulated macrophages. OC12-HSL also decreases the production of interleukin IL-12 but significantly increases that of IgG1 and IgE (Telford *et al.* 1998). This last AHL induces an increased synthesis of IL-8 in human lung fibroblasts (Smith *et al.* 2001) and the acceleration of apoptosis in macrophages and neutrophils (Tateda *et al.* 2003). Overall, the wealth of information on OC12-HSL-induced interkingdom signaling contrasts with the paucity of data on the perception of other AHL by animals (for review, see Hughes and Sperandio 2008). This comes in contrast with the situation described above for plants in two ways. First, plants respond differentially to various AHLs, while so far studies involving animals only describe responses to OC12-HSL. Second, while AHL sensing by plants is not dependent upon the integrity of the AHL molecules, that of animals strongly depends upon it.

QQ AS A CONSEQUENCE OF PHYSICAL CONSTRAINTS

A featured example of chemical degradation of QS signals is lactonolysis of AHL compounds, a phenomenon that should also affect all lactone derivatives, including the *Streptomyces* butyrolactone signals. Lactonolysis, i.e. the opening of the lactone ring by addition of a water molecule, occurs spontaneously in aqueous solutions (Byers *et al.* 2002; Yates *et al.* 2002). It is strongly favored at high temperature and under alkaline pH, and can be reversed in acidic pH solutions. Under laboratory conditions, short-chain AHLs are more prone to degradation than long-chain AHLs are (Yates *et al.* 2002), and half-life of *N*-hexanoyl-homoserine lactone (C6-HSL) varies from over 21 days (pH 5.5, 4°C) to less than 30 min (pH 8.5, 37°C) (Byers *et al.* 2002; Delalande *et al.* 2005). These environmental parameters may therefore exert a strong control on the half-life of QS signals produced by bacteria when present in soils, water and plant and animal hosts. Indeed, animal and plant hosts are able to increase temperature and pH upon infection, therefore generating physical conditions promoting inactivation of AHL QS signals (Felix, Regenass and Boller 1993; Boller and Felix 2009).

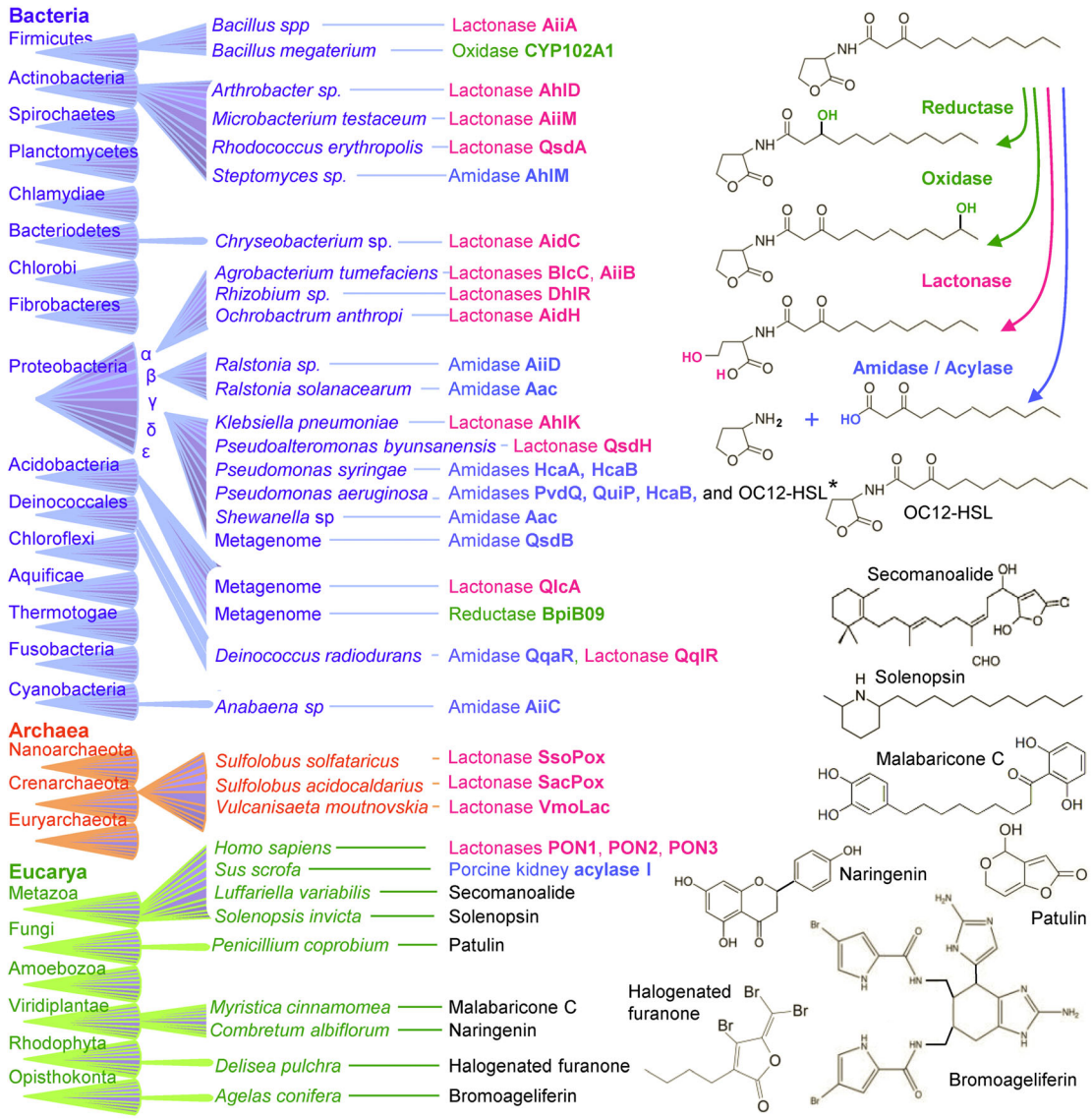

Figure 2. Diversity of QQ enzymes and QSIs. The production of QQ enzymes and QSIs is exemplified in different species of bacteria, archaea and eukaryotes. Enzymatic reactions are indicated at the top right of the figure: reductase and oxidase in green, lactonase in pink and amidase/acylase in blue color. QSIs are indicated in black fonts. The asterisk indicates that long-chain AHLs such as C12-HSL and C14-HSL may act as QSIs in short chain (C6-HSL) mediated QS signaling.

QQ ENZYMES: ORIGIN, DIVERSITY AND MODE OF ACTION

The first reports of an enzymatic degradation of a QS signal dealt with that of AHL by soil bacterial isolates of *Variovorax* and *Bacillus* genera (Dong *et al.* 2000; Leadbetter and Greenberg 2000). Since then, numerous enzymes involved in AHL degradation or modification have been reported. They represent four catalytic classes: the lactonases that open the homoserine lactone ring (e.g. Zhang, Wang and Zhang 2002; Uroz *et al.* 2008), the amidases (also referred as amidohydrolases or acylases) that cleave AHLs at the amide bond and release fatty acid and homoserine lactone (Lin *et al.* 2003), the reductases that convert 3-oxo-substituted AHL to their cognate 3-hydroxyl-substituted AHL (Bijtenhoorn *et al.* 2011a, b) and cytochrome oxidases that catalyze oxidation of the acyl chain (Chowdhary *et al.* 2007). They occur in bacteria, archaea and eukaryotes (Fig. 2). Interestingly, some bacteria are able to cleave their own AHL signal, such as *Agrobacterium* and *Pseudomonas*. Besides AHLs, other QS signals can be biologically degraded (for a review, see LaSarre and Federle 2013): they include 3-OH-PAME involved in the regulation of virulence in *Ralstonia* (Shinohara, Nakajima and Uehara 2007), the DSF produced by *Xanthomonas* (Newman *et al.* 2008) and the PQS by *Pseudomonas* (Pustelny *et al.* 2009). Diketopiperazines can also be degraded by microbial activities in a non-specific manner, i.e. irrespectively of their QS signal function (Kanzaki *et al.* 2000).

Amino acid sequence and architecture of the AHL-degrading enzymes are diverse, especially for the lactonases, as four lactonase families are known: the metallo-β-lactamase-like lactonases, the phosphotriesterase-like lactonases, the paraoxonases and the α/β-hydrolase fold lactonases. The metallo-β-lactamase-like lactonases display a unique fold like AiiA from *Bacillus thuringiensis* and AiiB from *Agrobacterium tumefaciens* (PDB codes 2A7M and 2R2D; Liu *et al.* 2005, 2007). Other members of this family such as BlcC—formerly AttM, AhlD and QlcA have

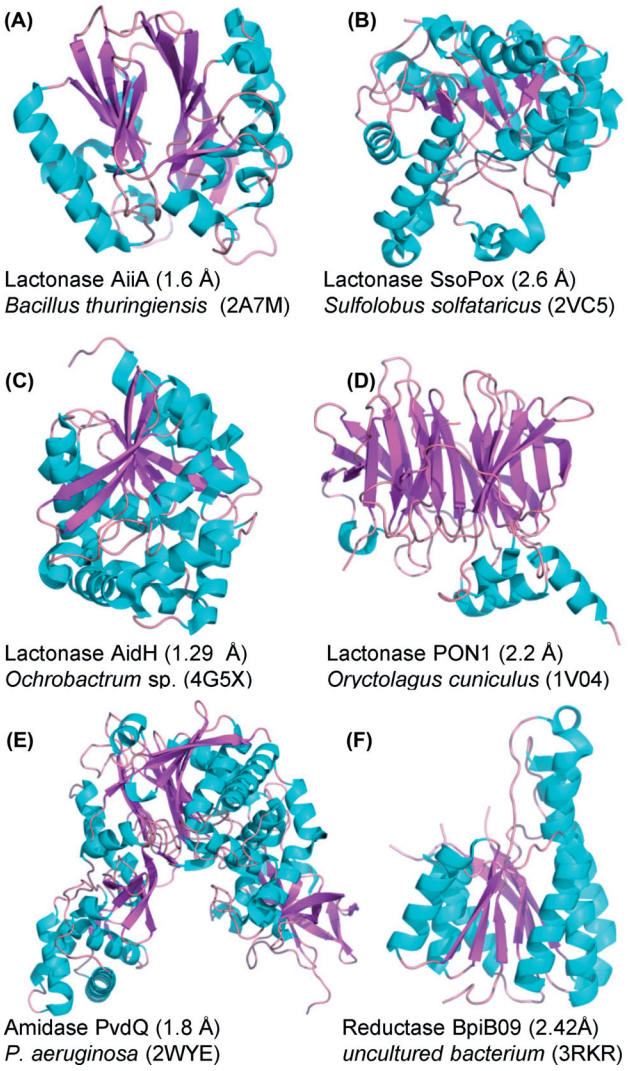

Figure 3. Structure of different enzymes degrading QS-signal AHLs. Ribbon representations of different QQ enzymes that belong to the families of the metallo-beta-lactamase like lactonase (**A**), the phosphotriesterase-like lactonase (**B**), the D-a/b hydrolase lactonase (**C**), the paraoxonase (**D**), the Ntn-hydrolase fold amidase (**E**) and NADP-dependent reductase (**F**). For all enzymes, the resolution and PDB code are indicated in brackets.

been identified in cultured bacteria and metagenomes (Dong *et al.* 2000; Zhang, Wang and Zhang 2002; Carlier *et al.* 2003; Park *et al.* 2003; Riaz *et al.* 2008). The fold of the phosphotriesterase-like lactonases consists in a TIM barrel as detected in SisLac from *Sulfolobus islandicus* and SsoPox from *S. solfataricus* (PDB codes 4G2D and 2VC5, Elias *et al.* 2008; Hiblot *et al.* 2012). This family encompasses other enzymes, such as QsdA and VmoLac (Uroz *et al.* 2008; Hiblot *et al.* 2013). The paraoxonases (extensively studied in mammals) adopt a six-bladed β-propeller fold like PON1 (PDB code 1V04, Harel *et al.* 2004; Ben-David *et al.* 2012). Despite different folds (Fig. 3), these three families share a similar catalytic mechanism using metal ions and key active-site similar architectures that have converged (Elias and Tawfik 2012). Indeed, the lactone substrate binds to the metal cation via its carbonyl oxygen, making the carbonyl carbon more electrophilic. An attacking water molecule is deprotonated either by one active-site metal or by a residue acting as a base. The resulting tetrahedral intermediate is subsequently broken (with protonation of the alkoxide leaving group) to give the hydrolyzed product. However, it should be noted that the catalytic mechanisms of these lactonases differ in detail. The structure of a α/β-hydrolase fold lactonase named AidH from the bacterium *Ochrobactrum* has been recently determined (PDB code 4G5X; Gao *et al.* 2013) and shows no metal-binding motif HXHXDH. Therefore, a novel catalytic mechanism was proposed which differs from that of other lactonases.

Known amidases/acylases are the porcine kidney acylase I and bacterial enzymes such as AiiD, PvdQ, AhlM, AiiC and QuiP that belong to the amidohydrolase cluster of the beta-lactam acylases (Huang *et al.* 2003, 2006; Lin *et al.* 2003; Park *et al.* 2005; Romero *et al.* 2008). The first crystal structure of an amidase, the PvdQ enzyme from *P. aeruginosa*, was reported in 2010 (PDB code 2WYE, Bokhove *et al.* 2010). The enzyme exhibits a typical α/β-heterodimeric Ntn-hydrolase fold with an unusually large hydrophobic-binding pocket adapted to the long acyl chain of the AHL substrates. Its catalytic mechanism proceeds via a covalently bound intermediate.

The first crystal structure of a QQ reductase, BpiB09 from a metagenomic library, has been published (PDB code 3RKR, Bijtenhoorn *et al.* 2011a, b). BpiB09 belongs to the so-called classical short-chain reductase family, the members of which contain a dinucleotide cofactor-binding site. The cytochrome P450 oxidase CYP102A1 of *B. megaterium* catalyzes the oxidation at the ω-1, ω-2 and ω-3 C atoms of the substrate acyl chain (Chowdhary *et al.* 2007). To date, no crystal structure of an oxidase has been determined.

Several studies pointed out functional, structural and phylogenetic proximities of QQ enzymes and xenobiotic/antibiotic-degrading enzymes (Elias and Tawfik 2012; Bar-Rogovsky, Hugenmatter and Tawfik 2013; Tannières *et al.* 2013). These could result from a convergent evolution process driven by structural similarities between signal and toxic molecules, or from diversification from common ancestors which had acquired additional catalytic properties as novel substrates emerged. The reconstruction of the paraoxonase phylogeny, including eukaryotic and bacterial PONs, and evaluation of their enzymatic properties suggest that inactivation of QS signal would be an ancestral function of PON enzymes which vertebrates would have acquired by horizontal transfer from bacteria (Bar-Rogovsky, Hugenmatter and Tawfik 2013).

