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Engineering Acyl-Homoserine Lactone-Interfering Enzymes Towards Bacterial Control

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Abstract

Enzymes able to degrade or modify acyl-homoserine lactones (AHL) have drawn considerable interest for their ability to interfere with the bacterial communication process referred to as Quorum Sensing (QS). Many proteobacteria use AHL to coordinate virulence and biofilm formation in a cell density-dependent manner, thus AHL-interfering enzymes constitute new promising antimicrobial candidates. Among these, lactonases and acylases, have been particularly studied. These enzymes have been isolated from various bacterial, archaeal or eukaryotic organisms and have been evaluated for their ability to control several pathogens. Engineering studies on these enzymes were carried out and successfully modulated their capacity to interact with specific AHL, increase their catalytic activity and stability or enhance their biotechnological potential. In this review, special attention is paid to the screening, engineering and applications of AHL-modifying enzymes. Prospects and future opportunities are also discussed with view to developing potent candidates for bacterial control.

Introduction

Over the last two decades it has become evident that bacteria are social microorganisms with the ability to coordinate their behavior in a cell density-dependent manner (1). This communication, referred to as Quorum Sensing (QS), relies on the synthesis, diffusion and detection of small signaling molecules, also known as autoinducers (AI) (**Fig. 1A**)(2, 3). Thanks to this cell-to-cell communication process, bacteria can collectively adapt their behavior as AI accumulate proportionally to cell density and orchestrate gene expression depending on AI concentration. QS thus enables bacteria to regulate mechanisms that are beneficial above a certain population threshold but are non-effective and may be deleterious at low cell density. A wide variety of chemical molecules has been integrated into bacterial communication, with Gram-negative bacteria mainly using acyl-homoserine lactones (AHL) (4, 5). AHL chemical structure includes a homoserine lactone ring with an acyl chain that can vary in length or functionalization (**Fig. 1B**). AHL have been largely studied because they are involved in the regulation of many bacterial traits including virulence (6, 7), biofilm formation (8) or tolerance to antimicrobials (9, 10) and are used by many human pathogenic bacteria, including antibiotic resistant bacteria, during their infection process (11–13). Several antibiotic resistant strains were flagged by the WHO as research priority targets. This list includes various Gram-negative bacteria with AHL-mediated virulence (e.g. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*) (**Fig. 2**) (14). Interfering with AHL signaling is thus considered a potential way to decrease bacterial virulence and to strengthen the available antimicrobial arsenal (15, 16). This strategy, also known as Quorum Quenching (QQ) can be achieved using various compounds including natural or synthetic QS inhibitors (QSI) able to compete with AHL, sequestering antibodies or degrading enzymes (17). AHL-interfering enzymes have been extensively investigated as they do not need direct contact with bacteria conversely to QSI. Indeed, they can catalytically degrade AHL without the need for entering cells, being less invasive than QSI and may further show bactericidal effects (18–20). Main representatives of such enzymes are lactonases and acylases (**Fig. 3**). These enzymes have been isolated from a wide variety of prokaryotes, archaea or eukaryotes. Lactonases catalyze the opening of the lactone ring, while acylases remove the acyl chain from the homoserine lactone moiety (**Fig. 1B**) (21, 22). AHL degrading lactonases from different protein families were reported including metallo-β-lactamase, phosphotriesterase-like lactonase (PLL) or paraoxonase. Conversely, acylases active towards AHL mainly belong to the Ntn-hydrolase family (20). The biochemical properties of these enzymes have been investigated including their kinetic properties, stability and ability to control microbes both *in vitro* and *in vivo* for various applications, ranging from medical devices to animal health and agriculture (18, 20). In addition, several protein engineering approaches have been considered to increase activity, enhance stability or change AHL selectivity. Besides acylases and lactonases, other AHL-interfering enzymes were also reported including oxidoreductases or esterases albeit their activity and engineering were more rarely studied (20).

Considering the global antimicrobial resistance concern, QQ offers a new approach to counteract bacterial virulence while not

challenging survival of bacteria and limiting their adaptation. In this review, a focus on QQ enzymes and their engineering is presented. Rational, semi-rational and random mutagenesis approaches are reviewed along with screening methodologies. Kinetic characterization and stability of engineered variants are discussed in comparison with native enzymes and the potential applications of these biocatalysts are further examined.

