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Biotechnological applications of quorum quenching enzymes

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ABSTRACT

Numerous bacteria use quorum sensing (QS) to synchronize their behavior and monitor their population density. They use signaling molecules known as autoinducers (AI's) that are synthesized and secreted into their local environment to regulate QS-dependent gene expression. Among QS-regulated pathways, biofilm formation and virulence factor secretion are particularly problematic as they are involved in surface-attachment, antimicrobial agent resistance, toxicity, and pathogenicity. Targeting QS represents a promising strategy to inhibit undesirable bacterial traits. This strategy, referred to as quorum quenching (QQ), includes QS-inhibitors and QQ enzymes. These approaches are appealing because they do not directly challenge bacterial survival, and consequently selection pressure may be low, yielding a lower occurrence of resistance. QQ enzymes are particularly promising because they act extracellularly to degrade AI's and can be used in catalytic quantities. This review draws an overview of QQ enzyme related applications, covering several economically important fields such as agriculture, aquaculture, biofouling and health issues. Finally, the possibility of resistance mechanism occurrence to QQ strategies is discussed.

1. Introduction

Bacterial communication, referred to as quorum sensing (QS), is the molecular mechanism by which bacteria sense their overall population density, allowing them to synchronize their behavior [1]. Bacteria produce small molecules known as autoinducers (AI's) which are secreted in the environment and can be perceived by specific receptors within neighboring cells. This mechanism regulates gene expression patterns [2]. The response of microorganisms to QS is organism-dependent, but some traits are commonly regulated through QS, such as: production of antibiotics, exopolysaccharides, or exoenzymes, expression of secretion systems, swarming motility, and biofilm formation.

This review first summarizes the main aspects of bacterial QS and its implications in virulence and biofilm formation. Consequently, disrupting QS is particularly promising to modify bacterial behavior and moderate their undesirable traits. Different strategies have been considered for this purpose, including the use of QS inhibitors (QSI's) or quorum quenching (QQ) enzymes. Special attention is then dedicated to applications involving QQ enzymes in various fields such as agriculture, animal and human health, and antifouling. The phosphotriesterase-like lactonase (PLL) family is then discussed as many of these enzymes have been found in extreme environments conferring attractive biotechnological capabilities. In addition, the possibility of resistance mechanisms to QQ strategies is discussed. The strengths and the weaknesses of this approach are emphasized in light of recently published research.

2. Quorum sensing

Several autoinducers have been identified as QS molecules. Gram-positive bacteria mainly use autoinducing peptides (AIP's), also referred to as peptide-pheromones, which are specific to species and strains. Gram-negative bacteria use different types of QS systems: (i) acyl homoserine lactones (AHL's), also known as autoinducer-1 (AI-1), are mostly produced by Gram-negative bacteria: it is a molecule composed of a lactone ring and an aliphatic chain whose length and nature may vary (e.g. *Pseudomonas* spp., *Acinetobacter* spp., *Burkholderia* spp.), (ii) autoinducer-2 (AI-2), a

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furanosyl-borate diester which is found in a wide range of both Gram-negative and Gram-positive bacteria (e.g. *Vibrio* spp., *Pectobacterium* spp.), (iii) AI-3 (epinephrine and norepinephrine) are commonly found in human opportunistic pathogens (e.g. *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., *Salmonella* spp.). Other molecules such as (iv) fatty acids (*Xanthomonas* spp.), (v) esters (*Ralstonia* spp.), (vi) α-hydroxy-ketones (*Legionella* spp., *Vibrio* spp.) or (vii) quinolones (*Pseudomonas* spp.) have also been reported [3–9]. Numerous Gram-negative bacteria utilize more than one QS system and may combine these systems either in additive models [10,11], or in hierarchical models when one system induces a second one [12], or with distinct or partially overlapping systems [13]. Considering the variety of signals and complexity of signaling networks, QS is a sophisticated communication system used by bacteria to sense their population density and their surrounding environment [14].

Bacterial pathogens represent increasing concern to human health due to the rapid dissemination of antibiotic-resistant strains. Infections with these pathogens result in increased lethality risks and greater costs for health care systems. In several bacterial pathogens, QS is involved in the switch between commensal, or saprophytic lifestyles, to pathogenic cycles. This is the case for *Pseudomonas aeruginosa* which is naturally present in water and humid environments. Moreover, *P. aeruginosa* is an opportunistic human pathogen that can proliferate in wounds; in such confined environments, QS signals accumulate and QS is triggered leading to the expression of virulence factors and the development of disease [15].

Bacterial pathogens also represent great financial burdens in industries other than health care. For example, bacterial infections of plants result in significant economic losses in agriculture [16]. The most widespread plant bacterial pathogens were recently listed according to their economic or scientific impact (e.g. *Ralstonia solanacearum*, *Xanthomonas* spp., *Pseudomonas syringae* pv., *Erwinia amylovora*) [17,18]. All of the selected bacteria use complex regulation networks where QS plays a central role to induce virulence. Additionally, fish or crustacean bacterial pathogens (e.g. *Vibrio* spp. [19,20]) have economic impacts in aquaculture, causing losses in livestock and contaminations that may be spread to humans.

QS also regulates the formation of biofilms. Biofilms are a specific mode of life where bacteria adhere to a surface and stick together. They build communities embedded in extracellular polymeric substances mainly made of DNA, proteins and polysaccharides that confer protection to environmental stresses (UV, desiccation, antimicrobial compounds). Biofilms are particularly challenging as they can be formed on a wide range of surfaces, biotic or abiotic, and they contribute to the virulence and resistance of bacteria affecting numerous industries, spanning health care (contamination of medical devices), fisheries, and the oil industry [21–25].