QQ ENZYMES: BIOLOGICAL ROLES

QQ lactonase to prevent AHL-mediated toxicity

AiiA, the prototypic AHL lactonase, has been identified in several strains of members of the *Bacillus* genus (Dong *et al.* 2000, 2002). Because AHLs are generally not used as QS signals in Gram-positive bacteria, the ecological role of this lactonase in *Bacillus* remains an intriguing question. As with most lactonases, AiiA hydrolyzes a large number of substrates with acyl chain length ranging from 4 to 12 carbon atoms, with or without oxosubstitution at carbon 3. Interestingly, the AHL OC12-HSL, as well as a spontaneously degradation product formed in aqueous environments, namely the tetramic acid 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione, was reported to be potent antibacterial molecules. The bactericidal activity was observed against several Gram-positive strains but not against Gram-negative bacteria (Kaufmann *et al.* 2005). Furthermore, the tetramic acid exhibits a potent iron-binding capability. *Bacillus* strains that express the lactonase AiiA appear to be insensitive to OC12-HSL because the opening of the lactone cycle prevents

the formation of the tetramic acid derivative. These findings can be related to the observed fitness reduction of an AiiA mutant strain of *B. thuringiensis* in the rhizosphere of pepper plants (Park *et al.* 2008b). Overall, these observations suggest that the ecological role of AiiA in Gram-positive bacteria could be more oriented toward detoxification than quenching only. Based on the observation that these bacteria are sensitive to AHLs and AHLs derivatives, it is tempting to propose that in *Bacillus* AHLs act as antibiotics and AHL-lactonase-encoding genes as antibiotic resistance genes.

QS-signal clearing by the *Agrobacterium* QQ lactonases

In *A. tumefaciens* strains C58 and R10, a unique LuxI-LuxR system, named TraI-TraR, is encoded by the Ti plasmid and controls, via the QS-signal OC8-HSL, both the amplification (i.e. the increase of the copy number) and horizontal transfer (conjugation) of this plasmid from a donor (virulent) to a recipient (avirulent) bacterial cell (Fuqua and Winans 1994; Hwang *et al.* 1994; Pappas and Winans 2003; Lang *et al.* 2013). Opines, such as octopine in strain R10 and agrocinopines A and B in strain C58, stimulate the transcription of the *traR* gene, the product of which dimerizes when associated with two OC8-HSL molecules (Zhang, Wang and Zhang 2002). The TraR-OC8-HSL complex stimulates the transcription of QS-regulated genes, including *traI*. Hence, QS occurs only in an opine-rich environment such as a tumor and directly contributes to the dissemination of the Ti plasmid.

Two cytoplasmic AHL-lactonases AiiB and BlcC (formerly AttM) have been discovered in *A. tumefaciens* (Zhang, Wang and Zhang 2002; Carlier *et al.* 2003). The structure of the lactonase AiiB has been solved (Liu *et al.* 2007). The AiiB-encoding gene is borne on the Ti plasmid of the virulent strains C58 and S56 as well as on the 185-kbp plasmid of the commercialized *A. radiobacter* biocontrol agent K84. The BlcC lactonase is encoded by the At plasmid of several virulent (e.g. A6, TT111, S56 and C58) and avirulent (e.g. CFBP5771 and CFBP5621) strains (Khan and Farrand 2009). The occurrence of *blcC* and *aiiB* was investigated by a PCR-based search among 600 *Agrobacterium* isolates collected from tomato and tobacco rhizospheres. This approach revealed that over 90% of the isolates harbored a *blcC* gene and were able to inactivate the supplemented AHL (Haudecoeur *et al.* 2009a).

Over the last decade, *A. tumefaciens* has been the bacterial model in which the regulation and function of the QQ lactonases have been the most intensively investigated. Some of these studies indicated that AHLs cannot be used as a sole C or N sources by *A. tumefaciens* (Carlier *et al.* 2004; Chai *et al.* 2007). A clearing function (rather than a recycling one) may therefore be proposed to describe the biological role of the lactonases. Neither AiiB nor BlcC lactonases are regulated by QS signals such as OC8-HSL or other AHLs (Carlier *et al.* 2004; Haudecoeur *et al.* 2009a). The transcription of the *aiiB* gene is stimulated in the presence of agrocinopine-enriched plant extracts (Haudecoeur *et al.* 2009a) and thus appears to be coregulated with the other Ti plasmid-encoded QS genes that are the AHL-sensor TraR, AHL-synthase TraI and anti-TraR regulator TraM. The expression of the *blcC* gene, which belongs to the *blcABC* operon (formerly *attKLM*), is enhanced in the presence of succinic semialdehyde (SSA), gamma-hydroxybutyrate (GHB), gamma-butyrolactone (GBL), gamma-aminobutyrate (GABA) and salicylic acid (Carlier *et al.* 2004; Chevrot *et al.* 2006; Yuan *et al.* 2008). However, *in vitro* both SSA and GHB, but neither GBL nor GABA, prevent DNA binding of the transcriptional repressor BlcR at the promoter of the *blcABC* operon (Chai *et al.* 2007). GABA and GBL should therefore be converted to SSA and GHB, respectively, to exert a role in the transcription of *blcABC*. The conversion of GBL into GHB and that of GHB into SSA is operated by BlcC and BlcB, respectively (Carlier *et al.* 2004; Chai *et al.* 2007). The enzyme required for the conversion of GABA into SSA is most likely a transaminase, but remains unidentified in *Agrobacterium*.

BlcC-inducing compounds, such as GABA and salicylic acid, accumulate in *A. tumefaciens*-infected plants and *A. tumefaciens*-induced plant tumors (Deeken *et al.* 2006). The ABC-transporter Bra and periplasmic-binding protein Atu2422 are required for the uptake of GABA and therefore for the GABA-induced expression of *blcABC* (Chevrot *et al.* 2006; Haudecoeur *et al.* 2009b). Remarkably, free amino and imino acids such as alanine, proline and valine antagonize the uptake of GABA, hence the GABA-induced degradation of OC8-HSL (Planamente *et al.* 2010). Because proline accumulates in the mature plant tumor, this imino acid would be the most probable natural antagonist of GABA signaling in *A. tumefaciens* (Haudecoeur *et al.* 2009b). Noticeably, the expression of the GABA-sensor Atu2422-encoding gene is repressed by the small RNA AbcR1 (Wilms *et al.* 2011), but a correlation between the AbcR1 activity and expression of the lactonase BlcC remains to be demonstrated. In a mutant defective for the GABA-sensor Atu2422, an alternative GABA-sensor Atu4243 may be used for the importation of GABA (Planamente *et al.* 2012).

Several studies analyzed the conjugation frequency of the Ti plasmid using donor strains in which the lactonase-encoding genes *aiiB* and *blcC*, as well as regulatory gene *blcR*, were mutated. *In vitro* and *in planta*, the *aiiB* and *blcC* mutants transfer their Ti plasmid slightly earlier than does a wild-type strain (Haudecoeur *et al.* 2009a; Khan and Farrand 2009). In contrast, a *blcR* (formerly *attJ*) mutant that constitutively expresses the BlcC lactonase exhibits a strongly delayed Ti-plasmid transfer (Zhang, Wang and Zhang 2002; Khan and Farrand 2009). All these reports highlight that the lactonase-mediated moderation of plasmid Ti transfer remains transitory and suggest that lactonases only control the accumulation of QS signals at an early stage of the development of plant tumor. Similarly, the antiactivator TraM also contributes to delay QS signaling (Fuqua, Burbea and Winans 1995; Qin, Su and Farrand 2007). A model of the lactonase role is proposed in Fig. 4.

A recurring question is why *A. tumefaciens* needs to prevent QS-regulated transfer of Ti plasmid at the early stage of the infection process? Considering that the activation of the type IV secretion system involved in the conjugal transfer of the Ti plasmid could be costly for the cells, a possibility exists that the Ti-plasmid donor cells would first multiply in the tumor niche until, being dominant, they activate the Ti plasmid transfer machinery. Consistent with this view, several weeks after infection, when aging tumors produce opines at a high level, the control exerted by the lactonases and antiactivator TraM on conjugation is released. Several non-exclusive mechanisms have been proposed to explain this phenomenon (for a review, see Lang and Faure 2014). First, the simplest one suggests that the kinetics of OC8-HSL-synthesis overcomes that of the lactonase-mediated OC8-HSL-degradation (Khan and Farrand 2009; Haudecoeur *et al.* 2009a). Secondly, the affinity of OC8-HSL for the sensor TraR would be much higher than it may be for the lactonases (Zhu and Winans 1999; Liu *et al.* 2007). Thirdly, the OC8-HSL molecules that are bound to TraR would be protected from a lactonase activity (Khan and Farrand 2009). Taken together, the second and third arguments may explain why lactonases cannot completely obliterate QS signal and prevent the transfer of the Ti plasmid. Fourthly, the expression of the lactonase-encoding genes could be tightly controlled by plant tumor-derived compounds such

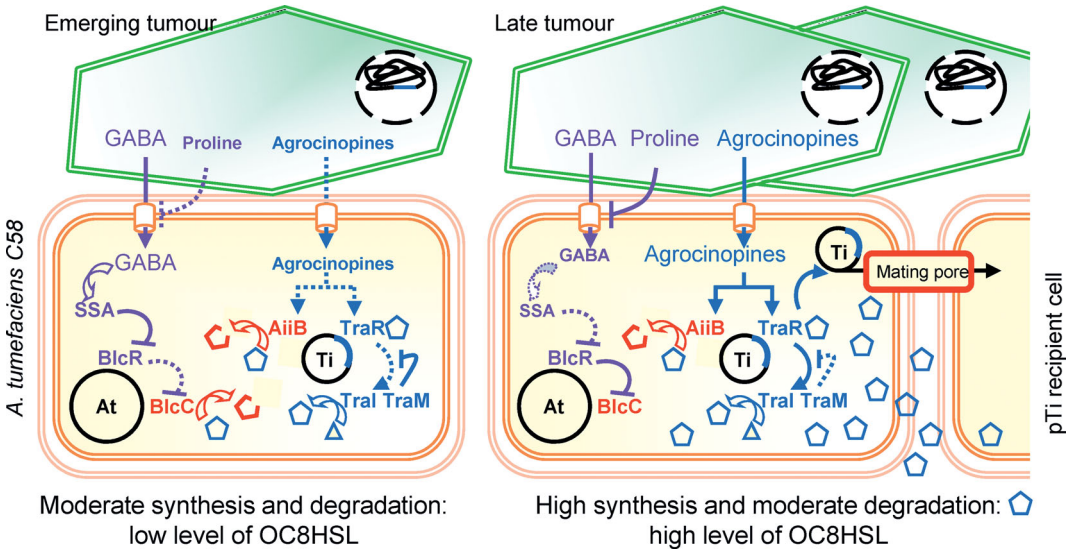

Figure 4. QS and QQ in *A. tumefaciens*. The *A. tumefaciens* C58-plant host model aggregates data from different papers cited in the text, although some plant compounds (e.g. salicylic acid, SSA or GHB) which, in addition to GABA, activate the expression of the lactonase BlcC, are omitted for clarity. The emerging tumor state refers to the first 2 weeks after infection, while the late tumor state to the 4-week (or more) tumor. In emerging tumors, agrocinopines start to accumulate and moderately stimulate the TraI-mediated biosynthesis of QS-signal OC8-HSL, which is repressed by the anti-activator TraM. At the same time, agrocinopine-induced AiiB-lactonase and GABA-induced lactonase BlcC cleave OC8-HSL signals which are unbound to TraR. As a consequence, the QS-regulated transfer of the Ti plasmid to recipient cells is a rare or undetectable event. In late (aging) tumors, agrocinopines are produced at a high rate and strongly activate OC8-HSL synthesis which overcomes anti-TraR activity of TraM and AiiB-mediated OC8-HSL degradation. Proline accumulates and antagonizes the transport of GABA, hence limiting the expression of BlcC. These conditions are permissive for QS transfer of plasmid Ti to non-virulent bacterial populations surrounding the plant tumor. Legends: Letter size of the words GABA, Proline and Agrocinopines refers to their relative abundance in the plant tumour at both states; plain and dashed lines to the active and attenuated regulatory pathways in *A. tumefaciens* C58; blue pentagons are OC8-HSL, red open pentagons are lactonase-cleaved OC8-HSL.

as free proline that antagonizes the GABA-induced expression of BlcC-encoding gene (Haudecoeur *et al.* 2009b).

In addition, the possibility that a lactonase exerted some functions unrelated to QS—as seen above in *Bacillus* sp.—should not be excluded. Unlike the AiiB lactonase which exhibits a high selectivity for AHL (Liu *et al.* 2007), the BlcC lactonase can hydrolyze several lactones, including GBL, that is converted to GHB. The two other genes which are part of the *blcABC* operon encode the conversion of GHB to SSA, and that of SSA to succinic acid (Carlier *et al.* 2004; Chai *et al.* 2007). While GABA accumulated at a high level in plant tumor (Chevrot *et al.* 2006; Deeken *et al.* 2006), the presence of GBL or GHB in plant tumor remains unknown.

QS-signal recycling by the *P. aeruginosa* QQ amidases

The virulence of *P. aeruginosa* depends on the production of cell-associated and secreted components, such as pili, flagella, lipopolysaccharide, siderophores, proteases and toxins, all of which contribute to the efficient invasion and colonization of host tissues, and formation of biofilms. Intricate regulatory pathways control the production of the virulence factors, biofilm maturation and swarming motility (for a review, see Jimenez *et al.* 2012). These regulatory pathways involve several signal molecules such as the two AHLs, OC12-HSL and C4-HSL, and 2-alkyl-4-quinolones (AQ) such as 2-heptyl-3-hydroxy-4-quinolone, also termed the *Pseudomonas* quinolone signal (PQS) and its precursor 2-heptyl-4-quinolone (HHQ) (Latifi *et al.* 1995; Diggle *et al.* 2003, 2007b). The synthesis and perception of OC12-HSL and C4-HSL are driven by two LuxI-LuxR systems, LasI-lasR and RhlI-RhlR, respectively. Together with AHLs, LasI-lasR and RhlI-RhlR modulate the expression of over 300 genes (Schuster *et al.* 2003; Wagner, Gillis and Iglewski 2004). The orphan LuxR homolog QscR, which is able to bind OC12-HSL and forms homodimers and heterodimers with LasR and RhlR, prevents the premature activation of the QS-regulated genes (Chugani *et al.* 2001; Ledgham *et al.* 2003; Lintz *et al.* 2011).