QQ enzymes screening methods to identify improved AHL-active variants

One of the bottlenecks in enzyme engineering research is the ability to develop effective screening or selection methodologies to identify and isolate desirable mutants (24, 25). From high-throughput screening (HTS) strategies that allow the assaying of large libraries (26–28), to medium or low-throughput procedures focused on small but high-quality libraries (29–31), many approaches have been developed. To identify relevant QQ enzymes or enhanced variants, various techniques including the use of reporter cells or *in vitro* assays were considered.

Reporter cells

Numerous different reporter strains have been developed and engineered. Based on AHL sensing, they can be used to screen AHL-modifying enzymes. Most reporter systems rely on the same principle as the QS paradigm: AHL diffuse freely through cell membranes, bind and activate a specific response regulator which in turn binds to its target promoters activating QS gene expression (**Fig. 4A**). Usually in reporter strains, the gene coding for the QS regulator is cloned together with one of its target promoters (most commonly the promoter of the cognate AHL synthase) upstream of a reporter gene or operon such as luciferase (32–34), β-galactosidase (35, 36) or fluorescent marker (*e.g.* GFP) (37, 38) (**Fig. 4A**). Exogenous AHL and cell lysates containing putative QQ agents are incubated together prior to the addition of the reporting system which, in turn, senses and responds proportionally to the quantity of remaining AHL. Reporter strains mostly differ by their ability to respond to a variety of structurally different AHL, as a function of the chosen regulator, and in the type of the reporter gene used. Luminescence, fluorescence and β-galactosidase activity are convenient for miniaturized screening (*e.g.* in microplate format). However, choosing β-galactosidase requires a biochemical assay to obtain quantitative results, while luminescence and fluorescence reporters can be directly measured (39).

One of the most common reporter strain used for screening, namely *Chromobacterium violaceum* CV026, does not require any exogenous sensing plasmid. Indeed, this strain is impaired (by transposon insertions) in AHL synthesis (40), but its endogenous QS system is functional and QS activation leads to the production of the purple pigment violacein that acts as an antibiotic at high cell density. Therefore, it can be used for screening purposes and responds best to exogenous AHL with chain lengths ranging from C4-HSL to C8-HSL including OC6-HSL and OC8-HSL (41–43). In particular, *C. violaceum* CV026 was used to screen a metagenomic library of 250,000 clones from a hypersaline soil located in Spain with a pool strategy of 50 clones per well, which resulted in the identification of a single lactonase, called HqiA which had no homology with any known lactonase or acylase. HqiA shared homology with enzymes from the cysteine hydrolase (CSHase) group as isochorismatase-like and N-carbamoylsarcosine amidase-like enzymes but more studies are required to unravel its function and mode of action (44).

Another type of reporter system is based on the expression of a β-lactamase (*i.e.* a β-lactam antibiotic degrading enzyme) and the expression of a β-lactamase inhibitor under regulation of a QS sensitive promoter (45). When AHL are present and not degraded by the QQ enzyme, the β-lactamase inhibitor is expressed, resulting in the death of the host strain grown in presence of the β-lactam antibiotics. Upon selection of an effective QQ enzyme, AHL are degraded, the β-lactamase inhibitor is not expressed and β-lactamase can degrade the antibiotic allowing the growth of the host cells (45). This biosensor strain was used to identify three improved variants of *Bacillus sp.* AiiA, screening an estimated library of $5x10^5$ clones (45).

Nevertheless, all these reporter systems suffer some drawbacks leading to the identification of false positive clones during the screening steps.

One of these drawbacks is due to the instability of AHL in cell lysates undergoing alkaline hydrolysis independently from QQ enzymes. To limit AHL hydrolysis, specific buffers have been established using *Agrobacterium tumefaciens* A136 β-galactosidase reporter strain (46). A second origin of false positive clones is the inverse proportionality between the efficiency of QQ and the read-out of the reporter of these turn-off assays. Indeed, the higher the activity of the QQ enzyme is, the more hydrolyzed AHL are and the lower the expression of the reporter gene is. This can limit the identification of best candidates and increase the number of false positives. This inverse proportionality can be reversed by expressing the reporter gene under the control of a sense/antisense RNA system. Such a reporter strain using fluorescence as readout was used for the selection of improved AiiA variants by screening an estimated library of $4.1x10^5$ clones leading to the identification of 200 improved variants with a true-positive frequency of 76% (47). Finally, a third reason for the identification of false positive clones is the use of live reporter cells whose growth rates may be hampered by other compounds present in the cell lysates. To prevent this problem, *in vitro* assays have also been developed.