Interfering with QS is an attractive strategy to inhibit biofilm formation and limit the pathogenicity of bacteria. This strategy was first described in 2000 through the identification of an enzyme that degrades AHL QS signal molecules [26]. Two QQ strategies can be distinguished: (i) to prevent bacteria from producing or perceiving QS signals and (ii) to degrade QS signals. The first strategy is mainly based on the identification of molecules QSI's by screening natural compounds that will inhibit QS by different means. Halogenated furanones are one of the most common families of QSI's and they were first isolated from a red macroalga, *Delisea pulchra* [27]. Further studies showed that they both target AHL's or AI-2 mediated QS with distinct modes of action: they reduce the stability or binding affinity of the LuxR regulator and they inhibit the synthase, LuxS, by covalent interaction to prevent AI-2 synthesis [28–30]. Many screens have already been performed to identify such molecules. Most results were obtained in laboratory conditions but few direct applications using QQ compounds have been described. Following the example of QSI's, QQ enzymes have also been investigated for their ability to disrupt QS without the need to enter the bacterial cell. Among these, AHL-lactonases, acylases, or oxidoreductases have proved to display QQ activities. The next section is focused on the description of QQ biotechnological applications.

3. Applications

3.1. Plant pathogens

Bacterial plant pathogens rely on sophisticated regulation networks to synchronize the infection process and induce specific virulence factors when in contact with the host plant. Besides the perception of plant signals or nutrient availability, QS plays an essential role in the establishment of the pathogenic cycle. Therefore, QQ strategies are now considered as possible alternatives or complementary strategies to the use of pesticides [17]. Depending on the bacterial pathogens, different QS signaling molecules are produced: AHL's for *Agrobacterium tumefaciens*, *Dickeya* spp., *Erwinia* spp., *Pantoea* spp., *Pectobacterium* spp. and *P. syringae*; AI-2 for *Erwinia* spp., *Pantoea* spp., *Pectobacterium* spp., 3-hydroxypalmitate methyl ester (3-OH-PAME) for *R. solanacearum*, and diffusible signal factors (DSF family) for *Xanthomonas* spp. and *Xylella fastidiosa* [31]. Most of these signals can be degraded by QQ enzymes: an esterase produced by the soil bacterium *Ideonella* sp. 0-0013 degrades 3-OH-PAME from *R. solanacearum*, the enzyme CarAB (a carbamoyl phosphate synthetase) produced by several *Pseudomonas* spp. degrades DSF signals. Lactonases or acylases are produced by many organisms to degrade AHL signals [32,33].

Some soil bacteria such as *A. tumefaciens* or *Bacillus* sp. naturally produce lactonases to degrade AHL's [26,34,35]. For example *Bacillus thuringiensis* was shown to produce a lactonase, called AiiA, which degrades the AHL's produced by *Pectobacterium carotovorum*, thereby reducing its pathogenicity on potato slices [36]. In order to improve the efficiency of the *B. thuringiensis* lactonase AiiA, a fusion with a secretive protein was generated to enhance the dispersion of the lactonase in the environment, resulting in an increased tolerance to *P. carotovorum* on potato [37]. Since the 1960s, *B. thuringiensis* is commonly used as a biological pesticide against insects due to its natural ability to produce endotoxins lethal to moths, butterflies or mosquitoes [38]. Currently, its use against bacterial pathogens in fields has, to our knowledge, not been reported.

Another QQ strategy was also tested against bacterial plant pathogens: some plants were genetically modified using bacterial genes from *Bacillus* spp. or *A. tumefaciens* to produce lactonases. The first transgenic lines were reported in 2001, transforming tobacco and potato lines with the *aiiA* gene from *Bacillus*. The resulting transgenic lines showed an increased tolerance to *P. carotovorum* with symptoms only appearing after inoculation with very high bacterial concentrations [39].

These results showed that QQ has been used as a successful approach to protect plants from bacterial pathogens in laboratory conditions. Nevertheless, this demonstration was only achieved using plant GMO producing lactonases. QQ enzymes that may be used to treat and protect plants from bacterial infections is an attractive alternative to genetically modified plants but is however impaired by the poor stability of enzymes. To circumvent this issue, the development of environmentally stable and chemical-resistant enzymes is crucial.

Another possible drawback in the use of QQ strategies for pest control could be the impact on beneficial or symbiotic bacteria that are naturally found in the environment. The ecological impact of

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tobacco lines expressing the lactonase AttM from *A. tumefaciens* was shown to be minimal, as no major difference was recorded between the root microbiota of transgenic and WT tobacco lines [40]. Nevertheless, if the bacterial populations were not impacted, some functions of bacteria using AHL-mediated QS might have been altered. This may prove incompatible with the use of *Pseudomonas* spp. as biocontrol agents. Indeed *Pseudomonas* spp. produce antibiotics and antifungal agents under control of AHL-mediated QS and using QQ strategies may prevent their beneficial effects [41]. To date, all experiments were performed in laboratory conditions with all interacting partners being inoculated simultaneously and at relatively high concentrations. The situation in the field is obviously different and further studies are needed to assess the impact of QQ and balance its drawbacks against its beneficial impact; controlling QQ enzyme specificity might be a way to modify this balance.

3.2. Aquaculture

According to the Food and Agriculture Organization (FAO), aquaculture is the farming of aquatic organisms in both coastal and inland areas involving interventions in the rearing process to enhance production. Economically, world aquaculture production represented about 97 million tonnes in 2013 (live weight) with an estimated value of 157 billion USD and 575 aquatic species registered [43]. Bacterial infections comprise a significant constraint to the development of aquaculture in the world, involving billions of USD in annual losses [44,45]. In the United States in 2012, infectious diseases are a top limiting factor that accounts for approximately 45% of losses in aquaculture [46]. The use of disinfectants and antibiotics has only limited success in treating or preventing aquatic diseases [44,45]. In developed countries, such as in the US, Canada or Norway, antibiotics have been restricted to limit the selection for resistant human pathogens [47]. However, the situation is much more problematic in countries with no or less stringent controls [47]. For example, in Chile, more than 385 tonnes of antibiotics were used in 2007 to produce a yield of 300,000 tonnes of Atlantic salmon [46]. It is estimated that about 1500 tonnes of tetracycline and 478 tonnes of florfenicol were used in salmon aquaculture in Chile between 2000 and 2007, and 950 tonnes of quinolones between 2000 and 2008 for this purpose [48]. More importantly, massive use of antibiotics in aquaculture systems is leading to rapid evolution and spread of multiple antibiotic resistant strains that could potentially threaten human health security in significant ways [49,50]. As a consequence, numerous resistant human pathogens were isolated from aquacultures [51]. Various approaches are used to reduce contamination risks, such as the prevention of pathogen transmission between farms, stress reduction, or increasing hygiene [44]. Biological methods such as the utilization of probiotics [52], bacteriophage therapies, or immunostimulants were also investigated to prevent fish infections [44,53]. Immunostimulants are able to increase non-specific and/or specific immune response. Among these, β-glucans, alginate, or ergosan have been studied for their capacity to stimulate innate immune resistance or to enhance physiological and immunological factors [54,55]. Vaccines are also employed to control the majority of fish pathogens [56]. The methods of administration are immersion, injection, or addition in the food [57]. Injection is preferentially used, but is laborious and not effective for small or young fish [58]. However, none of these methods seem to significantly solve the problems of bacterial infections.