Remarkably, *P. aeruginosa* is able to degrade and assimilate its own AHL signals via amidase and lactonase activities, but only the amidase encoding genes *pvdQ* (PA2385), *quiP* (PA1032) and *hacB* (PA0305) have been characterized (Huang *et al.* 2003, 2006; Wahjudi *et al.* 2011). PvdQ, which is the first identified AHL amidase in *P. aeruginosa*, was demonstrated to be non-essential for AHL assimilation by this bacterium but remains crucial for the catalysis of one of the final steps of pyoverdine maturation and contributes to virulence and swarming motility under iron limitation conditions (Bokhove *et al.* 2010; Jimenez *et al.* 2010; Drake and Gulick 2011). A *quiP* mutant is defective in the use of OC12-HSL as a sole carbon and energy source (Huang *et al.* 2006), but in rich medium a *pvdQ quiP* double mutant accumulates AHL to the same level as the wild-type strain (Wahjudi *et al.* 2011). The role of QuiP in the turnover of AHL in the course of interactions with animal hosts is still unknown (Huang *et al.* 2006). The third amidase, HacB, is active toward long-chain AHL such as OC12-HSL. The *hacB* simple mutant and *pvdQ quiP hacB* triple mutant accumulate AHL to a higher level than the wild type and *pvdQ quiP* double mutant. Moreover, an *hacB* mutant shows a slightly increased capacity to kill *Caenorhabditis elegans* when compared with the *P. aeruginosa* PAO1 wild-type strain (Wahjudi *et al.* 2011), demonstrating a role of the amidase HacB in *P. aeruginosa* virulence. The expression of the *hacB* gene is not regulated by QS and the molecular and environmental factors that control its expression *in vitro* and in infected hosts remain undetermined. The *hacB* gene in *P. aeruginosa* was identified by sequence similarity with the AHL-cleaving amidases HacA and HacB of *P. syringae* (Shepherd and Lindow 2009). In this plant pathogen, however, the role of these two amidases remains unknown.

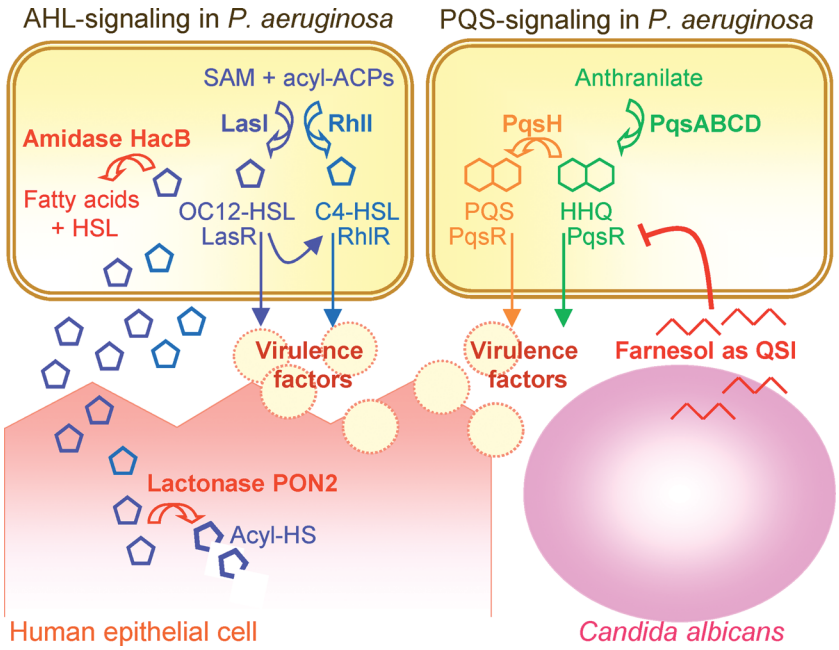

Figure 5. QS and QQ in *P. aeruginosa*. This scheme exemplifies some QS and QQ actors during human infection by *P. aeruginosa* and *C. albicans*. While the AHL-QS and PQS-QS pathways are connected, they are arbitrary represented in separate *P. aeruginosa* cells. During skin and lung infection, the AHL-synthases LasI and RhlI convert SAM and acyl-ACPs in OC12-HSL and C4-HSL, respectively. The complexes between OC12-HSL and the sensor LasR, and C4-HSL and the sensor RhlR activate the transcription of several genes encoding virulence factors as well as AHL synthases LasI and RhlI. The complex LasR-OC12-HSL also stimulates the transcription of *rhlR* gene. In *P. aeruginosa*, the amidase HacB (but also PvdQ and QuiP) may contribute to AHL recycling by converting AHLs into fatty acids and homoserine lactone (HSL). The major human lactonase expressed in airway epithelial cells and keratinocytes is the paraoxonase PON2 which opens the AHL ring and releases acyl-homoserine (Acyl-HS). The precursor of the PQS-signaling pathway is anthranilate which is converted into HHQ by the enzymes encoded by the *pqsABCD* operon. Then, PqsH converts HHQ into PQS. HHQ and PQS bind the sensor PqsR and the complexes regulate the expression of virulence factors. Among human microbiota, the yeast *C. albicans* may coexist with *P. aeruginosa* and produce farnesol. Farnesol may act as QSI and binds PqsR, hence disturbs PQS-regulated genes, including those encoding PQS synthesis. See the main text for additional roles of AHLs, PQS and farnesol.

QS-signal degradation by mammalian paraoxonases

Several eukaryotes, including plants and animals, express enzymes that are able to inactivate QS signals. None of the plant enzymes have been characterized so far. Consequently, only animal ones have been investigated and among these, the mammalian paraoxonases have received the most attention. In Fig. 5, three QQ mechanisms, which may affect QQ in *P. aeruginosa*, are shown.

Paraoxonases are a group of three enzymes (PON1, PON2 and PON3), highly conserved in vertebrates and, in particular, in mammals. In humans, the three *PON* genes are located on the long arm of the chromosome 7. *PON1* and *PON3* are mainly expressed in liver and kidney cells where they are secreted into the serum, while *PON2* is expressed in various tissues (Mochizuki *et al.* 1998; Draganov and La Du 2004; Stoltz *et al.* 2007). The name paraoxonase originates from their ability to hydrolyze paraoxon, a metabolite of parathion (Aldridge 1953). Paraoxonases exhibit a broad substrate range (Draganov and La Du 2004) including AHLs. PON2 efficiently cleaves AHLs and also exhibits an arylesterase activity (Draganov *et al.* 2005). Purified PON1 and PON3 show a lower activity toward AHLs, but they accept a wide range of substrates, including organophosphates, arylesters, gamma-lactones and delta-lactones (Draganov *et al.* 2005). PON1—the structure of which has been solved (Harel *et al.* 2004)—is well known for its activity on organophosphorous insecticides (parathion, diazinon and chlorpyrifos) and fatty acids.

Several studies evaluated the implication of paraoxonases in defense against the pathogen *P. aeruginosa*. The serum and tracheal epithelial cells of mammals are able to efficiently inactivate long-chain AHLs, including OC12-HSL, but they exhibit a weak or null cleaving activity toward C4-HSL (Chun *et al.* 2004; Ozer *et al.* 2005; Yang *et al.* 2005). Pioneering research established a link between the paraoxonases and lactonase-mediated inactivation of AHL in mammals (Draganov *et al.* 2005; Khersonsky and Tawfik 2005; Ozer *et al.* 2005; Yang *et al.* 2005). In relation, mammalian cells overexpressing each of three PONs inactivate AHLs faster than non-transfected cells do (Ozer *et al.* 2005; Yang *et al.* 2005; Stoltz *et al.* 2007).

Among invertebrates, arthropods do not have PONs. The ectopic expression of human PON1 in *Drosophila melanogaster* reduces the OC12-HSL-mediated virulence of *P. aeruginosa*, decreases the superoxide anion level and alters the composition of the gut microbiota (Stoltz *et al.* 2008; Pezzulo *et al.* 2012). Using PON1-KO mice, Ozer *et al.* (2005) showed that the activity of PON1 suffices to explain most of the AHL lactonase activity in serum. Moreover, while serum collected from a wild-type mouse reduces *P. aeruginosa* biofilm development by 75%, serum from a PON1-KO mice only reduces biofilm formation by 15% (Ozer *et al.* 2005). In addition, using a QS-reporter *P. aeruginosa* strain in which the *qsc102* OC12-HSL-responsive promoter was fused to the reporter gene *lacZ*, the QQ role of PON1 in serum was confirmed (Stoltz *et al.* 2007). These experiments revealed the capacity of PON1-containing sera to quench QS-mediated biofilm formation by *P. aeruginosa in vitro*. By contrast, using a sepsis infection model in which the death of mice depended on the production of OC12-HSL by *P. aeruginosa*, the same authors observed an increased survival of PON1-KO mice as compared to that of wild-type mice (Ozer *et al.* 2005). This observation is the opposite of the expected result and remains unexplained. Non-exclusive

hypotheses have been proposed, such as the expression of compensatory mechanisms in PON1-KO mice that would increase protection against *P. aeruginosa* (priming effect or upregulated expression of PON2 and PON3).

The major PON expressed in human airway epithelial cells is PON2, while both PON2 and PON3 are expressed at a high level in murine tracheal cells (Stoltz *et al.* 2007). Three KO lines of mouse affected in PON1, PON2 and PON3 were constructed and used to measure OC12-HSL-degrading activity in cell lysates collected from tracheal epithelial cells. Only cell extracts of the PON2-KO mutant exhibited a dramatically reduced activity toward AHL, confirming the key role of PON2 in AHL-degrading activity of tracheal epithelial cells. Moreover, when a cell suspension of *P. aeruginosa* expressing the QS-responsive promoter *qsc102* fused to a *lacZ* gene was applied to a murine tracheal cell culture, a higher reporting activity was observed in the presence of PON2-KO murine cells as compared to that observed with cells of the wild-type mice. Taken together, these data suggest that PON2 may be a major player in AHL degradation and QQ by lung epithelial cells *in vitro*. The use of PON2-deficient mouse highlighted the involvement of this lactonase in *P. aeruginosa* clearance in liver, lungs and spleen, as well as AHL degradation capability of macrophages (Devarajan *et al.* 2013). Using a siRNA approach that targetted PON2 gene expression, the involvement of this lactonase in OC12-HSL degradation was also demonstrated in lysates of both hepatoma and endothelial cell lines (Teiber *et al.* 2008). A similar siRNA technique was used in keratinocytes to demonstrate the key role of PON2 in OC12-HSL degradation, control of *P. aeruginosa* proliferation and expression of virulence genes (Simanski *et al.* 2012). Recent work also revealed that the application of OC12-HSL on lung epithelial carcinoma cells (human A549 cells) downregulates PON2 mRNA levels, protein and hydrolytic activity of OC12-HSL through a Ca^{2+}-dependent mechanism (Horke *et al.* 2010). This feature suggests that the pathogen would be able to limit PON2-mediated OC12-HSL degradation. In other cell lines, such as macrophages, PON2 expression is not altered in response to OC12-HSL (Devarajan *et al.* 2013). However, all the above research was performed on cell cultures, and the role of PON2 remains to be explored in whole mice using double or triple PON mutants, as suggested by different authors. The expression of PON2 was also measured in cells collected from bronchoalveolar lavage fluid of children suffering from cystic fibrosis, who were infected, or not, with *P. aeruginosa* (Griffin *et al.* 2012). Gene expression of PON2 is approximately 2-fold lower in bronchial cells of *P. aeruginosa*-positive patients than in bronchial cells of *P. aeruginosa*-negative patients. In this experiment, the expression of the OC12-HSL-synthase encoding gene *lasI* of *P. aeruginosa* was also verified in cells from the infected patients. These results established a correlation between a decrease of PON2 expression and early childhood infection of *P. aeruginosa*.

Overall, the above data has led to the identification of four roles for QQ enzymes. The first one is related to the fine-tuning of QS functions (QS-signal clearing in *A. tumefaciens*), while the second one is recycling of QS signals (model *P. aeruginosa*). These occur mostly in microorganisms that produce QS molecules. The third one is the disturbance of QS signaling by an organism which does not produce QS signals, but may take advantage of QQ processes such as the hosts of QS-emiting pathogens. The fourth one is a detoxication role. It occurs in microorganisms that do not produce QS but are sensitive to the toxicity of some QS signals. However, this classification of QQ enzymes according to their biological roles remains mostly tentative. Indeed, the number of microbial models in which QQ enzymes have been clearly identified at the molecular and biochemical levels is low. Even lower is the number of models that have been investigated under environmentally sound conditions, and lower again and the number of systems in which the regulation of QQ functions has been investigated and identified. Aside from the exploration of novel QQ activities, and instrumental to generate novel paradigms, a major effort should therefore be continued toward the deciphering of QQ functions in a coherent biological context, at the cell, population, microbiota and holobiont levels.

QSIs: ORIGIN AND DIVERSITY

Identification of QSIs: from screening to synthesis

To search for QSIs, an obvious approach consisted of screening organisms (among which medicinal plants have been frequently investigated), cells, tissues and chemical libraries using various bacterial QS-signal biosensors (for a review, see Rai, Rai and Venkatesh 2015).

To screen libraries or to demonstrate the efficiency of the potential QSIs, different methods exist, dependent upon the scale of screening and targeted QS system. The easiest ones involve colorimetric tests with biosensors such as *Chromobacterium violaceum* strain CV026 which allow the study of QSIs that affect C4- to C8-AHLs-based QS systems (McClean *et al.* 1997). Similarly, *A. tumefaciens* strain NT1(pZLR4) allows the expression of β-galactosidase upon addition of exogenous AHL and the identification of QSIs that affect C8- to C16-AHLs-based QS systems (Cha *et al.* 1998). In the latter system, sensitivity is higher when AHLs are oxidized at carbon-3 or with the luminescent substrate beta-Glo instead of the X-Gal substrate (Kawaguchi *et al.* 2008). Other series of tests target known QS-regulated functions such as the motility of *Yersinia enterocolitica* (Atkinson *et al.* 2006) or pyocyanin (Reimmann *et al.* 1997) or pyoverdin (Adonizio, Kong and Mathee 2008) production in *P. aeruginosa*. Some QSIs studies are focused on biofilms formation. They rely on qualitative methods such as Congo red agar (Nasr 2012) or quantitative ones such as the Microtiter plate assay (Thenmozhi *et al.* 2009) or the crystal violet assay (Girennavar *et al.* 2008) to evaluate the characteristics of the biofilms.