In vitro assays

Various *in vitro* assays, based on the detection of acidification, absorbance or fluorescence were developed for isolating active AHL-interfering enzymes.

The hydrolysis of one lactone molecule leads to the generation of one proton resulting in the acidification of the medium. Consequently, enzyme kinetics can be monitored with a pH indicator molecule such as cresol Purple or bromothymol blue in a colorimetric assay (43, 48, 49). This assay can be miniaturized and performed in microtiter plates, but its high background levels make it challenging to use with cell lysates.

Using chromogenic or fluorogenic substrates is usually a fast and convenient method to screen enzyme libraries, yet not all substrates can be efficiently substituted by a chromogenic or fluorogenic one (50). Esterases, that also degrade AHL, can be identified using 5-bromo-4-chloro-3-indolyl caprylate (X-caprylate), an ester that turns blue when degraded or tributyrin. Both have been used to identify and engineer esterase Est816 from a metagenomic library from Turban Basin (China) (51, 52). Nevertheless, no stable chromogenic or fluorogenic substrate exists for lactonase-type enzymes.

In the absence of representative chromogenic substrate, other *in vitro* methods have been developed for the identification of acylases or lactonases. Using *A. tumefaciens* β-galactosidase biosensors, cell free lysates were prepared bulk and stored at -80°C (53). These lysates can be used with two substrates resulting in absorbance or in luminescence for more sensitive screens. This *in vitro* screen was used to identify AHL-producing strains and *luxI* homologs (*i.e.* AHL synthase genes) from a *Desulfovibrio* genomic library, but could easily be adapted to QQ enzyme identification by providing exogenous AHL (53).

Two biochemical assays have also been developed based on the detection of AHL degradation products using specific fluorescent compounds. The first one was based on the detection of *L*-homoserine using copper-calcein. Calcein is a fluorescent chemosensor that can bind various metals, and in complex with metals, is not fluorescent (54). The degradation products of AHL by lactonase and acylase are converted by autohydrolysis or a secondary enzyme to *L*-homoserine which competes for copper binding. Free calcein is generated and fluorescence signal is detected (54, 55) (**Fig. 4B**). This assay can be adapted to HTS and was first used to characterize three new lactonases identified by sequence alignment analyses and harboring an α/β hydrolase fold homologous to the QQ lactonase AidH (55). To prove its adaptability to HTS screenings, an *Escherichia coli* artificial library was created mixing lactonase and acylase expressing *E. coli* cells (in a 125:1 ratio) in order to identify acylase producing cells (55). The second *in vitro* fluorescent assay, also suitable for HTS, was developed recently and applies specifically to the identification of acylases as it requires primary amine formation. This assay relies on fluorescamine, a non-fluorescent reagent, that reacts with the primary amines of *L*-homoserine lactones forming a highly fluorescent complex (56) (**Fig. 3B**).

None of these two *in vitro* biochemical assays have been reported in QQ enzyme screenings with the exception of the ones used for their development and their proof of concept. Despite the development of turn-on assays and *in vitro* assays most QQ enzyme screenings are based on biosensor turn-off assays to select best candidates that are further characterized biochemically using pH-based assays. Screening QQ enzymes using turn-on assays might be an interesting strategy to quickly identify the most promising candidates.

Protein engineering techniques to enhance QQ enzyme activities

Combining protein engineering approaches with efficient screening procedures permits identification of improved or finely tuned enzymes (**Fig. 5**). Given the diversity of QS-using bacteria and their equally diverse signaling lactones, enzyme engineering offers an opportunity to develop efficient biocatalysts to tackle bacterial virulence issues and strengthens the antimicrobial arsenal.