QQ is an appealing strategy that might reduce bacterial infections with a limited possibility that resistances will develop [44]. Numerous Gram-negative bacteria possess a QS system, including major fish pathogens such as *Aeromonas* or *Vibrio* spp. These bacteria use an AHL-LuxR/LuxI QS-like system with LuxR-LuxI homologues and the signal molecules are specific to each bacterium. More precisely, *Aeromonas hydrophila* mainly uses a *N*-butyryl-L-homoserine lactone (C4-HSL) as signal molecule [59], whereas the *Vibrio parahaemolyticus* QS system is regulated by *N*-hexanoyl-L-homoserine lactone (C6-HSL or HHL) [60]. AHL-degrading enzymes were thus investigated for disrupting QS of fish pathogens. Purified lactonases were tested and in particular oral administration of AHL-lactonase from *Bacillus* sp. strain AI96 was shown to decrease *A. hydrophila* infection in zebrafish [59]. Similar results were observed using AHL-lactonase (AiiAB546), from *Bacillus* sp. B546 produced by *Pichia pastoris*, in common carp [61]. Another pathogen, *V. parahaemolyticus*, is responsible for significant infections in shrimp and involves gastroenteritis for people who consume infected shrimp. The pathogen colonization of Indian white shrimp was successfully reduced after injection into the abdominal cavity of AHL-Lactonase from *Bacillus licheniformis* DAHB1 [60].

The use of lactonase-expressing whole cells was investigated as an economically-friendly alternative. For example, *Tenacibaculum* sp. strain 20J was shown to degrade C4-HSL to C14-HSL and was successfully used to decrease *in vitro* concentration of AHL's produced by *Edwardsiella tarda* strain ACC35.1, a bacterium responsible for the Edwardsiella septicemia [62]. In order to select natural AHL's degrading microbial communities, shrimp fed with AHL's were used. One of the generated communities sampled from shrimp intestinal track was shown to protect a euryhaline rotifer from *Vibrio harveyi*. This microbial community proved to be able to degrade the *V. harveyi* HAI-1 autoinducer *in vitro* and *in vivo* [63].

Altogether these results suggest that QQ strategies are particularly attractive for limiting bacterial infections in the aquaculture industry. Moreover, QQ enzymes may be used in combination with prebiotics, probiotics, immunostimulants and vaccines to control and protect fish against a wide spectrum of pathogens.

It should be noted that biofilms in aquaculture pools act as reservoirs for the pathogenic bacteria that are responsible for recurring diseases [64], hence the importance of biofilm and biofouling treatments.

3.3. Antifouling

- Membrane bioreactors and filters

Membrane bioreactors (MBR) are used in many industries or processes to combine a classic bioreactor system with a membrane filtration step. This technology is widely used in wastewater treatment for the bacterial cleaning of soluble pollutants and the retention of microorganisms and solid particles [65]. Fouling is a major concern encountered in such systems due to the accumulation of biological material both onto and into the membrane. In wastewater treatment, the filtration membrane is quickly colonized by organisms, thus reducing the efficiency of the process. To circumvent this drawback, high pressure is often required which represents an important energy consumption [66], as well as increased cleaning frequency, causing additional costs.

In order to counter biofouling, several approaches have been developed, including chemical, physical and biological strategies, for either preventing formation or cleaning [67,68]. In this context, QQ has emerged as a promising technology to inhibit the early stages of biofouling development.

The presence of AHL-producing bacteria in membrane biofouling has been demonstrated, highlighting the presence of species potentially using QS [66]. AHL-degrading enzymes or QS inhibitors are particularly attractive for minimizing the consequences of biofouling. Obviously, this would not avoid using classic cleaning methods, but it could reduce their frequency and decrease

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overall maintenance costs.

A study using membrane filters has shown the potential of vanillin to reduce biofilm formation, presumably due to the QQ effect of the molecule [69]. Two more practical studies have described the QQ effect of *Piper Betle* extract to reduce biofouling in MBR [70,71]. Other QS inhibitors could be used to prevent biofouling, but such molecules are usually soluble and would pass through the membrane, causing a secondary contamination.

Another approach is to use QQ enzymes that could disrupt bacterial communication, thus limiting the formation of biofilms and reducing impairment of the filtering system. The efficiency of Porcine kidney acylase I was demonstrated and showed an increasing lifetime of the filter in presence of the enzyme as compared to the control [66]. Most enzymes offer the advantage to be efficient and more stable when immobilized. Former enzymes were thus considered for immobilization strategies onto nanofiltration membrane [72], as well as alginate capsules [73], magnetic ion-exchange resin [74], and magnetic mesoporous silica beads [75]. These all increased stability and efficiency of the enzyme. In order to avoid the immobilization step and to limit the process costs, whole bacterial cells producing QQ enzymes were investigated [76–80]. Indeed, by entrapping the cells in different kinds of systems, be it microvessels or beads, the continuous production of one or more lactonases or acylases was developed. All these studies showed a significant diminution in biofilm formation and an increase of the MBR efficiency at low filtering pressure. In both cases, the QQ did not seem to impact the depolluting efficiency of the wastewater bacteria, confirming the potential of the technology [73,78].