However, these tests cannot be used for high-throughput screening of QSIs. To optimize large screenings, other methods have been developed, such as the QSI selector system (Rasmussen *et al.* 2005). This system involves a plasmid that carries a construct consisting in a *luxR* homolog gene and a killing function controlled by a QS-regulated promoter. Consequently, bacteria harboring this plasmid can grow on AHL-containing medium only in the presence of efficient QSI molecules. A high-throughput screen was also established by Desouky *et al.* (2013) to highlight QSIs that target cyclic peptide-mediated QS in Gram-positive bacteria. It relies upon the use of both the *agr* and the *fsr* systems from *Staphylococcus aureus* and *Enterococcus faecalis*. It combines an *St. aureus agr* reporter strain that carries luciferase and green fluorescence protein genes under the control of the *agr* promoter, and a gelatinase-induction assay to examine the *E. faecalis fsr* QS system and look for QSis. A total of 906 Actinomycetes extracts were screened with this method and 4 of them showed QSI activity against the QS systems of both *St. aureus* and *E. faecalis*.

Library screenings have provided a large number of structurally diverse compounds (termed hits) that exhibited variable efficiency and selectivity. A massive screen of a library of 200 000 molecules resulted in the identification of two AHL QSIs which act at millimolar and nanomolar levels (Muh *et al.* 2006).

Another study of 16 000 synthetic compounds led to the identification of nine molecules with significant QSI activity at the micromolar range (Borlee *et al.* 2010). Screening from natural sources may also reveal some unexpected QSI activity of known compounds, such those of the plant alkaloid hordenine (N,N-dimethyltyramine) and human sexual hormone estrone and its structural relatives estriol and estradiol (Beury-Cirou *et al.* 2013).

From above, an efficient solution to identify QSIs can be based on drug design, i.e. the chemical synthesis of signal or signal precursor analogs, or analogs of previously identified QSIs. Through these bio-inspired approaches, numerous molecules have been found that antagonized AHL sensing, including thiolactones (McInnis and Blackwell 2011), cyclopentyamide (Ishida *et al.* 2007) and urea analogs (Frezza *et al.* 2006) as well as synthetic furanone derivatives (e.g. triazolyldihydrofuranones, Brackman *et al.* 2012) or obviously synthetic AHL derivatives (Smith, Bu and Suga 2003; for reviews, see Stevens *et al.* 2011; LaSarre and Federle 2013). Docking with LuxR-like proteins has also been performed (Ahumedo, Diaz and Vivas-Reyes 2010; Soulère *et al.* 2010), as well as computer-assisted screens, in the last case for the identification of AI-2 sensing inhibitors. Over 1.7 million molecules have thus been screened *in silico* using the crystal structure of the signal receptor LuxP with bound AI-2 (Li *et al.* 2008). This led to the identification of 12 QSIs, the most potent one in the *V. harveyi* biosystem being pyrogallol. Another subsequent study of 500 000 molecules allowed the discovery of seven QSIs, out of which three heterocyclic compounds were the most active (Zhu *et al.* 2012b). Similarly, Shen *et al.* (2006) and Malladi *et al.* (2011) also proposed several molecules which would be QSIs but their action on AI-2-regulated phenotypes has not yet been investigated.

Drug design has also been used to identify QSIs aimed at cyclopeptides in Gram-positive bacteria. In *St. aureus*, cyclopeptide QS signals, termed autoinducing peptides (AIPs), are encoded by the *agrD* gene as a propeptide, and exported outside the bacteria via AgrB, an integral membrane protein that also removes the N-terminal signal sequence. At high population density, the AIP is sensed by a two-component system that consists of a transmembrane receptor encoded by *agrC* that autophosphorylates upon AIP binding, and in turn transfers a phosphate residue to the response regulator AgrA (for a review, see Antunes *et al.* 2010). The phosphorylated AgrA, in association with the transcription factor SarA, indirectly activates the transcription of genes that encode the synthesis of secreted virulence factors. AIP analogs, in the form of truncated AIP thiolactone peptides, were synthesized and appeared to be potent inhibitors of AIP-regulated functions (Lyon *et al.* 2000).

Origin and biochemical nature of natural QSIs

QSIs are produced by a wide range of organisms, such as bacteria, fungi, plants or animals from terrestrial, marine or freshwater ecosystems (Skindersoe *et al.* 2008; Musthafa *et al.* 2010; Koh *et al.* 2013; Natrah *et al.* 2011b; Tello *et al.* 2011; Montgomery *et al.* 2013; examples in Fig. 2). However, while many organisms or tissue extracts exhibit QSI activities, active compounds have been fully characterized only in a limited number of cases. The biochemical nature of purified QSIs is highly diverse (Galloway *et al.* 2011; Stevens *et al.* 2011; LaSarre and Federle 2013) and, except for structural analogs of signal molecules that usually act as competitive inhibitors, no direct correlation exists between the molecular structure or chemical functional groups of the QSIs and the targeted actor in the QS pathway.

In bacteria, the production of QSI molecules has been reported in different genera such as *Bacillus*, *Halobacillus*, *Alteromonas* and *Pseudomonas* (Holden *et al.* 1999; Nithya, Begum and Pandian 2010; Nithya, Devi and Pandian 2011; Bobadilla Fazzini *et al.* 2013). Nevertheless, only a few bacterial QSIs have been well characterized, such as phenylethylamides and cyclo-L-proline-L-tyrosine (Teasdale *et al.* 2009, 2011; Rendueles, Kaplan and Ghigo 2013). Some strains of rhizobacteria among *P. fluorescens* or *Se. plymuthica* species produce unusual QSIs, i.e. volatile organic compounds (VOC) such as dimethyl disulfide, inhibiting NAHL-based QS regulation (Chernin *et al.* 2011). Fungi, especially species of the Ascomycota *Penicillium* genus produce QSIs, such as patulin or penicillic acid, that contain a furanone core and target the RhlR and the LasR proteins (Rasmussen *et al.* 2005). Some Basidiomycetes, such as *Tremella, Auricularia or Phellinus igniarius*, may also produce QSIs (Zhu *et al.* 2011, 2012a).

Among lower plant species, no QSIs have been identified to date in Bryophytes and Pteridophytes, in contrast to algae where numerous examples of QSIs have been reported. Indeed, many studies investigated QSIs in algae, and showed that freshwater and marine microalgae (Natrah *et al.* 2011a) as well as marine macroalgae (Kim *et al.* 2007; Jha *et al.* 2013) are QSI producers. In microalgae, QS-interfering activity might be isolate specific rather than species specific, and different microalgae from the same species might produce different QSIs (Natrah *et al.* 2011b). However, the main source of natural QSIs remains higher plants from very diverse species and all continents. They include many medicinal plants, vegetables and edible fruits. QSIs may be extracted from all types of plant tissues: roots and rhizomes, flowers, bark, leaves, stems, seeds and fruits (Vandeputte *et al.* 2010; Chong *et al.* 2011; Koh and Tham 2011; Abd-Alla and Bashandy 2012; Gimenez-Bastida *et al.* 2012; Jakobsen *et al.* 2012a, b; Packiavathy *et al.* 2013). The majority of the studies dealt with plant extracts from which the QSI molecules have been rarely isolated. Among identified QSIs, most are cyclic compounds, such as phenolic derivatives or nitrogen cyclics. Recently, Ahmad and Viljoen (2014) demonstrated the QQ activity of plant VOC, especially terpenes from essential oils, on AHL-based QS in *Chromobacterium*. Some non-cyclic compounds have also been detected, such as some organosulfur compounds or hypobromous acid which deactivates 3-oxo-acyl HSL molecules (Borchardt *et al.* 2001). The main chemical classes of natural QSIs are exemplified in Table 1. Interestingly, some molecules of natural origin act as QSI only in association. As an example, the simultaneous presence of two molecules from the red alga *Ahnfeltriopsis flabelliformis* is necessary to recover the inhibition of OC8-HSL-based QS observed with the total algal extract (Liu *et al.* 2008) in an *A. tumefaciens* reporter system.

Among known QSI-producing animals, most of them belong to marine ecosystems, even though Park *et al.* (2008a) showed that fire ants (*Solenopsis invicta*) produce an effective alkaloid QSI. The most common marine QSI producers are soft corals and sponges, but gorgonians, hard corals, bryozoans and ascidians also produce QSIs (Skindersoe *et al.* 2008; Tello *et al.* 2011). Amongst those, marine Bryozoans produce different brominated alkaloids, the ability of which to block AHL-regulated gene expression was demonstrated in different bacteria (Peters *et al.* 2003). Octocorals produce macrocyclic diterpenoid QSIs that act on the inhibition of biofilm maturation of Gram-negative and Gram-positive bacteria (Tello *et al.* 2011) while sponges produce diverse sesterterpenoids QSIs that act as biofilm modulators in Gram-negative bacteria (Skindersoe *et al.* 2008). Remarkably, meat or meat by-products (e.g. frozen fish, poultry washes, turkey patties) have been identified as unexpected sources of

Table 1. Chemical nature of natural eucaryotes QSIs.

Structural group	Chemical representant	Class	Examples	Produced by	Reference
Cyclic molecules	Phenolic derivatives	Phenolic aldehyde	Vanillin	*Vanilla planifolia*	Choo, Rukayadi and Hwang (2006) Ponnusamy, Paul and Kweon (2009)
		Tanins	Tanic acid	*Quercus*	Jones, Dang and Martinuzzi (2009)
		Flavonoids	Naringenin	*Citrus sinensis*	Truchado *et al.* (2012)
			Taxifolin	*Cedrus deodara*	Vandeputte *et al.* (2011)
			Eriodictyol	*Eriodictyon californicum*	Vandeputte *et al.* (2011)
		Phenylpromene	Methyl eugenol	*Cuminum cyminum*	Packiavathy *et al.* (2012)
	Nitrogen cyclics	Indole derivatives	Auxin 3-indolyacetonitrile	Cruciferous	Lee, Cho and Lee (2011)
		Alkaloids	Solenopsin A	*Solenopis invicta*	Park *et al.* (2008a)
			Betonicin	*Ahnfeltiopsis flabelliformis*	Kim *et al.* (2007)
			Brominated alcaloids	*Flustra foliacea*	Peters *et al.* (2003)
	Furanones	Halogenated furanones	4-Bromo-3-butyl-5-(dibromomethylene)-2(5H)-furanone	*Delisea pulchra*	Denys *et al.* (1993)
	Lactones	Unsaturated lactones	cinnamolide valdiviolide	*Drimys winteri*	Carcamo *et al.* (2014)
		Polyketide lactone	Patulin	*Penicillium*	Rasmussen *et al.* (2005)
Non-cyclic molecules	Organosulfur compounds	Isothiocyanates	Iberin	*Armoracia rusticana*	Jakobsen *et al.* (2012a)
			Erucin	*Brassica oleracea*	Ganin *et al.* (2013)
		Sulfonic acids	Isethionic acid	*Ahnfeltiopsis flabelliformis*	Kim *et al.* (2007)
		Disulfides	Ajoene	*Allium sativum*	Jakobsen *et al.* (2012b)
	Others	Acetaldehyde	Houttuynin	*Houttuynia cordata*	Wu *et al.* (2014)

QSIs, including long-chain fatty acids inhibiting AI-2-based QS in *V. harveyi* (Lu, Hume and Pillai 2004; Widmer *et al.* 2007). Noticeabely, the biochemical group 2(5H)-furanone appears to be present in many QSIs derived from animals (e.g. octocoral, sponges) and plants (e.g. algae, citrus). Thus, Skindersoe *et al.* (2008) proposed that QSI activities of furanones produced by eukaryotes may represent a conserved mechanism for communication between eukaryotes and bacteria. Janssens *et al.* (2008) also noticed the conservation of a 2(5H)-furanone moiety among many AHL-based QS system inhibitors of natural origin, strengthening this hypothesis.

QSIs: TARGETS AND MODE OF ACTION

QSIs may interfere with (i) the synthesis of the autoinducers, (ii) the cell-to-cell exchange of autoinducers and (iii) the perception and transduction of autoinducer signal through its interaction with sensor/transcriptional factors. In Fig. 6, the QS steps which may be targeted by QSIs are summarized and exemplified in four QS pathways involving the QS signals AHL, PQS, AIP and AI-2.

Inhibition of signal synthesis

The suppression of autoinducer production implies the inhibition of either the precursor synthesis or the activity of the QS-signal synthases themselves. AHL autoinducers are synthesized from two precursors, SAM and acyl-ACP. Because SAM and acyl-ACP are essential and ubiquitous metabolites, a complete inhibition of their synthesis is lethal for bacteria and thus not relevant for the development of antivirulence compounds. This is exemplified by the triclosan molecule which has been identified to limit the acyl-ACP biosynthesis. At high levels, triclosan kills bacteria while at a sublethal concentration it inhibits AHL synthesis (Hoang and Schweizer 1999). However, LuxI-type synthase activity could be inhibited by end products, reaction intermediates and/or substrate analogues. Thus, several analogs of SAM act as AHL synthesis inhibitors, such as butyryl-SAM and sinefungin (Parsek *et al.* 1999). From a chemical library of AHL antagonists, Chung *et al.* (2011) identified a competitive inhibitor (named J8-C8) of an acyl-ACP carrier that acts on the C8-HSL synthase TofI. Another screening revealed that the tryptophan derivative indole-3-acetic acid (i.e. the plant hormone auxin) inhibits the AHL synthase BmaI1 from *Burkholderia mallei* (Christensen *et al.* 2013).