Random engineering strategies

When HTS methods are available, large libraries can be created to select promising variants among many others. The most common technique generating large libraries is random mutagenesis using error-prone polymerase chain reaction (epPCR). This technique may be used to explore sequence space without requiring prior structural knowledge. AiiA from *Bacillus sp.* was for example engineered with an epPCR approach. Using the above mentioned β-lactamase-based assay, the single variant V69L, with 3.7-fold increase in catalytic efficiency (k_{cat}/K_M) for C6-HSL was obtained. This mutant was further improved, leading to the identification of double (V69L/I190F) and triple (V69L/I190F/G207V) mutants, with 7- and 6.1-fold enhancements in k_{cat}/K_M values for C6-HSL, respectively (45).

Another enzyme, namely MomL from the marine bacterium *Muricauda olearia*, with 10 times greater activity on C6-HSL than AiiA, was also engineered. Three rounds of epPCR were performed, resulting in the identification of I144V and V149A mutants with higher efficacy on C6-HSL and OC10-HSL (1.3- and 1.8-fold respectively) (43). By sequencing and analyzing the inactive mutants obtained in this screen, key residues for MomL lactonase activity have also been identified and would likely help to guide future engineering work.

Once identified in different variants, several beneficial mutations can also be combined in one enzyme. This approach was applied to the thermostable esterase Est816 active towards various AHL. Random mutagenesis first led to identification of two-point mutations (A216V and K238N) exhibiting increased k_{cat}/K_M values towards C8-HSL. Mutant A216V showed 6-fold enhancement in k_{cat}/K_M value resulting from an increased affinity *(i.e.* lower K_M value) compared to wild-type enzyme. Conversely, K238N mutation increased k_{cat} value by 8-fold while decreasing affinity. These mutations have been further combined leading to A216V/K238N variant with 3-fold enhancement in k_{cat}/K_M with C8-HSL compared to wild type Est816 (52). While A216 is close to the lactone ring and has a direct impact on ligand-substrate interactions, K238 is located on the enzyme surface, and its impact on activity is still to be understood.

Random mutagenesis approaches have been shown to increase native lactonase activities on specific substrates, but a single mutation can also modify an enzyme in such a way that hydrolysis of new substrates becomes possible. Engineering of a phosphotriesterase-like lactonase MCP, from *Mycobacterium paratuberculosis,* by random mutagenesis led to the isolation of an N266Y mutant showing improvement from 4 to 32-fold in k_{cat}/K_M values on usual substrates and able to hydrolyze C4-HSL and OC6-HSL, while no activity was detected on these substrates for the wild-type enzyme (57). The same approach was also applied to a thermostable PLL GKL from *Geobacillus kaustophilus*, revealing a quadruple mutant exhibiting better QQ ability than wild-type enzyme. Retro-engineering allowed identification of the double mutant E101G/R230C with global increase in catalytic performance towards AHL, with a 1.2- and 32-fold increase, respectively, for C6-HSL and OC12-HSL, and compared to GKL, a new ability to degrade C4-HSL. (33).

Random mutagenesis by epPCR was thus efficiently used to improve lactonase and esterase activities and led to the identification of residues playing key roles in AHL hydrolysis, generating

improved and promising QQ enzymes (**Fig. 5**). In most cases, beneficial mutations involved residues close to the enzyme active sites and activity modulation often results from an enhancement of hydrophobic interactions between mutated residues and AHL acyl-chains.

Rational design approaches

Based on 2D or 3D information, rational design is a powerful approach to limit the size of variant libraries and decrease screening efforts. Through analysis of protein sequence, overall structure, active site or catalytic mechanism, or molecular dynamics (MD), key amino acids can be identified to aid design (58).

Docking analysis and MD simulations have been used to enhance activity of AiiA in favor of substrates having a short acyl chain and to the detriment of that with long acyl chain. Computational docking of various AHL in the AiiA active site showed that C4-HSL binding could be favored when hydrophobic interactions with a short acyl chain were increased and space for long acyl chain was reduced. To this end, 15 mutants with hydrophobic residue substitutions have been constructed. Eight mutants showed increased k_{cat}/K_M values with C4-HSL, while mutant V69W showed more than 6-fold increase. Combining the best mutations, double and triple mutants were created, leading to a more than 10-fold activity increase on C4-HSL for F64W/V69W and F64W/V69W/A206F mutants. Improved enzymes have also been shown to proportionally lose activity on long-chain AHLs, substantiating the chosen design approach (59).