- Marine biofouling

Biofouling is also a major issue for structures in contact with seawater, such as boats, fish nets, or pipelines. Two related phenomenon usually occur on submerged surfaces: microfouling caused by microorganisms such as bacteria or protozoans, and macrofouling linked to algae or barnacles [81]. Biofouling is responsible for friction on boats, inducing excessive fuel consumption, increased maintenance costs, and generates considerable monetary losses annually [81,82]. Since tributyltin (TBT), an efficient antifouling molecule, has been banned due to its high toxicity, current solutions for biofouling prevention include paints and coatings, mainly containing copper as antifouling agent. The use of copper is also considered an environmentally unfriendly method but is still in use [82,83]. Antifouling paints and coatings save an estimated 60 billion USD annually [22].

In the quest for non toxic alternatives, enzymes have been considered for either preventing biofouling formation or destroying biofilms. Several reviews discuss the potential of these different enzymes and even consider actual patents for their incorporation into paints or coatings [81,82,84].

However, mature biofilms remain difficult to degrade and special attention is dedicated to prevent biofouling formation. In this respect, QS disrupting strategies would be of prime interest. First, it could reduce QS-regulated biofilm formation involved in microfouling but may also impact the attraction and fixation of macrobiofouling species. Different studies have demonstrated the influence of bacterial biofilm on the settlement of spores from algae or others [85–87]. A chemical approach would be to use QS inhibitors in the coating to prevent biofouling. In this way, kojic acid has been reported as a non-toxic QS inhibitor and is incorporated into painting, conferring the ability to inhibit both microfouling from bacteria as well as diatom *Amphora coffeaeformis* macrofouling over one month [87]. Even though QQ enzymes have been described in the literature as a solution for MBR fouling, no comparable work has been done yet for marine biofouling. The main constraint would undoubtedly be the lack of stability of enzymes within paints as well as their possible limited activity in seawater. QQ enzymes from extremophile organisms would constitute promising candidates as they usually are highly robust and may be active in non-conventional environments. Furthermore, compared with QS inhibitors, QQ enzymes would constitute an environmentally friendly solution as they may be active while incorporated irreversibly into paints or coatings, their action would be localized to the ship hull. Moreover, in case of releasing, enzymes could be degraded in the environment and would not bio-accumulate. QQ-based approaches, while restrictive as it does not prevent direct settlement of macrofouling species, would be a good complement of available approaches to reduce marine biofouling.

3.4. Medical devices

QS-induced bacterial infections are particularly problematic in medical environments. In the US, *P. aeruginosa* accounts for 7.5% of general healthcare associated infections. Other pathogens, such as *Acinetobacter baumannii*, *Proteus* spp., and *Serratia* spp., are overall responsible for 6.4% of the infections. These pathogens are often found with catheter associated urinary tract infections and ventilator associated pneumonia [88]. As such, healthcare facilities could directly benefit from research on AHL-targeted QQ. Furthermore, QS is responsible for a number of problematic complications such as antibiotic resistance, biofilm formation, competence, and virulence factor expression [89–91]. Hereafter are presented QQ-based devices, mainly based on enzymatic functionalization, to circumvent QS-mediated bacterial infections.

- Quorum quenching membrane

The immobilization of the hyperthermostable Phosphotriesterase-Like Lactonase (PLL) from *Sulfolobus solfataricus*, referred to as *Sso*Pox, onto nanoalumina membranes to quench bacterial communication was investigated [92]. About 95% of the enzyme was successfully immobilized with interactions tight enough to resist high ionic strength washes. The enzymatic activity after immobilization was 25% that of the free enzyme. The addition of membranes containing *Sso*Pox in bacterial cultures of *P. aeruginosa* PAO1 resulted in an overall decrease of pyocyanin expression and elastase activity. For the first time, the authors demonstrated that immobilization of QS-disrupting enzymes may be useful to decrease the virulence of bacterial pathogens and paved the way for the development of innovative medical devices.

- Functionalized catheter

The persistence of pathogens in catheters is quite problematic both in terms of costs and patient health [93]. To address this issue, a central venous catheter coated with the QSI 5-fluorouracil was developed and clinically evaluated [94]. The study was performed with a total of 960 adult patients in 25 US intensive care units and demonstrated that 5-fluorouracil coated catheter is a safe and promising alternative to catheters coated with silver sulfadiazine or chlorhexidine. Catheter functionalization with catalytic quenchers such as enzymes has also been investigated using a silicon catheter coated with multiple layers of an acylase from *Aspergillus melleus* [95]. The adherence of *P. aeruginosa* ATCC 10145 was assessed and showed to be strongly inhibited as compared to the non-treated control. Biofilm quantity was also reduced by about half in the acylase coated catheter, both in static and dynamic models. In itself, the coated catheter proved to be non-toxic against cultured skin fibroblast. Very recently, the development of functionalized urinary

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catheters combining both a QQ acylase and a matrix degrading α-amylase was reported. The acylase and amylase limited the biofilm formation of *P. aeruginosa* and *Staphylococcus aureus* respectively. This device was further evaluated in an *in vivo* animal model and was shown to delay biofilm development for up to 7 days [96].

- Topical treatment and dressing perspectives

A burn infection model on mice using *P. aeruginosa* PAO1 was developed to assess the efficiency of a purified lactonase from *Bacillus* sp. ZA12 [97]. Animals were burned to the third degree and infected with a lethal dose of 10^6 bacteria subcutaneously. Topical application with a lactonase-containing gel prevented systemic spread of *P. aeruginosa* through burned skin and reduced animal mortality. When the lactonase was combined with ciprofloxacin no mortality was observed, underlining the synergistic effect of the treatments. This report highlighted the efficiency of quenching enzyme by topical administration alone or in combination with antimicrobial treatments to prevent bacterial infection of wounds. Histological studies demonstrated that fewer damages and inflammatory markers were observable in tissues when the enzyme and antibiotics were used together. This would offer new perspectives for the investigation of enzymatically functionalized dressings and bandages (Fig. 1).