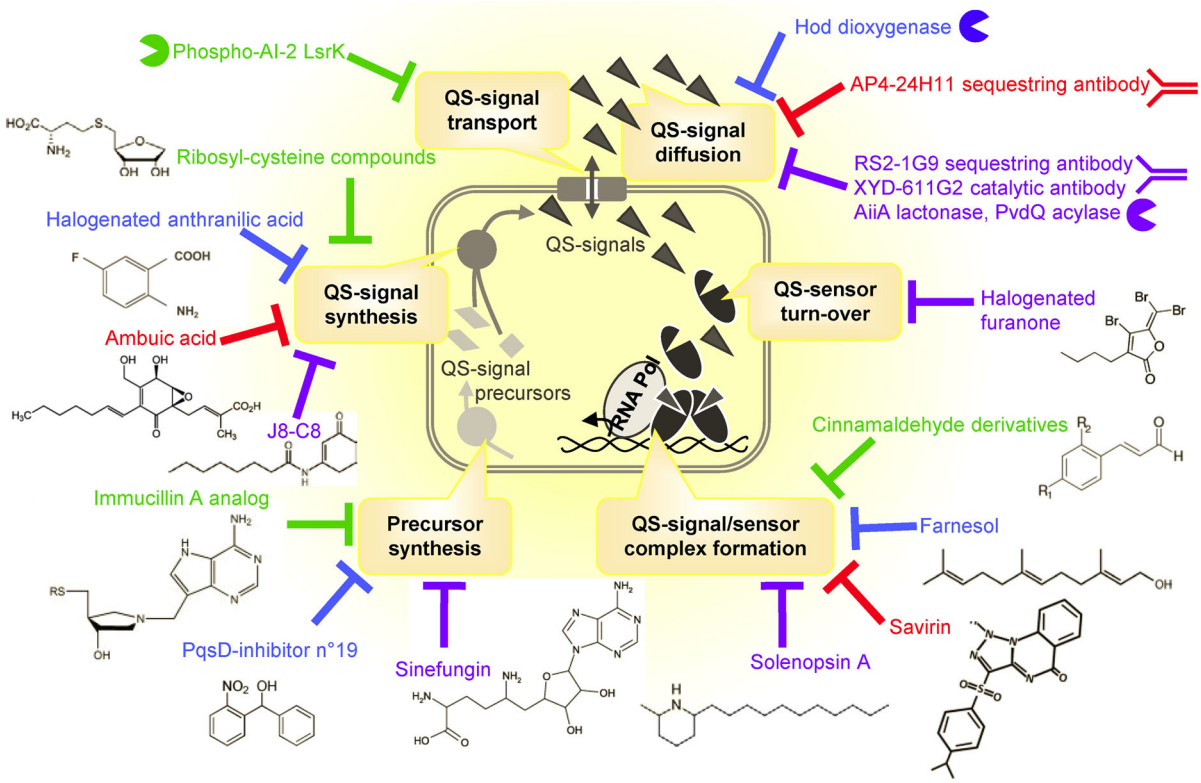

Figure 6. QQ targets in the AHL, AI-2, PQS and AIP QS pathways. The different QS steps targeted by QQ actors are indicated in the black boxes. For each of the illustrated compounds (QSIs, enzymes and antibodies), the color of the stop lines indicates which QS-signal pathway is affected: AHL (purple colour), AIP (red colour), PQS (blue colour) and AI-2 (green colour).

The AI-2 autoinducer DPD is synthesized from S-adenosylhomocysteine (SAH) by two enzymatic steps: the enzyme MTAN (5′-methylthioadenosine/S-adenosylhomo-cycteine nucleosidase) converts SAH in S-ribosylhomocysteine (SRH), then the LuxS metalloenzyme produces the AI-2 signal DPD (Pereira, Thompson and Xavier 2013). Transition state analogs of 5′-methylthioadenosine hydrolysis strongly inhibit the MTAN enzyme of several bacteria, while different immunocillin analogs corresponding to other transition states were identified as inhibitors of the synthesis of the AI-2 precursor SRH (Singh *et al.* 2005). Additional mechanisms are involved in the inhibition of LuxS. Some furanones may covalently modify and inactivate LuxS (Zhang *et al.* 2009), while different LuxS-substrate analogs may act as competitive inhibitors of LuxS (Alfaro *et al.* 2004; Shen *et al.* 2006).

PQS autoinducer is produced from anthranilic acid via 2-HHQ which is converted into PQS by the FAD-dependent monoxygenase PqsH. Upstream, PqsD mediates the conversion of anthraniloyl-coenzyme A to HHQ. In *Pseudomonas*, PQS synthesis may be weakly affected by methyl-anthranilate and halogenated anthranilate (Calfee, Coleman and Pesci 2001; Lesic *et al.* 2007). Storz *et al.* (2012) identified a new class of PqsD inhibitor compounds that led to a dramatic decrease of the concentrations of the PQS precursor HHQ and reduction of biofilms in *P. aeruginosa*. PqsD appeared to be an interesting target for QQ strategies. As a consequence, mechanistic studies were carried out to design efficient QSIs usable in therapeutic treatments (Hutter *et al.* 2014).

In Gram-positive bacteria, pro-AIP peptides are produced by AgrD, then processed and secreted as a mature AIP by the *trans*-membrane transporter/endopeptidase AgrB which removes of the N-terminal segment of the pro-AIP peptides. Some linear peptides containing a proline residue inhibit the cleavage by the AgrB peptidase and hence prevent the maturation of AIP. A fungal secondary metabolite, ambuic acid, also inhibits the biosynthesis of cyclic peptides in different Gram-positive species such as *St. aureus* or *Listeria innocua*, possibly via the inhibition of AgrB-like enzymes (Nakayama *et al.* 2009).

Inhibition of QS signal cell-to-cell exchange and transport

Aside from the enzymatic degradation of QS signal discussed in this review, different mechanisms and QSIs may contribute to limit the exchange of QS signals. For example, some molecules were identified as autoinducer sequesters. Apolipoprotein B sequesters the AIP-1 signal in *St. aureus*, hence prevents subsequent QS signaling through its receptor, AgrC (Peterson *et al.* 2008). Antibodies may also act as QS-signal sequesters, as shown for the antibody AP4–24H11 raised against AIP-4 in *St. aureus* (Park *et al.* 2007) and the antibody RS2–1G9 raised against OC12-HSL in *P. aeruginosa* (Kaufmann *et al.* 2006; see also the section 'antibody-based quorum quenching efforts' below). The antigens used to generate these monoclonal antibodies could therefore be proposed as potential vaccine antigens (Zhu and Kaufmann 2013). For example, Brady *et al.* (2011) delivered simultaneously four antigens, produced in various locations in a staphylococcal biofilm, as a quadrivalent vaccine with an additional antibiotic treatment. Vaccination, coupled with vancomycin, allowed a 67 and 82% reduction of clinical and radiographic, respectively, signs of infection, even though vaccination alone resulted in a non-significant decrease. Catalytic

antibodies were also screened for, but only a few were efficient such as that observed with XYD-611G2 that promoted the degradation of OC12-HSL (Marin *et al.* 2007).

Though AHL signals diffuse through membranes, AI-2 needs a transporter to enter the cells. In *Escherichia coli*, the cytoplasmic enzyme LsrK phosphorylates AI-2 following its transport into the cell. When the LsrK enzyme is introduced into bacterial cultures, it phosphorylates extracellular AI-2 signals. As a consequence, the negative charge of phospho-AI-2 prevents its transport into the cell (Roy *et al.* 2010). In Gram-positive bacteria, the final processing of AIP and its export from the cell are performed at the same time by the transmembrane peptidase AgrB. Thus, the AgrB inhibitors (see above) also prevent the reuptake of AIP.

Inhibition of QS-signal perception and response

Many, if not most, studies on QSIs reported the identification of QS-signal antagonists. They may prevent the binding of the QS signal with the receptor and/or modify the normal conformation of the receptor-signal complex, hence blocking its dimerization or interaction with the appropriate DNA region (gene promoter) or RNA polymerase.

Different natural and synthetic analogs of AHLs act as QSIs, such as thiolactones (McInnis and Blackwell 2011), lactams (Malladi *et al.* 2011), the solenopsin A alkaloid from *So. invicta* that targets the QS-system RhIR in *P. aeruginosa* (Park *et al.* 2008a), and the isothiocyanate iberin from horseradish that prevents the binding of C4-HSL to the RhlR regulator in *P. aeruginosa* (Jakobsen *et al.* 2012a). Remarkably, some QSIs may also act directly on LuxR-like receptors as for example, halogenated furanones, thus causing receptor-QSI complex to rapid proteolytic degradation (Manefield *et al.* 2002).

Some analogs (alkyl-substituted, cyclic and aromatic derivatives) of the AI-2 signal DPD exhibit a moderate capacity to inhibit QS signaling (Lowery *et al.* 2009a; Gamby *et al.* 2012). Thus, cinnamaldehyde analogs targeting LuxR in *Vibrio* have been found to inhibit AI-2 signaling (Brackman *et al.* 2008). In *V. harveyi*, detection of AI-2 involves two periplasmic proteins LuxP and LuxQ which constitute interesting targets to design QSIs. By structure-based virtual screening, about 10 potential inhibitors have been identified (Brackman *et al.* 2009; Zhu *et al.* 2012b) but their QSI activity remain to be tested *in vivo*.

In PQS-based QS of *P. aeruginosa*, PQS binds to the receptor PqsR (or MvfR) and enhances PqsR binding to the *pqsA* promoter (PqsA is involved in the synthesis of PQS signal). Some molecules, such as farnesol from *C. albicans*, inhibit PQS production in *P. aeruginosa* preventing *pqsA* transcription. The binding of farnesol to PqsR provokes a conformational modification of the receptor PqsR that prevents efficient binding with the promoter (Hornby *et al.* 2001).

In AIP-based QS, recognition and signal transduction involve a two-component system. The first element, AgrC, is a sensor kinase with an N-terminal transmembrane domain which senses the AIP signal and a C-terminal histidine kinase domain that phosphorylates upon ligand binding. The second component (AgrA) is a cytoplasmic response regulator which needs to be phosphorylated by AgrC to bind to the promotor regions and consequently enables the expression of QS-target genes. The search of antagonists directed at AgrC remains an important area of investigation (Lyon *et al.* 2002; Gorske and Blackwell 2006), including truncated or modified AIPs resulting in potent inhibitors for different Agr systems. However, from a high-throughput screen (24 087 compounds), one novel QSI has been identified and named savirin—*St. aureus vi*rulence *i*nhibitor—(Sully *et al.* 2014). Savirin blocks the transcriptional activity of AgrA and thus inhibits the Agr pathway in *St. aureus* and attenuates the tissue injury caused by *St. aureus*.

QSIs: BIOLOGICAL ROLES

When a QS signal acts as a QSI in *Ch. violaceum*

Chromobacterium violaceum is a Proteobacterium commonly found in soil and water and may be an opportunistic pathogen. In *Ch. violaceum*, QS controls biofilm formation and the production of cyanide, exoprotease and chitinase enzymes and that of the violacein pigment (McClean *et al.* 1997; Chernin *et al.* 1998). The production of the purple pigment violacein is an easily quantifiable QS-regulated function. The QS signal forms a complex with CviR, a cytoplasmic LuxR-type receptor that binds DNA to activate transcription of QS target genes. The active form of the LuxR-type protein is a homodimer, each monomer of which consists of two domains, a ligand-binding domain and a DNA-binding domain (Choi and Greenberg 1991; Hanzelka and Greenberg 1995). These transcriptional regulatory proteins bind directly to an AHL-molecule leading to stabilization of the protein-ligand complex, and then dimerize. These dimers bind directly to a DNA promoter element and either activate or repress transcription of downstream genes.

In some *Ch. violaceum* strains, such as strain ATCC31532 (McClean *et al.* 1997), the transcription of the *vioABCDE* gene cluster that encodes violacein synthesis is controlled by the QS-signal C6-HSL (Throup *et al.* 1995; Swem *et al.* 2009). However, in some other *Ch. violaceum* strains, such as strain 12472 (Stauff and Bassler 2011), C10-HSL acts as a QS signal controlling violacein production (Davis, Gustafson and Rosazza 1975). This observation suggests a diversification of the QS signal within *Ch. violaceum* populations. Bassler *et al.* evaluated whether cross-talk or an inhibition of QS-signaling may occur in a *Ch. violaceum* strain harboring the C6-HSL-type regulator CviR (Swem *et al.* 2009; Chen *et al.* 2011). In particular, they tested the CviR response to several AHL molecules with acyl chains of various lengths, performing genetic, biochemical and structural analysis. CviR is agonized by C4-HSL and C6-HSL, partially agonized by C8-HSL, but antagonized by C10-HSL, C12-HSL and C14-HSL. The antagonistic activity of AHLs with longer acyl chains as C10- and OC10-HSL, C12- and OC12-HSL C14- and OC14-HSL was also demonstrated by McClean *et al.* (1997).

The antagonistic long-chain AHLs bind CviR in the pocket normally occupied by C6-HSL but two mechanisms are responsible for the disruption of the transcriptional activation by CviR according to the length of the acyl chain (Fig. 7). The first one involves C10-HSL and targets the interaction between the AHL-CviR complex and RNA polymerase, while the second one involves C12-HSL or C14-HSL and consists in preventing the AHL-CviR complex from binding DNA. The first C10-HSL-mediated mechanism has been revealed by protein–protein interaction analysis. Bacterial two-hybrid experiments showed that the CviR/C6-HSL complex interacts directly with the C-terminal domain of the RNA polymerase α-subunit. A weak interaction was observed in the presence of C8-HSL, while no interaction was observed in the presence of C10-HSL. These results indicate that C8-HSL induces a conformational change in the CviR protein that allows the binding to DNA, but slightly modifies its interaction with RNA polymerase, and so attenuates its transcriptional activating potential. The extension of the acyl chain by two additional carbons (C10-HSL) increases the magnitude of

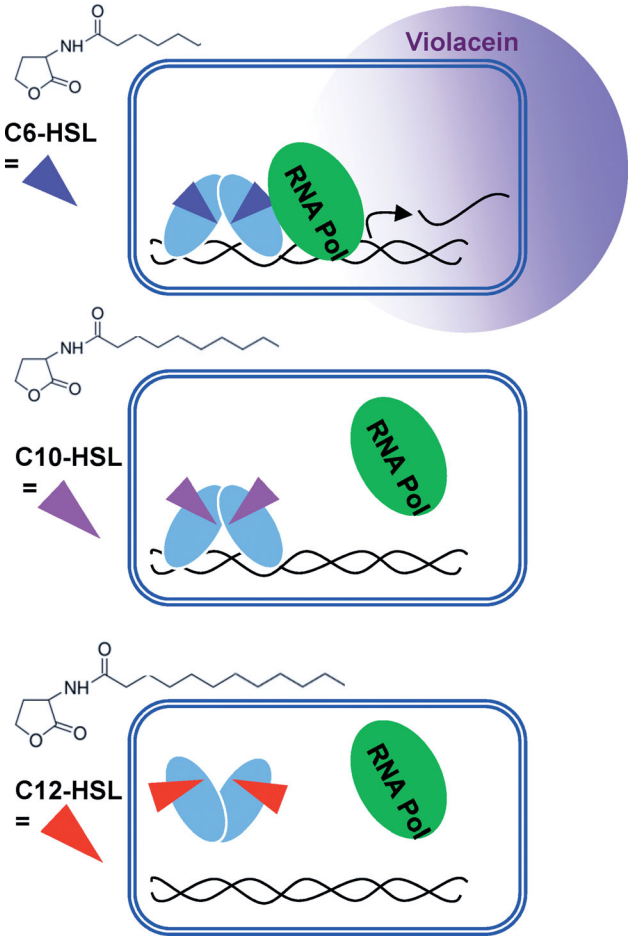

Figure 7. QS and QQ in *Chromobacterium*. Upper panel: C6-HSL binds the LuxR-like sensor CviR that dimerizes and stabilizes the RNA polymerase (RNA Pol) to promote the expression of the violacein synthesis genes. Central panel: C10-HSL stabilizes the CviR homodimer, but does not promote the association with the RNA Pol, hence the lack of expression of the violacein operon. Lower panel: C12-HSL (or C14-HSL) bound CviR but prevent DNA binding hence the expression of the violacein operon.