Other lactonases with different scaffolds have also been rationally engineered. *Sso*Pox is a hyperthermostable PLL from *Saccharolobus solfataricus*, hydrolyzing a broad range of lactones. This enzyme was shown to be resistant to many deleterious conditions including high temperature, solvents, denaturing agents or sterilization (60, 61). Its tremendous thermostability is also appealing for engineering purposes and may help to buffer the damaging effect of beneficial mutations that are often detrimental to stability (62). *Sso*Pox active-site structure analysis identified a residue (W263) at the beginning of the loop 8 (**Fig. 3B**), that impacts enzyme flexibility and specificity (34). This residue was exhaustively mutated and kinetic parameters for the best *Sso*Pox WT substrate (OC10-HSL) and the worst substrate (OC12-HSL) have been determined. Mutations W263I and W263V respectively improved OC12-HSL degradation by 45-fold and 54-fold, while all mutations decreased OC10-HSL activity. Interestingly, all mutations also increased lactonase activity towards -δ and -γ lactones, some of them such as γ-butyrolactone being potentially involved in *Streptomyces* sp. signaling (63, 64). Mutations of the W263 residue were thus demonstrated to strongly alter *Sso*Pox specificity and activity, while mutants conserved great stability (as measured by melting temperature) allowing the W263I variant to resist harsh industrial conditions (60). Among the diverse bacterial AHL, *Burkholderia cenocepacia* mainly uses C8-HSL. In order to specifically target this pathogen, an acylase from *P. aeruginosa,* PvdQ, was engineered (38). This enzyme, originating from *P. aeruginosa*, specifically degraded AHL from C11-HSL to OHC14-HSL and was thus an ideal candidate for enzyme engineering towards the degradation of C8-HSL (22). Structural analysis revealed an unusually large active-site pocket fostering the binding of long acyl chain lactones. Following molecular docking of C8-AHL into the active site, 12 residues interacting with the acyl chain were selected for *in silico* exhaustive mutagenesis. Computational analysis led to the design of 18 mutants for further kinetic characterization. Two single mutants (L146W and F24Y) exhibited a great increase in activity for C8-HSL. By combining these two mutations, the mutant F24Y/L146W was increased in C8-HSL activity by 4.3-fold, while reducing OC12-HSL degradation by a 3.8-fold. This shifted preference suggests an accommodation of the active site towards the targeted substrate and implies that PvdQ would not be an ideal candidate to engineer a broad range degrading enzyme.

In order to enhance lactonase activity, information obtained from random mutagenesis can further contribute to a successful rational design approach. The structure of the previously described Est816 A216V/K238N obtained after random mutagenesis, was solved and used for *in silico*

docking with an AHL. Observing that the L122 residue side chain was interfering with the AHL acyl chain, mutation L122A was considered and yielded the mutant (L122A/A216V/K238N) with 21.6-fold enhancement towards C8-HSL compared to wild-type Est816 (52). Furthermore, these three improving mutations did not impact Est816 great thermostability (**Fig. 5**). GKL variant E101G/R230C obtained by random mutagenesis was also intensively investigated through rational engineering. Residues 101 and 230 have been targeted by site-directed mutagenesis leading to a novel double mutant, E101N/R230I, able to hydrolyze OC12-HSL with 2-fold increase compared to E101G/R230C, for 72-fold total improvement towards this lactone as compared to wild-type enzyme (33). Mutations of E101 residue are altering the lactone ring positioning by enhancing a critical loop flexibility while mutations of R230 are modulating the position of the attacking hydroxide nucleophile, resulting in a more efficient nucleophile attack angle (65).

The promiscuous activities of enzymes (i.e. their ability to use substrates other than those for which they evolved) are also an interesting starting point for a rational design experiment. PLL are well known to have latent phosphotriesterase activity and it has been demonstrated that phosphotriesterase (PTE) quickly diverged from PLL (66, 67). This specificity was used to considerably enhance lactonase activity from *Brevundimonas diminuta*'s PTE. The PTEΔ7-2/254R mutant, obtained by deleting a few residues specific to PTE , has increased activities on both C4-HSL and OC6-HSL of more than 2,000-fold, with a k_{cat}/K_M value around 10^4 M^{-1} s^{-1} (68). This study demonstrates the power of rational design to trace back evolution and open up a new range of possible future efficient lactonase design by using PTE.