- Aerosolization

The *in vivo* use of an exogenous, engineered hyperthermostable lactonase derived from *Sso*Pox, was reported in a rat pulmonary infection model. The purified enzyme was administered concomitantly to an infection by *P. aeruginosa* PAO1 resulting in a mortality drop, from 75% to 20%, 48 h post infection [99]. The enzyme was also shown to be able to reduce, *in vitro*, the biofilm formation of *P. aeruginosa* PAO1 by up to 65% as compared to the untreated condition. The aerosolized lactonase proved to be efficient to diminish the expression of QS dependent virulence factors and would be particularly relevant for the treatment of cystic fibrosis related infections.

As previously described, many studies have reported the potential of QQ strategies to inhibit bacterial virulence and limit biofilm formation of a wide range of bacterial strains and the corresponding applications are summarized in Table 1. Among these, the use of AHL degrading enzymes was particularly emphasized. Such enzymes may be used in a broad spectrum of applications, from agriculture, bioprocess and anti-fouling, to human and animal healthcare. However, many issues have to be addressed to strengthen their potential. Large-scale production, stability, tolerance to industrial processes, or storage have to be investigated as they represent major constraints to the use of enzymes for biotechnological purposes. In this context, phosphotriesterase-like lactonases appear to be particularly relevant as many of these may be isolated from extreme environments and display both high activity and large substrate specificity. The possible emergence of resistance phenomenon to QQ strategies has also to be considered.

4. Phosphotriesterase-like lactonases (PLL's)

4.1. Highly promiscuous enzymes

PLL's are natural lactonase (EC 3.1.1.25) enzymes with promiscuous catalytic activity against organophosphates (e.g. paraoxon). These enzymes are strongly related to phosphotriesterases (PTE's), constituting their most probable ancestor [100–102]. They belong to the amidohydrolase superfamily and their 3D-structure is formed by a $(\beta/\alpha)_8$-barrel fold, shows an active site containing two metal cations involved in catalysis and coordinated by four histidine residues, as well as a carboxylated lysine and an aspartic acid [103]. The bi-metallic center participates to the catalysis as a Lewis acid involved in the activation of a water molecule into a hydroxide anion that subsequently acts as a nucleophile to attack the substrate. Two subfamilies, PLL-A's and PLL-B's, were identified according to their sequence similarities as well as the length of the

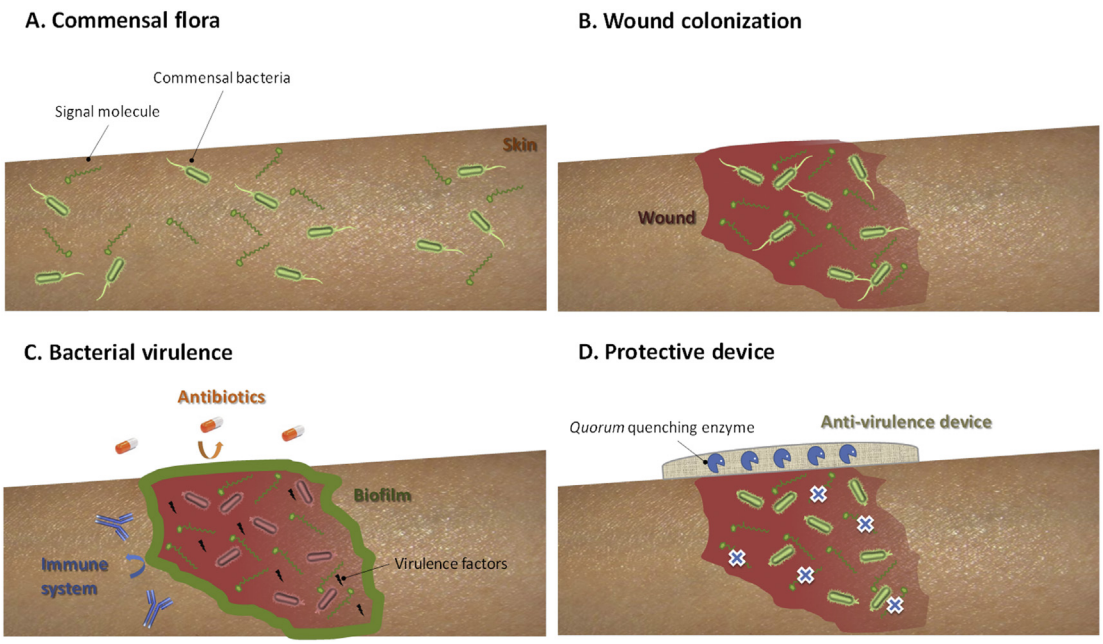

Fig. 1. Enzyme-functionalized anti-virulence device and mechanism of action. **A**) Commensal bacteria are naturally present on healthy skin and use signal molecules to communicate without being virulent. **B**) In case of wound, the bacteria have a favorable medium for their growth and start their colonization step. **C**) When bacterial concentration is over a certain threshold the bacteria adapt their behavior and start being virulent by reducing motility [98], synthesizing a biofilm and secreting virulence factors **D**) Enzyme-containing devices hydrolyze QS signal molecules and prevent infection by decreasing virulence factor secretion, biofilm synthesis and motility.

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Table 1
Summary of QQ applications.

Field	Application	Quencher	Reference
Agriculture	Transgenic lines of tobacco and potato	AiiA lactonase from *Bacillus* sp.	[39]
	Transgenic lines of tobacco	AttM lactonase from *A. tumefaciens*	[40]
Aquaculture	Oral administration in zebrafish	AI96 lactonase from *Bacillus* sp.	[59]
	Oral administration in common carp	AiiAB546 lactonase from *Bacillus* sp.	[61]
	Injection in indian white shrimps	Lactonase from *B. licheniformis* DAHB1	[60]
Antifouling			
MBR	Reduce biofouling in MBR	P. betle extract	[70,71]
	Increase filter lifetime	Porcine kidney acylase	[66]
	Nanofiltration membrane	Porcine kidney acylase	[72]
	Alginate capsules	Porcine kidney acylase	[73]
	Magnetic ion-exchange resin	Porcine kidney acylase	[74]
	Magnetic mesoporous silica beads	Porcine kidney acylase	[75]
Marine biofouling	Coating	Kojic acid	[87]
Medical devices	Nanoalumina membrane	PLL *Sso*Pox from *S. solfataricus*	[92]
	Coated catheters	5-fluorouracil	[93,94]
		Acylase from *A.melleus*	[95]
		Acylase from *A. melleus* and α-amylase from *B. amyloliquefaciens*	[96]
	Topical treatment	Lactonase from *Bacillus* sp. ZA12	[97]
	Aerosol	PLL *Sso*Pox from *S. solfataricus*	[99]