the conformational change. Upon binding, C10-HSL locks CviR into a conformation incapable of interaction with the RNA polymerase even though the CviR complex formed with C10-HSL binds DNA as efficiently as those formed with C6-HSL or C8-HSL. Remarkably, C10-HSL is such an efficient antagonist that it prevents the expression of virulence of the bacterium toward nematodes, as judged from killing assays with *Ca. elegans* and *Ch. violaceum* (Swem *et al.* 2009). The second mechanism that involves C12-HSL or C14-HSL as antagonists has been revealed by protein–DNA interaction analysis. Purified CviR bound to C12-HSL or C14-HSL displayed no ability to bind DNA. DNA gel mobility shift analyses revealed that these antagonists bind the CviR receptor that adopts a conformation unsuitable for binding DNA. Structural analysis of the LuxR homodimer bound to an antagonistic synthetic AHL analog shows that the DNA-binding domain of each monomer is positioned below the ligand-binding domain of the opposite monomer in a crossed-domain conformation. This conformation designs a 60Å space between the DNA-binding helices, twice the 30Å separation required for operator binding, thus preventing the expression of QS-target genes. This crossed-domain conformation would explain the antagonist effect observed with C12-HSL or C14-HSL (Chen *et al.* 2011).

The above example clearly highlights the subtlety of QS-mediated bacterial communication where close members of a QS-signal family may act not only as QS agonists (cross-talk), but also as QSIs that use different QQ mechanisms according to the length of the acyl chain (C12-HSL or C14-HSL).

When halogenated furanones control biofilm formation: from marine to human microbiota

The first marine QSIs were isolated from the red macroalga *Delisea pulchra* that appears to have developed natural defense mechanisms to prevent microbial biofouling of its surfaces (Givskov *et al.* 1996). Secondary metabolites of the halogenated furanones class, which are found at the surface of this alga, exhibit the antifouling activity (Dworjanyn, de Nys and Steinberg 1999). de Nys *et al.* (1993) reported the isolation of over 20 halogenated furanone compounds from *D. pulchra* extracts. The concentrations of the different furanones range from 10 to 250 ng cm^{-2} at the surface of the alga. These halogenated furanones are structurally similar to AHLs, though furanones have a furan ring instead of a homoserine lactone ring. Both AHL and furanones possess a non-polar aliphatic carbon part, attached to a relatively polar moiety.

The majority of the studies on natural *D. pulchra* furanones involved the (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. This molecule exhibits high inhibitory activities on AHL signaling (Rasmussen *et al.* 2000; Hentzer *et al.* 2003) and AI-2 signaling (Ren, Sims and Wood 2001). Particularly, the QSI effect of natural *D. pulchra* furanones was evidenced on different bacterial species and QS-regulated functions (Rasmussen *et al.* 2000; Manefield *et al.* 2001) such as the bioluminescence produced by *V. fischeri* and *V. harveyi*, swarming motility of *Se. liquefaciens*, *Proteus mirabilis* and *Es. coli*, biofilm formation in *Es. coli*, virulence and OHHL-regulated carbapenem production in Pectobacterium *carotovorum*, exoenzyme production by *Se. liquefaciens*, *V. harveyi* and *P. aeruginosa*, and siderophore biosynthesis in *P. putida*.

At a mechanistic level, the natural furanones act differentially on AHL- and AI-2-based QS signaling. In the case of the AI-2-based QS processes, natural brominated furanones covalently modify and inactivate LuxS, binding to thiol groups of cysteine residues of the LuxS protein (Zang *et al.* 2009). As a consequence, the enzyme can no longer synthetize the AI-2 signal. In the case of the AHL-based QS processes, the half-life of *V. fischeri* LuxR is reduced up to 100-fold in the presence of halogenated furanones. These furanones interact with the LuxR protein causing conformational changes that enlist the furanone–LuxR complex for a rapid proteolytic degradation (Manefield *et al.* 2002). In *V. harveyi*, Defoirdt *et al.* (2007b) have postulated that furanones block the QS process by decreasing the DNA-binding activity of the transcriptional regulator protein LuxR. In the presence of furanones, LuxR cannot bind DNA, due to either an alteration of the structure of the DNA-binding domain or that of the regions involved in dimer formation. Both phenomena hinder the ability of LuxR to bind to target gene promoter sequences (Defoirdt *et al.* 2008).

Structure-function analyses revealed that a conjugated exocyclic vinyl bromide on the furanone ring is required to inhibit *Es. coli* biofilm formation (Han *et al.* 2008). Two structural constraints critical for the QS inhibitory activity were also identified: the presence of an exocyclic double bond at the carbon 5-position and an acetyl or hydroxyl group at the carbon

1`-position (Kjelleberg *et al.* 1997). The synthesis of several compounds based on the structure of natural products showed that the alkyl side chain, common to the *D. pulchra* furanones, does not play a critical role in QQ activity (Hentzer *et al.* 2002). The synthetic addition of variable-length acyl side chains on other natural furanones revealed that the short alkyl chains have a better QQ activity than the longer ones (Kim *et al.* 2008).

In order to identify furanone QSI target genes, Hentzer *et al.* (2003) carried out a transcriptomic analysis in the bacterium *P. aeruginosa* using a synthetic derivative (termed C30) of natural furanones. This study revealed that 1.7% of all *P. aeruginosa* genes were affected by C30, the expression of these genes being mainly repressed (91%). Approximately 30% of repressed genes have previously been reported as QS-controlled virulence factors. The genes activated in response to C30 encoded mainly transporters (e.g. ABC, MFS, multidrug efflux). A comparative analysis of the QS regulon and C30 target genes showed that 80% of the furanone-repressed genes were indeed controlled by QS, even though the major QS regulators (*lasRI*, *rhlRI*) were not transcriptionally affected.

QSIs were identified in all kingdoms of living organisms but predominantly in plants. Even though many plant extracts showed QS inhibition activity, only a few QSI molecules were identified. These identified QSIs exhibit an important structural diversity. Excepted for QS signal analogs that can act as competitive inhibitors of the signal sensing, no correlation between the structure of the QSIs and the targeted steps of the QS regulation pathway has been demonstrated. In this respect, the *Ch. violaceum* example highlights the complexity of the mechanisms implicated in QS interference by QSIs. Thus, in the same species, the signal molecule of a strain may act as QSI in another strain and two structurally related molecules may interfere with QS via two different mechanisms.

Overall, even though QSIs are produced by many organisms and are present in many ecosystems, the real impact of their action in ecosystems has rarely been studied. Nevertheless, QS inhibition by QSIs is an interesting case of interkingdom interaction. In marine ecosystems, for instance, QSIs (such as the furanones) may affect different AHL-based QS regulatory processes, including those detected in pathogenic bacteria of the aquatic environment. In these marine ecosystems, many QS-regulated functions have been described in bacteria. Rationally, marine organisms may have evolved QQ enzymes or/and QSI mechanisms to suppress this communication, to control bacteria, or even eukaryotes that sensed QS signals, for association or competion. As a consequence, the study of a simplified, confined ecosystem (e.g. a single coral or sponge) could be very informative in terms of understanding of the regulation of QSI production and impact on the ecosystem, for instance when biotic and abiotic conditions are changing.

Though still speculative, a similar comment can be made with respect to QSIs of fungal origin that may inhibit the production of antifungal compounds by competing bacteria with plant pathogens, the pathogenicity of which relies on QS. A possibility exists that potent QSIs produced by some plants may contribute to the resistance of the plant to these pathogens.

From above, the view that QSIs can be used in agronomical, aquaculture or water processing environments has emerged. However, the use of natural QSIs may present some limits, due to a weak stability or efficiency in the targeted environment, or to a possible toxicity for higher organisms. A way to bypass these limitations may be to design, based on natural QSIs, synthetic molecules for novel biotechnical applications.

QQ-BASED ANTIBACTERIAL TREATMENTS IN MEDICINE

Toward an integrated use of QQ processes

In 2014, the report entitled 'Antimicrobial resistance: global report on surveillance' by the World Health Organization reveals that antibiotic resistance is a major threat to public health around the world (World Health Organization 2014). If effective antibiotics have been one of the pillars of modern medicine, allowing us to live longer and healthier lives, the massive use and misuse of antimicrobials in medicine and farming has increased the number and types of resistant organisms. Hence, in addition to efforts to prevent infections and change antibiotic usages, the search for new treatments is crucial. Thus, the interesting paradigm of anti-virulence has emerged. Antivirulence aims at targeting virulence functions and behaviors (including biofilm formation) rather than the viability of the pathogens. QQ-based strategies belong to these antivirulence approaches (Fuqua and Greenberg 2002).

Many QSI studies dealt with the opportunistic pathogen *P. aeruginosa*. They yielded evidences of QQ *in vitro* and *in vivo* using different animal models such as *Ca. elegans* (Bijtenhoorn *et al.* 2011a), *Dr. melanogaster* (Chugani *et al.* 2001; Stoltz *et al.* 2008; Cady *et al.* 2012), *Galleria mellonella* (Imperi *et al.* 2013) or rodents (Christensen *et al.* 2007; Hoffmann *et al.* 2007; Bjarnsholt *et al.* 2010; Nidadavolu *et al.* 2012). To date, only a few pilot clinical trials have been conducted, with promising results such as those using azithromycin as a QSI in the treatment of ventilator-associated pneumonia (Van Delden *et al.* 2012). However, some difficulties in this trial yelded positive results only in a subgroup of patients. In addition, to reduce the expression of virulence functions, QSIs may reduce biofilm formation in *P. aeruginosa*. Biofilms are complex microbial structures which limit the efficiency of antibiotic molecules and that of immune cells to reach their microbial targets. As a consequence, the alteration of these biofilms makes bacterial cells more susceptible to treatments. The combination of tobramycin and QSI compounds (furanone or ajoene) exemplified the efficient association of QSIs and antibiotics that allows an increased clearance of *P. aeruginosa* in mice (Christensen *et al.* 2012). As a general rule, however, therapeutic use of QSI on humans as a single treatment seems less conceivable than utilization in integrated therapeutic strategies with antibiotics or antibiotics plus biofilm dispersing agents as nitric oxide, hybridized or not on the QSIs (Kutty *et al.* 2013).

In addition to QSIs, a special effort had been made to study and engineer the susbtrate spectrum and stability of the QQ enzymes. A direct administration of QQ enzymes to patients or animals has been proposed by several authors. This approach constitutes an emerging area in the development of treatments targeting QS pathogens. Several thermostable and stabilized enzymes have already been used: lactonases from Firmicutes such as *Bacillus* (Cao *et al.* 2012) and hyperthermophilic Archaea such as *Sulfolobus* (Del Vecchio *et al.* 2009; Hiblot *et al.* 2012) and amidases such as the PvdQ enzyme from *P. aeruginosa* (Wahjudi *et al.* 2013). Native QQ enzymes have also been engineered to enhance their stability and substrate specificity (Koch *et al.* 2014; Luo *et al.* 2014). Some *in vivo* assays were performed to protect insects from *Bu. cenocepacia* (Koch *et al.* 2014) and zebrafish from *Aeromonas hydrophila* (Cao *et al.* 2012). A combination of QQ lactonase and antibiotic (ciprofloxacin) was tested to prevent infection against *P. aeruginosa* in a murine burn wound model (Gupta, Chhibber and Harjai 2015). However, in an attempt to facilitate the use of QQ enzyme in human treatment against *P. aeruginosa*,

an interesting approach allowed the formulation of a freeze-dried powder for spray inhalation (Wahjudi *et al.* 2013). This formulation remains to be tested in a biological model.

Besides *P. aeruginosa*-mediated infection, an environment where ecological and medical considerations can be studied is the oral cavity. This part of the human body constitutes a true ecosystem that hosts over 700 microbial species. Good health of the oral cavity depends upon the equilibrium of the highly structured multispecies microbial community. As a consequence, this strict organization of the bacterial microbiote does not favor the use of bactericide compounds as a way to fight infections. Dental caries, gingivitis and periodontal diseases are among the most common bacterial infections in humans, possibly leading to tooth loss. These pathologies also act as a risk factor for other ones, such as cardiovascular or respiratory diseases, and preterm birth (Marsh 1994). At the microbial level, dental caries and periodontitis are associated with the transition from a multispecies, mainly beneficial community to a pathogenic community chiefly constituted of members of the species *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Treponema denticola*, *Tannerella forsythia*, *Streptococcus mutans,* among others.

In the complex ecosystem of the oral cavity, QS may play a key role in the regulation of bacterial communication, competition and cooperation. Thus, AIPs (especially CSP, i.e. competence-stimulating peptide) from streptococci and AI-2 from Gram-positive- and Gram-negative bacteria appear to be involved in both intraspecies QS and a variety of interspecies interactions across oral species (Guo, He and Shi 2014). Thus, Frias, Olle and Alsina (2001) showed that *Fusobacterium nucleatum*, *Po. gingivalis* and *Pr. intermedia* produce AI-2, while Novak *et al.* (2010) showed that *Ag. actinomycetemcomitans*, a species associated with aggressive forms of periodontitis, regulates the expression of virulence factors and biofilm development by an AI-2-dependent QS system. Jang *et al.* (2013a, b) demonstrated that AI-2, secreted by *F. nucleatum,* increases the colonization of periodontopathogens *(Po. gingivalis, T. denticola, Ta. forsythia)* in oral biofilms. Furthermore, the concentration of AI-2 appears to be critical with respect to the bacterial equilibrium, as a low AI-2 concentration (pM to nM) is optimal for the growth of commensals whereas high levels of AI-2 accelerate the growth of pathogens and reduce the growth of commensal bacteria (Rickard *et al.* 2006). As a consequence, Kolenbrander *et al.* (2010) proposed that AI-2 may be a modulator for the transition of multispecies microbial communities from a mainly beneficial community to a pathogenic community.