Besides improving activities, rational design can be used to improve protein solubility or expression in a heterologous host. The human paraoxonase huPON2, able to hydrolyze various lactones, has been considered for health-related applications. Unfortunately, this cell-membrane protein is difficult to express in soluble form because of an excessive self-aggregation. huPON2 has then been engineered to enhance its solubility and facilitate its recombinant expression. Three highly hydrophobic helixes, unlinked to lactone hydrolysis, have been replaced by hydrophilic polypeptide linkers, leading to two mutants (D2 and E3) with higher soluble expression (6.2 and 3.2 mg per liter of culture). The latter have then been fused to maltose binding protein (MBP) to lead to a final protein yield of 320 and 200 mg L^{-1} of culture for MBP-D2 and MBP-E3, respectively, while MBP-huPON2 has only been expressed in its insoluble form (69).

Rational design appears as a useful tool to directly target enzyme hotspots in order to enhance lactonase activity. Both random mutagenesis using ep-PCR and rational design experiments resulted in new enzyme variants with strongly enhanced activities (**Fig. 5**). While random mutagenesis improved activities by an order of magnitude, rational design led to close to a hundred-fold improvement, relying on mutations that have a direct interaction with the substrate in the active site. These engineered QQ enzymes, able to block bacterial communication with high efficiency constitute promising candidates that can be used in a broad spectrum of applications. However, it has been observed that engineering studies focus on catalytic efficiency improvement, while stability and solubility of the protein are rarely considered. Only few studies measured the loss in stability generally induced by mutations (**Fig. 5**). This should be considered in forthcoming studies to yield easily expressed and resistant QQ enzymes, usable in industrial processes.

Biotechnological applications

Going beyond kinetic and structural characterization, numerous engineered enzymes were evaluated for their application potential. As QS regulates various bacterial phenotypes associated with virulence, QQ may find application in several fields, including human and animal health or agriculture. Furthermore, biofilm formation is also primarily governed by QS, so that disruption of bacterial communication is of primary interest in limiting biofouling problems and preventing biofilm impact on human health.

Human health

QS plays an important role in pathogenicity of numerous invasive bacteria such as *P. aeruginosa* or *A. baumannii* (13, 17, 18). Due to rising

antibiotic resistance, alternative and/or complementary therapeutic strategies are required (82). QQ is an appealing approach to tackle bacterial virulence *in vitro* and *in vivo,* without compromising bacterial survival, and QQ enzymes have been isolated and engineered towards this end.

*Sso*Pox mutant W263I was obtained through rational engineering and was shown to efficiently decrease virulence factor production of *P. aeruginosa* clinical isolates, especially pyocyanin, protease and biofilm formation with higher efficacy than chemical QS inhibitors (*i.e.* furanone C-30 and 5-fluorouracil) (83, 84). *Sso*Pox W263I variant reinforced antibiotics and bacteriophage treatments against *P. aeruginosa in vitro* and *in vivo* using an amoeba infection model with *Acanthamoeba polyphaga* (85) and was further shown to alter the regulation of the CRISPR-Cas system in *P. aeruginosa* clinical strains, thereby potentially modifying their ability to compete with phages. In fact, this finding is consistent with the previous observation that the CRISPR adaptation system is induced by QS at high population density (86–88). Finally, this variant also reduced biofilm formation, violacein production and down-regulated CRISPR-Cas genes of *C. violaceum,* a tropical aquatic bacterium responsible for rare but frequently fatal infections in animals and humans (86, 89, 90). *In vivo*, *Sso*Pox W263I was also proved to protect rats from *P. aeruginosa* infection in a pneumonia model. Fifty hours after infection, 75% mortality was observed in untreated rats, while mortality was reduced to 20% in treated animals (91). QQ effects were also obtained in *A. baumannii*, with another engineered enzyme, GKL E101G/R230C, able to reduce biomass, thickness and surface of biofilm formed by this pathogen (92, 93).