two characteristic loops 7 and 8. PLL-A's have been shown to degrade AHL's as well as oxo-lactones, while PLL-B's are specific to oxo-lactones [104]. As such, both PLL-A's and -B's may be considered for QQ strategies and PPL-A's are particularly promising considering their broad substrate promiscuity. Furthermore, PLL's are attractive because this family encompasses numerous representatives from extreme environments. These enzymes exhibit high thermal stability and robustness that is desirable in biotechnological applications. Among these, *Gk*L (isolated from the thermophile *Geobacillus kaustophilus*) [105], *Sac*Pox (isolated from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*) [106], *Sis*Lac (isolated from the hyperthermophilic archaeon *Sulfolobus islandicus*) [107], *Sso*Pox (isolated from the hyperthermophilic archaeon *S. solfataricus*) [103,108–110], or *Vmo*Lac (isolated from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia*) [104,111], have drawn special interest. Regarding their intrinsic stability (e.g. T_m values of 106 °C and 128 °C for *Sso*Pox and *Vmo*Lac, respectively) some of these PLL's have been further considered for directed evolution experiments to improve their potential for QQ purposes [109,112,113].

PLL's have distinct sequences and structures from another family of QQ lactonases (QQL's) belonging to the metallo-β-lactamase superfamily (Fig. 2). This superfamily includes the above-mentioned enzyme AiiA from *B. thuringiensis* [114,115]. Although these proteins share neither sequence nor structural similarity, their active sites show striking similarities [102].

4.2. *SsoPox a promising candidate for QQ applications*

One of the best characterized PLL's so far is *Sso*Pox. This enzyme, isolated from the hyperthermophilic archaeon *S. solfataricus*, was initially investigated for its ability to hydrolyze phosphotriesters (widely used as pesticides and chemical warfare agents) [116]. *Sso*Pox is an extremely stable enzyme [108], active over a wide range of temperatures (10–100 °C) and pH values (5.0–9.0), paving the way for a wide panel of biotechnological applications [116]. Moreover, it is a very proficient lactonase, including at room temperature. The 3D-structure of *Sso*Pox was reported and was found as being a distorted $(\beta/\alpha)_8$ barrel-fold [117]. Conversely to other known phosphotriesterases displaying a similar structural organization, *Sso*Pox differs by the length of two loops. Loop-7 and loop-8 are shorter and longer than other reported PTE's, respectively. These modifications

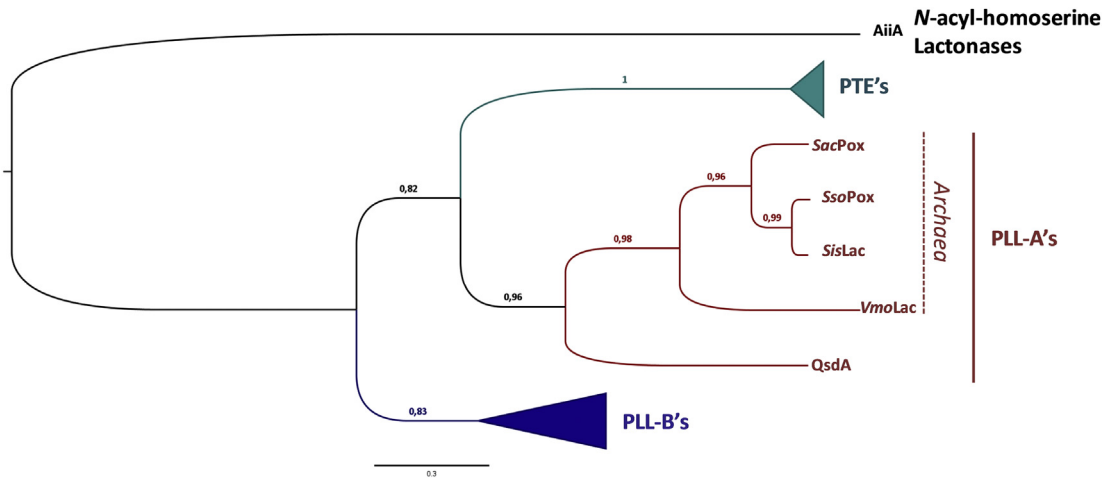

Fig. 2. Phylogenetic tree of PLL's and enzyme-related families. Webtool www.phylogeny.fr/simple_phylogeny.cgi was used for sequence alignment and phylogeny and the tree was obtained with FigTree v1.4.0. Sequence used for the analysis were: AiiA (P0CJ63), PTE's (Q93LD7, P0A434), *Sac*Pox (V9S7Z1), *Sso*Pox (Q97VT7), *Sis*Lac (C4KKZ9), *Vmo*Lac (F0QXN6), QsdA (B1N7B5), PLL's-B (A4IN23, Q5KZU5, Q9RVU2).

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are responsible for the creation of a hydrophobic channel that perfectly accommodates the lactone substrate (Fig. 3).

Various lactones were assayed with the wild-type enzyme including AHL's, γ-lactones, or δ-lactones (Fig. 4) [100,109,118]. Catalytic parameters were determined and k_{cat}/K_M values up to $8.0 \times 10^4\ M^{-1}\ s^{-1}$ were reported, suggesting that *Sso*Pox is a natural proficient lactonase. Protein engineering strategies were also considered to further increase its catalytic efficiency towards lactone substrates. Residue W263 was particularly considered as it is located in the active site and is involved in enzyme loop flexibility, mediating its promiscuity [109]. Saturation mutagenesis experiments were performed and led to the identification of extremely efficient variants (e.g. *Sso*Pox-W263I) with k_{cat}/K_M values up to $5.8 \times 10^6\ M^{-1}\ s^{-1}$ at room temperature. About twenty lactones with various chemical structures are known to be hydrolyzed by *Sso*Pox and/or its variants underlining the wide promiscuity of these enzymes (Table 2). Furthermore, *Sso*Pox and its variants exhibit a strong tolerance to proteases, surfactants, and also organic solvents [109,110].