The above data suggest that AI-2-mediated QS systems may be valuable targets for the development of new therapies to control periodontal pathogen population and biofilm growth. In agreement with this idea, recent assays with furanone compounds and D-ribose allowed the inhibition of biofilm and co-aggregation processes (Jang *et al.* 2013a, b). In addition, several patents have been registered. Patent n°US8617523, deposited by the Colgate-Palmolive Company, describes oral care compositions and methods for inhibiting growth and formation of oral biofilm using carbonate QSIs, while patent N°US20120189710 describes a garlic-based formulation of QSI with antibacterial and anti-inflammatory components. This QSI product is distributed under license in the UK and is currently used by dentists.

Another interesting strategy does not directly interfere with QS but uses the chemical structure of a signal molecule to create antimicrobial peptides (AMPs). Based on the addition of a targeting peptide (antibody, signaling peptides, and so on) to an existing broad-spectrum AMP, a Specifically Targeted AntiMicrobial Peptide (STAMP) can be generated. It is active against particular bacterial species or strains, and has the potential to eliminate, in a specific way, pathogens while preserving the normal healthy flora. *Streptococcus mutans*, a producer of CSP, has been implicated as a primary pathogen involved in the formation of dental caries. This is why Eckert *et al.* (2006) used CSP as a STAMP targeting domain to mediate the specific delivery of an AMP domain in *Str. mutans*. This class of STAMP can eliminate *Str. mutans* grown in liquid or under a biofilm state without affecting closely related, non-cariogenic oral bacteria. The most successful study deals with STAMP C16G2 from C3-Jin Inc. included in a mouth rinse: C16G2 exhibited antimicrobial activity directed against *Str. mutans* (Eckert *et al.* 2006; Sullivan *et al.* 2011). Furthermore, the induced reduction of *Str. mutans* population generates a rise in the pH of dental plaque that stimulates the growth of non-pathogenic bacteria, and decreases the demineralization process (Eckert, Sullivan and Shi 2012). The phase 2 clinical trial of the C16G2 molecule, formulated as mouth wash or as a gel, has been approved by the FDA and is currently in progress. This approach is a promising way to fight other diseases that require the elimination of specific pathogens without damage to the existing healthy flora. In this respect, the patent relative to C16G2 also contains additional STAMPs directed at *P. aeruginosa* to fight pathologies caused by this bacterium (Eckert *et al.* 2014).

Antibody-based QQ efforts

Immunologically, QS signals are regarded as haptens, i.e. low molecular weight molecules that are poorly immunogenic. The production of antibodies, therefore, involved the use of hapten-carrier protein conjugates to elicit the synthesis of hapten-specific antibodies. To increase the *in vivo* stability of the hapten, and to allow 'grafting' to the carrier protein, haptens may not be AHLs but analogs. In a pioneer study, 4-methoxyphenyl amide AHL analogs were used to raise antibodies in mice. One of these, RS2–1G9, exhibited the ability to inhibit OC12-HSL-mediated QS signaling in *P. aeruginosa* after the measurement of the activity of reporter-gene fusions and the production of pyocyanin. The production of this toxin was reduced to approximately half of its nominal value in cultures performed in the presence of RS2-1G9 (Kaufmann *et al.* 2006). Interestingly, RS2-1G9 exhibits some specificity toward AHL, as it did not recognize C4-HSL produced by *P. aeruginosa*. An analysis of the antibody/antigen structure revealed the complete encapsulation of the cyclic group of the AHL analog (here, a lactam) and that of the first six carbon units of the C12 chain in the antibody. It also revealed that the absence of a 3-oxo group in the C4-HSL molecule could lead to the loss of a strong hydrogen bond interaction which, when combined with the lack of 11 van der Waals' contacts observed with the 3-oxo-C12-lactam analog, explain the absence of recognition of the C4-HSL molecule (Debler *et al.* 2007). Using a similar approach and a squaric monoester monoamide AHL analog as a hapten, Marin *et al.* (2007) reported that the antibody they raised inhibited ca. one third of the pyocyanin production by strain PAO1, by catalyzing the opening of the lactone ring. Antibody directed at OC12-HSL can therefore both sequester this molecule and inactivate it by lactonolysis.

The validity of the antibody-based quenching strategy was strengthened using cell and animal models. The above-described RS2-1G9 antibody protected murine macrophages from the cytotoxic effects of OC12-HSL and also prevented the activation of a mitogen-activated protein induced by the same AHL (Kaufmann *et al.* 2008). In another assay, mice repeatedly

injected with OC12-HSL-BSA conjugate produced specific antibodies in their serum. When challenged with *P. aeruginosa* PAO1, all control mice that were not treated with the OC12-HSL-BSA conjugate died within 2 days post-challenge while 36% of the immunized mice survived at day 4. Furthermore, the levels of pulmonary tumor necrosis factor alpha and the blood and lung concentrations of OC12-HSL of the immunized mice were significantly lower than those of control mice (Miyairi *et al.* 2006). In relation with the increasing interest in AHL-targeting antibodies, a novel series of AHL-targeting antibodies were recently developed, directed at various AHL molecules. The active antibodies produced efficiently recognized OC10-HSL and C8-HSL and cross-reacted with other AHL molecules. These antibodies much more efficiently recognized the open lactone ring forms of the AHL, i.e. OC10-HL (homoserine lactone), C10-HL and C8-HL (Chen *et al.* 2010). In another experiment, sheep-mouse chimeric monoclonal antibodies were raised toward unsubstituted, O- and OH-C12-HSL and used in two animal assays: a *Ca. elegans* slow-killing assay and a mice assay (Palliyil *et al.* 2014). Over 4 days, the survival rates of *Ca. elegans* treated with the antibodies increased from 15% (control, untreated) up to 60%. Mice treated with antibodies exhibited significantly prolonged survival rates (up to 83% survival 7 days postinfection) when compared with those of untreated mice (0% survival 2 days post-infection).

Considering the promising results obtained with AHLs, antibody-based quenching strategies have also been developed toward other QS signals. Antibodies directed to the *P. aeruginosa* PQS QS signal were raised in mice and female rabbits using PQS analog coupled to BSA as the antigen. The produced sera recognized PQS and HHQ, but not anthranilic acid or OC12-HSL (Pathak 2012). Inhibition assays of PQS regulated functions by the anti-PQS antibodies yielded erratic results—possibly due to the low availability of PQS. However, when the anti-PQS antibodies were used in combination with anti OC12-HSL antibodies, a synergistic effect was observed.

In Gram-positive bacteria, QS regulation involves cyclic peptides known as AIPs. In *St. aureus*, AIP-based QS is determined by the *agr* genes and controls the expression of virulence genes (George and Muir 2007). These genes code for the synthesis of (i) cell-surface proteins such as protein A and fibronectin-binding proteins and (ii) secreted proteins that include enzymes (e.g. hemolysins and proteases) and toxins (e.g. toxic shock syndrome toxin and enterotoxin B). Using the structure of one of the *St. aureus* AIPs, Park *et al.* (2007) designed an analog of this cyclopeptide to raise antibodies upon coupling to two carrier proteins. These antibodies drastically reduced the production of α-hemolysin by *St. aureus* grown *in vitro* to a level where no hemolytic activity could be detected on blood agar plates. In addition, α-hemolysin might be responsible for the observed apoptosis of Jurkat T cells post-challenge by *St. aureus* culture supernatants. Antibody-treated *St. aureus* supernanants were partially or almost completely unable to induce apoptosis markers in Jurkat T cells. Protein A expression was also significantly increased by the presence of the antibodies, a result consistent with the inhibition of protein A synthesis at elevated bacterial cell concentrations. Remarkably, these antibodies were also used in mice models to evaluate whether they may prevent *St. aureus*-induced skin abscess formation. Subcutaneous injections of bacterial suspensions with or without the antibody were made in the flank of hairless mice. The results demonstrated that skin injuries were abrogated in mice that received the antibody, even when high amounts (i.e.10^8 cfu) of bacteria were injected subcutaneously.

All the above data demonstrate the interest of the antibody-based QQ strategies, as these have been validated by experiments in animal models. In a post-antibiotic research era, this approach fits well the novel therapeutic strategies directed at animal and human pathogens that aim at jamming cell communication to deregulate key pathogenic functions (see for instance Yano *et al.* 2011 or Ricard 2012).

QQ APPLICATIONS IN AQUACULTURE, AGRICULTURE AND ENGINEERING

In aquaculture

The global decline of fish supplies and the growth of the world's population that triggered an increasing fish consumption combined with the improvement of the 'domestication' of aquatic animals has led to the dramatic development of aquaculture. The world's aquaculture production expanded at an average annual rate of 6.2% in the period 2000–12 (9.5% in 1990–2000) to reach a global production of 90.4 million tonnes, currently becoming the fastest growing food producing sector in the world. With aquaculture intensification, ecological and animal health problems have, however, emerged and developed.

Bacterial diseases are one of the most critical problems in commercial aquaculture. Vibriosis cause high mortality rates in almost any type of aquacultured organisms from mollusks to crustaceans to fish (Defoirdt *et al.* 2007a) and can be transmitted by algae, rotifer and *Artemia* cultures used for feeding. The strict regulations relative to the usage of antibiotics (in Europe and North America) and the emergence of antibiotic resistance promoted the development of other disease control strategies, such as QQ.

Since the 1970s, the integration of microalgae has been empirically carried out in the culture devices of fish and shellfish in coastal open water- and land-based systems, though the first demonstration of the anti-QS activity of the alga *D. pulchra* (via the furanones produced by this species) dates from 1996 (Givskov *et al.* 1996). In relation, in 2006, Defoirdt *et al.* demonstrated that natural and synthetic brominated furanones were able to protect brine shrimps (*Artemia franciscana*) from pathogenic isolates of *Vibrio* (*V. harveyi*, *V. campbellii* and *V. parahaemolyticus*) through the disruption of AI-2-based QS (Defoirdt *et al.* 2006). As these compounds are toxic toward higher organisms such as the rainbow trout (Rasch *et al.* 2004), brominated thiophenones have been synthesized and found to also protect brine shrimp larvae from *V. harveyi* at 2.5 μM while a severe toxicity was noticed only at 250 μM (Defoirdt *et al.* 2012). Natrah *et al.* (2011b) evaluated the QS inhibitory activity of freshwater and marine microalgae using three reporter strains responding to unsubstituted, oxo- and hydroxyl-substituted AHLs, including the AHL produced by the aquaculture pathogen *V. harveyi*. They identified five marine and one freshwater algal strains of interest. The most promising one (*Chlorella saccharophila*) inhibits QS-regulated gene expression in all reporter strains and could be used as a biocontrol agent in aquaculture farms.

Another disease control strategy consists in using organisms or extracts with autoinducer degradation capacities. Different microbial communities or strains with autoinducer degradation activity were discovered, such as (i) microbial communities from the gut of the shrimp *Penaeus vannamei*, with HAI-1 degradation activity that improves the growth rate of rotifers challenged with *V. harveyi* (Tinh *et al.* 2007); (ii) microbial

communities from the gut of the fish *Dicentrarchus labrax* L. and *Lates calcarifer* with AHLase activity, used as biocontrol agents in prawn (*Macrobrachium rosenbergii*) larviculture (Nhan *et al.* 2010); and (iii) *Bacillus* sp. QSI-1, from the gut of the fish *Carassius auratus*, with AHLase activity allowing an increased survival rate of infected zebrafishes (*Danio rerio;* Chu *et al.* 2014). Patents such as that of Otero, Romero and Roca (2013) and market products such as AquaStar Hatchery of BIOMIN that contain *Bacillus* strains producing AHLase are deposited or available. The ability to incorporate biocontrol bacteria in the rearing water or by bioencapsulation in the feed stock (*Artemia nauplii* for example) is one advantage of this strategy.

If AHL-degrading bacteria should be considered as biocontrol organisms to fight bacterial fish disease (Chu *et al.* 2014), the consequences of their use could be deleterious on invertebrates. As it was demonstrated for *Ulva* sp. and *Acrochaetium* sp., where AHLs from biofilms act as chemoattractants and environmental cues for the settlement of zoospores, a preference to settle on biofilms is also exhibited by larvae of several invertebrates such as oysters (Zhao, Zhang and Qian 2003), mussels (Yang *et al.* 2007) or bay barnacles even if the implication of AHLs has been proved only for the latter species in the laboratory (Tait and Havenhand 2013). Because an efficient settlement of larvae is an important stage in invertebrate aquaculture, the use of AHLase systems that can exert a negative effect on AHL concentrations and biofilm formation could eventually be a problem.

Other strategies may therefore be proposed, such as the use of probiotic bacteria. The species *Phaeobacter gallaeciensis*, an α-Proteobacteria of the family *Rhodobacteraceae*, appears able to control vibriosis (D'Alvise *et al.* 2013). In addition, Garcia, D'Alvise and Gram (2013) showed that a combination of such a probiotic bacterium and a QQ strategy could be beneficial because the inhibitory activity of *Phaeobacter* on *V. anguillarum* is independent of the QS system in aquaculture environments.

In crop cultures

At this time, the main plant protection strategies targeting QS of plant pathogens involved QS-signal-degrading enzymes and QSIs. QS-degrading enzymes may be expressed by plants or microbial biocontrol agents. To the best of our knowledge, all these approaches have been evaluated under laboratory conditions, and no field assays are reported in the literature. While several plants are able to take up and respond to QS signal (Mathesius *et al.* 2003; Schuhegger *et al.* 2006; Palmer *et al.* 2014; Sieper *et al.* 2014), only a few of them (e.g. clover and birdfoot deervetch) are known to exhibit QS-signal-degrading activities (Delalande *et al.* 2005; Götz *et al.* 2007). At this time, the plant QS-degrading enzymes and encoding genes are still unknown and plant selection and breeding based on the QS-signal-degrading character are therefore still in their infancy. However, the transfer of some bacterial genes encoding QS-signal-degrading enzymes, such as the *Bacillus* AiiA and *Agrobacterium* AttM lactonases, to different plants was achieved (Dong *et al.* 2001; Ban *et al.* 2009; Vanjildorj *et al.* 2009; D'Angelo-Picard *et al.* 2011). In laboratory assays, a lower level of symptoms and, in some cases, an absence of symptoms induced by QS-producing pathogens such as *Pectobacterium,* were observed in lactonase-expressing plants. Interestingly, transgenic plants expressing the lactonase AttM do not significantly alter diversity of the root-associated bacterial populations (D'Angelo-Picard *et al.* 2011), a feature that suggests a limited impact of such strategies on the dynamics of the plant microbiote.