Similarly, acylases have also been assayed for potential therapeutic use. The engineered QQ acylase PvdQ F24Y/L146W (38) was proved to reduce the virulence of *Burkholderia cenocepacia*, both *in vitro* and *in vivo*. By pre-incubating the bacteria and the variant before injection in *Galleria mellonella* larvae drastic enhancement in survival was obtained as compared to larvae treated with wild-type enzyme, nearly 100% of larvae survived with PvdQ F24Y/L146W and around 20% of larvae survived with PvdQ wild-type (38). One of the limitations of the therapeutic use of QQ enzymes is their potential to trigger an immune response (69). To that end, the huPON2, as previously described, has been engineered for QQ purposes to benefit of minimal immunogenicity and a soluble expression. huPON2 D2 and huPON2 E3 variants have been obtained and tested on swimming and swarming motilities in *P. aeruginosa* PAO1. Inhibition of *P. aeruginosa* motilities has been observed, however huPON2 D2 and huPON2 E3 variants were not better than AiiA lactonase (69).

Engineered enzymes have also been immobilized for anti-virulence purposes. *Sso*Pox W263I was immobilized in polyurethane coating and was able to decrease virulence factors of *P. aeruginosa* PAO1 (83). AiiM, a wild-type lactonase isolated from *Microbacterium testaceum* StLB037, was overexpressed in *E. coli* DH5α as a recombinant protein with maltose binding protein tag (MBP-AiiM) for its purification process and was then successfully incorporated in polyvinyl alcohol fibres, by electrospinning. MBP-AiiM was capable of quenching QS-dependent prodigiosin production in *Serratia marcescens* AS-1 (94, 95).

Aquaculture

Besides human health, QQ offers a promising approach to counteract bacterial infections in animals. Aquaculture, for example, suffers from a large number of bacterial diseases responsible for multi-billions US dollar losses annually (96). Gram-negative bacteria, including *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* are responsible for numerous diseases in a large variety of marine animals (97–99). Antibiotics are largely used to control bacterial infection in fish farming, however these antibiotics have great impact on host and environmental bacteria leading to resistant pathogen emergence (100). Thus, native and engineered QQ enzymes constitute an appealing strategy for aquaculture (17, 18).

Many different AiiA-like lactonases have been assayed as proof of concept of QQ efficiency to limit diseases in aquaculture. AiiA lactonase from *Bacillus thuringiensis* has been shown to disturb *Vibrio harveyi* QS. In the presence of AiiA, *V. harveyi* luminescence was decreased by 85%

(101). Another AiiA, from *Bacillus licheniformis* DAHB1, was able to decrease Indian white shrimp mortality rate from 80 to 23% after 5 days of infection by *V. parahaemolyticus,* both bacteria and enzyme were administered by injection in the abdominal cavity (102). The lactonase AiiA, from *Bacillus* sp. B546, was used to reduce *A. hydrophila* mortality in common carp, decreasing the mortality at 4 days down to 54% compared to 79% without enzyme (103). Furthermore, another AiiA, from *Bacillus* sp. AI96, decreased mortality rate in zebrafish by the oral administration down to 20% as compared to 60% without enzyme (104).

These promising results, based on wild-type lactonases, show that the use of engineered enzymes that offer better stability, efficiency or immobilization potential merit further testing to potentially replace antibiotic use in aquaculture. Moreover, testing in conditions closer to real world situations will also be needed.

Agriculture

Bacterial infections not only affect human or animals, but also plants. Agricultural ecosystems are impacted by numerous bacterial plant pathogens, for instance *Pectobacterium carotovorum* responsible for soft rot on various hosts such as potatoes or Chinese cabbages (105, 106), *Erwinia amylovora* responsible for fireblight (107, 108), or *Agrobacterium tumefaciens* causing crown gall disease (109, 110). These and other diseases have a significant economic impact on agriculture and on the quantity and quality of food (111, 112), hence the interest of the QQ approach to control diseases caused by bacterial plant pathogens.