About ten structures, either in *apo* or *holo* form, are now available in the protein database (www.pdb.org). Among these, variant *Sso*Pox-W263I was found to be particularly efficient for lactone hydrolysis and has been co-crystallized with the substrate analogue *N*-decanoyl-L-homocysteinethiolactone (C10HTL) (Fig. 3). This variant is of utmost interest for QQ investigations as it is both highly efficient for lactone hydrolysis and is widely promiscuous towards a large range of substrates. Moreover, *Sso*Pox-W263I retains an impressive thermostability ($T_m = 88\ °C$), albeit lower than the wild-type, and, owing to its properties, might be suitable for a large panel of applications in the various domains afore-mentioned. Altogether, thermostable lactonases are particularly appealing for biotechnological investigations as these enzymes could be more readily compatible with material sciences, such as incorporation into coatings, materials, paints and polymers, and to develop innovative, non-toxic and environmental-friendly alternatives against bacterial infections and biofilms.

5. QQ strategies and resistance

Antibiotics have been widely used over the past decades for treating chronic and acute bacterial infections. Antibiotics induce a strong selection pressure on bacteria by either killing them or inhibiting their growth. However, the intensive use of antimicrobial agents has led to the emergence of adaptive resistance that considerably limits their efficiency and is associated with treatment dose increase [119–121]. QQ has emerged as a promising therapeutic alternative as it can be used to inhibit both the secretion of virulence factors and the formation of biofilm [122], but does not kill bacteria [123–125]. Therefore, QQ strategies are believed to induce a milder selection pressure. However, recent evidence suggests that the effect of QS disruption on bacterial growth was dependent on the culture medium used (*i.e.* nutrient-rich or not) [126], and might thereby introduce a selection pressure, albeit milder than a biocide strategy, and select for resistant bacteria [127,128]. Using QS-disrupted variants, studies have shown that bacterial resistance to QS may arise. Mutations increasing efflux of C-30, an efficient QQ furanone, as well as compensatory mutations were observed as mechanisms to overcome QS disruption [129,130]. Moreover, "Social cheaters", (*i.e.* bacteria that ceased production of quorum regulated factors), were reported [131]. Such QS-insensitive mutants might interfere with QQ efforts [132], but recent experimental studies suggest that QQ resistance would spread slowly, as these mutants were found to be less fit than their counterparts [133].

The emergence of resistance to QQ strategies will certainly depend on the actual used strategy. The use of QQ enzymes is possibly the least resistant-prone of all QQ strategies because enzymes can act remotely and do not need to enter the bacterial cells. Moreover, contrary to QSI's that need to bind to a target protein (the signaling receptor), QQ enzymes act independently. Putative resistance mechanisms to QQ enzymes have been proposed [127,128] and suggest that bacteria may evolve for an increased production of the autoinducer molecule (AHL) to counteract the hydrolysis by QQ enzymes. This could be bypassed by increasing the total enzymatic activity in the environment. Another resistance scenario would consist of modifications to the chemical structure of the autoinducer. This possibility is reduced by the fact that QQ enzymes are naturally broad spectrum enzymes and can be engineered for altered specificity. Another mechanism would consist of the selection of modified LuxR receptors with tremendous affinity for the autoinducer, or with improved response to AHL [134,135], therefore the QQ enzymes would not be active enough at these low concentrations of autoinducers. In this case, enzyme engineering may offer solutions to produce enzymes with higher affinity for AHL's.

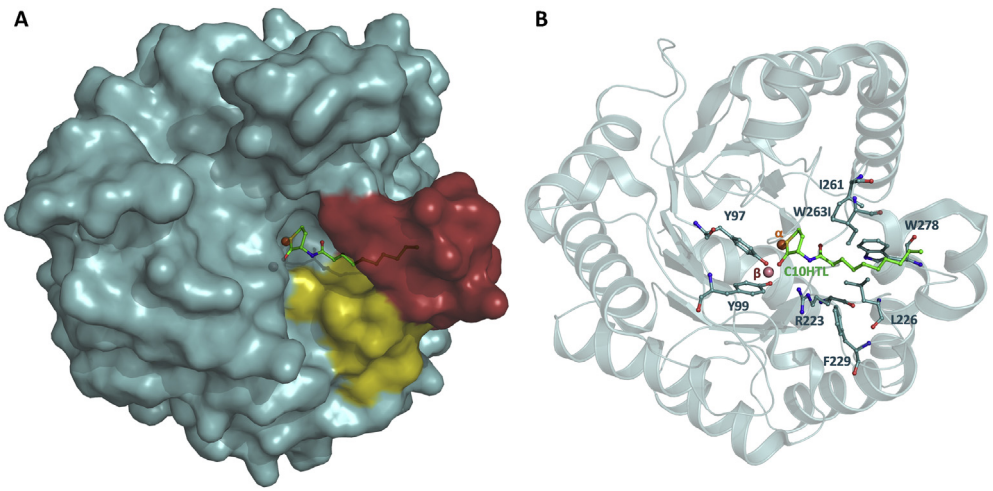

Fig. 3. Representation of *Sso*Pox variant W263I structure (PDB ID: 4KF1) bound with substrate analogue C10HTL. (**A**) Structure is shown as surface and loop-7 and loop-8 are emphasized in yellow and red respectively. (**B**) C10HTL is shown in green stick, surrounding residues are emphasized by blue sticks and divalent cations are drawn as spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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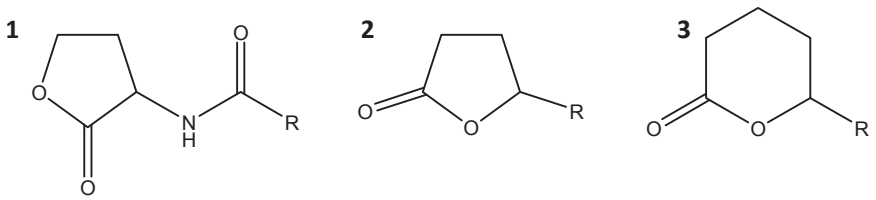

Fig. 4. Chemical structures of *Sso*Pox lactone substrates. (**1**) Acyl-Homoserine Lactones, (**2**) γ-lactones, (**3**) δ-lactones.