Some QSIs and QS-degrading microbes were also used to disrupt QS-regulated virulence in plant pathogens, including the soft-rot *Pectobacterium* spp. (Faure and Dessaux 2007). However, the efficiency of a given QSI depends on the tested pathogens. As a consequence, QSIs have to be studied and characterized for each of the targeted pathogens (Rasch *et al.* 2007; des Essarts *et al.* 2013). QS-degrading bacteria belonging to *Bacillus* and *Rhodococcus* genera, which are common inhabitants of soil and potato rhizospheres, have been especially studied as they are efficient AHL-degrading bacteria. These bacteria do not inhibit the growth of the *Pectobacterium* pathogens, but inactivate their QS signals, hence abolishig the *Pectobacterium*-induced symptoms (Uroz *et al.* 2003, 2008; Dong *et al.* 2004). In an *aiiA*-defective mutant of *B. thuringiensis*, the degradation of the QS signals by the lactonase AiiA and potato-tuber protection against *Pectobacterium* are completely abolished (Dong *et al.* 2004). *Bacillus thuringiensis* is of a special interest because this species is already commercialized as an insecticidal agent. In *Rhodococcus erythropolis*, several activities (lactonase, amidase, reductase) are involved in QS-signal inactivation (Uroz *et al.* 2005, 2008) but only the *qsdA* gene, coding for a lactonase, has been characterized so far (Uroz *et al.* 2008). In a *qsdA*-defective mutant of *R. erythropolis*, QS degradation and potato-tuber protection against *Pectobacterium* are weakly or not affected depending on the analyzed strain (Uroz *et al.* 2008; Barbey *et al.* 2013).

A complementary strategy developed in the biocontrol of plant pathogens is biostimulation. It is based on the observation that growth and root colonization of the introduced and native *R. erythropolis* populations may be stimulated by elective carbon sources, such as gamma-caprolactone and gamma-heptanolactone (Cirou *et al.* 2007, 2012; Barbey *et al.* 2012). Noticeably, *R. erythropolis* strains and enzymes are already used as anti-biofouling agents (Oh *et al.* 2012, 2013), as well as bioremediation agents in relation with their fuel and xenobiotic degradation capability (de Carvalho and da Fonseca 2005).

Plant protection strategies targeting QS of the plant pathogens may however have some drawbacks. In some *Pseudomonas* strains used as biocontrol agents, QS positively regulates the expression of plant growth-promoting functions, e.g. production of antibiotic and antifungal molecules directed at fungal pathogens. In addition, a study raised questions about the interference between the biocontrol agents that produce and degrade QS signals, and experimentally evaluated their incompatibility (Molina *et al.* 2003).

Anti-biofouling

Biofouling can be defined at the attachment of one or more organisms to a surface in contact with water. This phenomenon poses serious technological and economical problems in various domains or processes such as naval transportation, aquaculture, the petroleum industries, medical devices, bioreactors or water distribution networks and wastewater plants (a non-exhaustive list; for recent reviews see for instance Fitridge *et al.* 2012; Feng, Wu and Yu 2013; Harding and Reynolds 2014). Bacteria are among the most common biofouling organisms, a feature related to their capability to generate biofilms containing one or more species. In fact, early antifouling strategies relied on physical cleaning, or the use of antibacterial compounds (e.g. cooper salts or metal—a technique already known by the Phoenicians, 1500–300 BC), detergents (e.g. benzalkonium chloride, and sodium dodecylsulfate), aldehydes (e.g. formaldehyde and gluteraldehyde), oxidizing agents such as hydrogen peroxide, ozone, chlorinated compounds (i.e. bleach or chlorine), or

diverse biocides such as tributyltin. All these compounds, used in large amounts or in confined environments, pose serious health and environmental problems due to their toxicity and ecotoxicity. For instance, tributyltin was largely used in antifouling paints for ship hulls in the 1980s and 1990s, and has been identified as the probable major pollutant responsible for the decline of oyster production in marine farms in France during these decades. Further reports on the role of tributyltin in sexual dedifferentiation of gasteropods ('imposex') led numerous countries worldwide to prohibit the use of this molecule in ship paints (for a review, see Dafforn, Lewis and Johnston 2011). Because bacterial biofilm formation is, in part, controlled by QS, various QQ strategies have been developed. Valuable QSI molecules with anti-biofilm activity have been identified in the laboratory, such as the halogenated furanones isolated from the red alga *D. pulchra* (Rasmussen *et al.* 2000).

Being exposed to biofouling, other marine organisms thus constitute a source of antifouling molecules. *Flustra foliacea*, a marine colonial animal of the Bryozoa phylum, produces a set of ten brominated alkaloids, two of which exhibit QSI activity (Peters *et al.* 2003). More generally, Skindersoe *et al.* (2008) reported that out of 284 extracts of marine organisms tested, 23% were active in a LuxR-based screen. Of these, 36 appeared to be active in a *P. aeruginosa* screen (Skindersoe *et al.* 2008). In a similar manner, Dobretsov *et al.* (2011) investigated 78 products isolated from marine and terrestrial organisms, and found that kojic acid, an oxo-pyrone, prevented biofouling in glass plate assays. More recently, derivatives of the diterpenes knightine isolated from *Eunicea knighti* of the Eumetazoa phylum were shown to inhibit bacterial biofilm formation at lower concentrations than does kojic acid (Tello *et al.* 2012). In spite of a relative abundance of data, and the existence of related patents, anti-QS strategies have not been widely translated into innovative products such as antifouling paints in the marine industry.

Membrane filtration is another field in which biofouling is a concern. The food industry and freshwater or wastewater treatment plants are places where biofilm formation on membranes alters their functioning. Several reports described the occurrence of a large number of AHL molecules in biofilm cakes on membrane devices and linked their presence to biofilm formation (e.g. Lim *et al.* 2012). These observations prompted the use of bacteria that degrade QS signals or that of QSI molecules to prevent biofilm formation. Among these, a *Rhodococcus* sp. strain or a recombinant *Es. coli* strain that both efficiently degraded AHL yielded promising results in wastewater treatment procedures (Oh *et al.* 2012, 2013), whereas vanillin associated with a cellulose acetate membrane was found to prevent biofilm in reverse osmosis apparatus (Kappachery *et al.* 2010; Ponnusamy *et al.* 2013). Other groups reported the use of Piper betle (also known as betel) extracts (Siddiqui *et al.* 2012) as an anti-QS agent to mitigate membrane biofouling. Lastly, enzymes immobilized on the membrane, such as acylase (Kim *et al.* 2011; Jiang *et al.* 2013), also permitted a reduction of biofilm formation on these membranes. The development or QQ-based strategies directed at the prevention of biofouling in membrane devices is a relatively recent field of applied research, as most studies have been published within the last five years. These strategies are only in a preliminary phase and several questions on the exact nature of biofilms both in terms of implicated microbes and composition of the extracellular matrix remain, but undoubtedly this field bears high hopes for future applications (for a review, see Malaeb *et al.* 2013).

EMERGING RESISTANCE TO QQ STRATEGIES

QQ strategies are appealing in large part because it is assumed that they apply a limited selective pressure toward the development of resistance, especially in pathogens, considering that virulence traits are generally not essential for bacterial survival (Rasko and Sperandio 2010). However, the possible development of resistance has been suggested in several recent articles. Defoirdt, Boon and Bossier (2010) noted that the expression of QS regulatory genes (i.e. those coding the synthesis and perception of the QS signals) varied within strains of the same species and argued that there is a risk of resistance if this variation induces fitness differences under QQ conditions.

Several reports showed that QS and QS disruption did not affect bacterial growth, but these involved studies performed in rich media, under laboratory conditions where QS-regulated genes are not essential for growth (Defoirdt, Boon and Bossier 2010). In some cases, however, a cost of production of the QS signal was observed (Diggle *et al.* 2007a) and it was found that the cell density of a *lasI* or a *lasR* mutant of *P. aeruginosa* may be 1.5 times higher than that of the wild-type bacteria in rich medium. Under conditions where QS is needed for growth via the production of an extracellular protease, the *lasI* and *lasR* mutants grew much less efficiently than the wild-type strain. A related investigation, based on experimental evolution, revealed that an emerging subpopulation of *lasR* mutants of *P. aeruginosa* was detected after ca. 100 generations of the wild-type strain under culture conditions that require the QS-regulated production of a protease for growth (Sandoz, Mitzimberg and Schuster 2007). Both observations can be related to the ecological concepts of public goods and cheaters. Within this frame, cheaters avoid the cost of cooperation, i.e. the production of the public goods such as QS signals and proteases, but still harvest the benefits (Keller and Surette 2006). The *lasR* mutants that appeared in the experimental evolution experiment do not express QS-regulated genes and thus limit the associated metabolic burden, while they can take advantage of the peptides and amino acids liberated by the protease activity. A recent analysis nevertheless suggested that the spread of resistance to QSIs may be limited by the facts that (i) a population of QSI-sensitive bacteria treated with a QSI would eventually not produce sufficient signal to activate the QS system of a small number of QSI-resistant cheaters and (ii) the signal-independent resistant organisms may not be automatically more fit than sensitive ones when growth was dependent on the group-beneficial QS-regulated production (Gerdt and Blackwell 2014). Another study, based solely on modelization, suggests that the combinations of QSIs—such as a LuxI inhibitor and a non-comptetitve LuxR inhibitor—may be the most robust anti-QS strategy (Anand, Rai and Thattai 2013). In the past five years, several excellent reviews and opinion papers summarize the hypotheses and work that addressed the ecological aspects of QS regulation in relation with a possible resistance to anti-virulence strategies (e.g. Defoirdt, Boon and Bossier 2010; Garcia, D'Alvise and Gram 2013; Zhu and Kaufmann 2013).

The observation that QS inhibition may have some unexpected and adverse effects was reported in a study by Koehler *et al.* (2010) that involved patients whose respiratory tract was colonized by *P. aeruginosa*, and azithromycin, an antibiotic devoid of bactericidal activity on this bacteria, but interfering with QS. As observed *in vitro* by Sandoz, Mitzimberg and Schuster (2007), in the absence of treatment, *lasR* mutants that exhibited reduced virulence spontaneously appeared in the pseudomonad population. However, the advantage of lasR mutants was lost and virulent wild-type isolates predominated during

azithromycin treatment (Koehler *et al*. 2010). This demonstrated that the antivirulence strategy may in some cases increase the prevalence of more virulent genotypes *in vivo*. This is especially true when QSIs also have antibiotic-like properties or affect antibiotic resistance. Some anti-QS compounds may therefore exert an additional and indirect selective pressure toward antibiotic resistance if those molecules are present.

Interestingly, the development of resistance toward QSIs has been investigated at the molecular level in *P. aeruginosa* (Maeda *et al*. 2012; Moore *et al*. 2014). The above-mentioned brominated furanone C30 (see section on halogenated furanones and biofilm formation), closely related to that produced by the red algae *D. pulchra*, was used to supplement *P. aeruginosa* cultures at concentrations that do not inhibit growth in rich media, i.e. when an intact QS system is not needed for growth (Maeda *et al*. 2012). Mutants were isolated that resisted C30. These were affected in two regulatory genes, *mexR* and *nalC*, that control the multidrug efflux pump MexAB/OprM. The *mexR* mutant and the wild-type strains are equally virulent, as judged from *Ca. elegans* fast killing experiments, but only the *mexR* mutant retained an elevated virulence when the assay was performed in the presence of C30. It could be argued that the selection phase experiment was performed under conditions where C30 plays an antibiotic-like role, by preventing growth of the bacteria. Under this condition, it is not surprising to observe that a mechanism allowing resistance to antibiotics, i.e. an increased efflux system, was selected.

As a rule, and in addition to the enhanced efflux activity described above, three other drug resistance mechanisms are known: the degradation or modification of the drug, the overexpression of the target to 'dilute' the drug or the modification of the target to render it insensitive. With respect to the last point, two results need to be mentioned. First, a mutation in a *luxR*-like gene may lead to a modification in the signal-binding site that may cause the sensor protein to become insensitive to the inhibitor, or to turn the inhibitor into an activator that will induce the expression of QS-regulated genes (Koch *et al*. 2005). Second, the degradation of a QSI has been reported by Maeda *et al*. (unpublished, quoted in Garcia-Contreras, Maeda and Wood 2013), who found that C-30 can be catabolized by *P. aeruginosa* strain PA14.

CONCLUSIONS

Surprisingly enough, from the large amount of literature that describes the identification of QQ activity in organisms and tissue extracts, only a few QQ actors have been finely characterized at a molecular level. Only a few were also experimentally evaluated with respect to their biological role in the organism where they came from, the mechanisms of action supporting QQ activity or the use as antibacterial treatments under realistic conditions. In line with the last point, since their discovery in the 1930s, antibiotics have been massively used and proved to be extremely efficient to fight infections. On the dark side, it is quite likely that QQ components (enzymes, QSIs, antibodies) will not be as efficient under 'real life' conditions as antibiotics were and are. On the bright side, the literature strongly suggests that resistance to QS inhibition may appear, but probably at a much lower level than what has been seen for conventional antibiotics, essentially because QQ generated a selective pressure only under conditions where QS is essential, whereas antibiotics generate a very strong selective pressure under all environmental conditions (Defoirdt, Sorgeloos and Bossier 2011). Multitherapies appear as promising approaches against pathogens for limiting their proliferation, virulence and resistance emergence.

A parallel can be drawn here with agricultural issues. The use of phytosanitary compounds has also proved to be highly efficient in fighting pests and weeds, increasing crop quality and yield, but their usage on a large scale—both geographically and over time—has led to the development of resistance (and pollution). A mainstream agronomical trend promotes the development of environmentally friendly approaches, based on a use of novel technical itineraries, biological control agents that do not target the viability of pests, and combined use of molecules and processes. This is also the case for QSIs and QQ enzymes that may be (should be) used as mixes (Anand, Rai and Thattai 2013) or in conjunction with other bioactives molecules such as biostimulating agents (Cirou *et al*. 2007). However, with respect to QQ strategies, a number of problems remain unsolved, such as targeting and delivery of the enzymes or molecules, evaluation of the cytotoxicity and more globally adverse effect of QQ enzymes and QSIs at population, organism, cellular and subcellular levels. These points, along with the above interrogations on the possible developement of resistances, are certainly interesting trails for future research on QQ.

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