6. Conclusion and perspectives

Although it may affect bacterial fitness, QS disruption is a promising strategy to substitute or at least supplement antibiotics. Many studies have shown that biofilm is associated with antibiotic and antimicrobial agent resistance for a wide range of bacteria [21,136–141]. Biofilm formation governs many mechanisms involved in antibiotic resistance, such as limited penetration of the antibiotic, horizontal gene transfer within the bacterial community and changes in gene expression that may influence resistance [142,143]. QS disruption often results in decreased biofilm formation, therefore QQ could be an efficient tool for the restoration of

Table 2
Catalytic parameters of *Sso*Pox wild-type and variants towards lactones with respective reaction conditions. Data were taken from Ref. [100,109,118].

Substrate	Enzyme	Condition	k_{cat} (s^{-1})	K_M (μ M)	k_{cat}/K_M (M^{-1} s^{-1})
3-oxo-C12 AHL (l)	WT	25 °C	1.01	456	2.22×10^3
	W263F	25 °C	0.41	146	2.81×10^3
	W263M	25 °C	ND	ND	ND
	W263L	25 °C	ND	ND	ND
	W263I	25 °C	1.8	17.8	1.01×10^5
	W263V	25 °C	3.0	24.7	1.21×10^5
	W263T	25 °C	6.44	137	4.70×10^4
3-oxo-C10 AHL (l)	WT	25 °C	4.52	143	3.16×10^4
	WT	25 °C + 0.1% SDS	ND	ND	1.96×10^2
	WT	25 °C + 0.01% SDS	0.75	243	3.09×10^3
	W263F	25 °C	3.96	288	1.38×10^4
	W263M	25 °C	ND	ND	ND
	W263L	25 °C	ND	ND	ND
	W263I	25 °C	0.6	1605	3.74×10^2
	W263V	25 °C	0.19	1346	1.41×10^2
	W263T	25 °C	0.11	1000	1.06×10^2
3-oxo-C6 AHL (l)	WT	25 °C	0.08	558	1.49×10^2
3-oxo-C6 AHL (r)	WT	25 °C	0.04	592	6.87×10^1
3-oxo-C8 AHL (l)	WT	25 °C	0.54	123	4.39×10^3
3-oxo-C8 AHL (r)	WT	25 °C	0.42	256	1.63×10^3
Undecanoic-δ-lactone (r)	WT	25 °C	7.38	94	7.86×10^4
	W263F	25 °C	66.5	135.2	4.92×10^5
	W263M	25 °C	71.2	161	4.42×10^5
	W263L	25 °C	56.8	219	2.59×10^5
	W263I	25 °C	58.0	<10	$>5.80 \times 10^6$
	W263V	25 °C	44.8	57	7.92×10^5
	W263T	25 °C	93.3	130	7.17×10^5
Undecanoic-γ-lactone (r)	WT	25 °C	4.95	2099	2.36×10^3
	WT	25 °C + 0.1% SDS	2.23	1250	1.78×10^3
	WT	25 °C + 0.01% SDS	0.46	94	4.89×10^3
	W263F	25 °C	4.63	373	1.24×10^4
	W263M	25 °C	4.25	334	1.27×10^4
	W263L	25 °C	3.92	371.8	1.05×10^4
	W263I	25 °C	1.94	361	5.37×10^3
	W263V	25 °C	5.64	1760	3.20×10^3
	W263T	25 °C	4.55	13	3.49×10^5
γ-butyrolactone	WT	25 °C	ND	ND	1.20×10^3
γ-heptanolide (r)	WT	25 °C	2.92	166	1.76×10^4
Nonanoic-γ-lactone (r)	WT	25 °C	5.54	2943	1.88×10^3
Dodecanoic-γ-lactone (r)	WT	25 °C	2.72	1220	2.23×10^3
δ-valerolactone	WT	25 °C	ND	ND	ND
Nonanoic-δ-lactone (r)	WT	25 °C	15.32	359	4.27×10^4
Dodecanoic-δ-lactone (r)	WT	25 °C	12.65	1678	7.54×10^3
ε-caprolactone	WT	25 °C	4.45	234	1.90×10^4
Dihydrocoumarin	WT	25 °C	7.32	1376	5.32×10^3
5-thiobutyl-γ-butyrolactone	WT	70 °C	29.0	80	3.60×10^5
	R223H	70 °C	0.42	273	1.54×10^3
	Y97W	70 °C	75.7	1540	9.58×10^5
5-thioethyl-γ-butyrolactone	WT	70 °C	9.0	15	7.00×10^5
5-thiohexyl-γ-butyrolactone	WT	70 °C	6.0	70	8.00×10^4

ND corresponds to an undetermined value.

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bacterial susceptibility to antibiotics or antimicrobial agents in biofilms [143].

QQ strategies, particularly catalytic quenchers, such as enzymes are appealing to develop new alternatives for QS-disruption and antifouling. Since stability is a major constraint that usually impairs enzyme utilization, intensive efforts have been dedicated to the isolation of robust enzymes from extreme environments. Among these, PLL's (particularly *Sso*Pox) are highly promising as they have already been successfully incorporated into devices while retaining their lactonase activity. Moreover, enzymes are highly attractive as these molecules are usually not toxic and may be integrated into various matrices without being released. The proofs of concept have been widely described and further investigations would obviously permit to develop concrete applications (e.g. medical devices, paintings, coatings …) in order to address the issues of bacterial virulence and biofouling. Furthermore, the applications of QQ enzymes are focused on the disruption of the AI-1-based QS mechanism. The quest for enzymes targeting AI-2, AI-3, or even AIP's is of utmost interest for extending the potential of QQ strategies to a wider panel of Gram-negative and Gram-positive bacteria.

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