Currently, no endogenous enzyme capable of quenching bacterial QS has been described in plants (17). Some plants, such as *Lotus corniculatus*, *Hordeum vulgare* (barley) and *Pachyrhizus erosus* (yam bean), have been shown to naturally degrade the QS signal, possibly by enzymatic degradation, but this has not yet been proven (113, 114). Conversely, soil bacteria are known to produce QQ enzymes, like AiiA from *Bacillus sp.,* AttM from *Agrobacterium tumefaciens* or QsdA from *Rhodococcus erythropolis* (115–117). This is the reason why the use of these bacterial enzymes is attractive. For instance, AiiA from *Bacillus subtilis* BS-1 and AiiA from *Bacillus amyloliquefaciens*, have been shown to reduce *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) soft rot symptoms on potato and carrot slices respectively (118, 119). Similarly, the variants MomL I144V and MomL V149A obtained by random engineering were able to decrease infection of Chinese cabbage by *Pcc*. Cabbage leafs have been infected with *Pcc,* in presence of MomL variants or controls, on a cut surface of the leaf, thus MomL variants permitted to greatly reduce decay areas (43).

Biofouling/Biocorrosion

Biofouling is a colonization phenomenon of immersed surfaces by aquatic organisms. This surface behavior detrimentally affects diverse activities such as wastewater treatment, marine transport or aquaculture. Wastewater treatment is well advanced in QQ applications, especially for protecting membrane bioreactors (MBR) from biofilm formation using whole bacteria (120, 121) or QQ enzymes. Mainly, the porcine kidney acylase was used to reduce biofilm formation in MBR systems. For instance, acylase wild-type immobilized in sodium alginate capsules reduced biofouling formation and improved filterability of MBR system (122). The same acylase immobilized on magnetically separable mesoporous silica particles was also capable of preventing fouling, as observed by confocal laser scanning microscopy, and enhancing filtration performance, determined by membrane permeability measurements (123). QQ enzymes in MBR systems have proven their efficiency, and engineered enzymes were further evaluated for their anti-biofilm property. Recently, a recirculating bioreactor with filtration cartridge containing bacteria expressing *Sso*Pox W263I in silica capsules was developed to assess the impact of QQ on complex bacterial communities. *Sso*Pox variant presence in capsules led to changes in bacterial communities and to biofilm inhibition (124). *Sso*Pox W263I immobilized in silica gel coating painted on steel plates was also able to reduce corrosion tubercles by 50% compared to controls after eight weeks of immersion in Duluth-Superior Harbour (Minnesota, USA) (125). Use of enzymes instead of, or as a complementation to biocides in paint is attractive, due to some biocides use restriction because of their negative impact on

the environment (126). With respect to both antifouling and anticorrosion strategies, enzyme engineering could offer suitable catalysts with high activity or stability to develop bioactive materials and coatings.

All these promising applications need further development, such as larger range of improved enzymes, upscale to industrial applications, experiments performed in more relevant situations of bacterial colonization and infection and also better controls to confirm enzyme action on target.

Concluding remarks and prospects

AHL-interfering enzymes constitute a promising alternative or complementation to classical antimicrobial treatments. This review highlights several recent progresses achieved thanks to enzymatic engineering approaches. Numerous efforts have been dedicated to isolate AHL degrading enzymes specifically using environmental samples from extreme environments, including enzymes that are sufficiently stable for real-world applications. Some of the identified QQ enzymes were further characterized and engineered for enhanced activities. The availability of these enzymes allowed to develop laboratory scale prototypes that now need to be turned into scalable and cost-effective solutions to reach preclinical tests and clinical trials. Considering the wide structural variety of AHL signals, the AHL-degrading enzyme stability, activity levels and substrate specificity are critical parameters to achieve the desired QQ effects. The importance of the latter property was recently investigated using the lactonases *Sso*Pox W263I and GcL from *Parageobacillus caldoxylosilyticus* in *P. aeruginosa* PA14 or clinical isolates, revealed that lactonases with distinct efficacy towards AHL, yielded drastically different quenching effects at both molecular and phenotypic levels, the broadly active enzyme being not necessarily the most efficient (84, 127). These results underscore the determinant role of enzyme specificity on QQ at a single specie level. It demonstrates that catalytic performance may not be used at the sole selection criterion and that specific screening have to be developed to assess the potential of QQ enzymes in specific systems. Therefore, more selective QQ enzymes as tools will help in the development of powerful and specific interference strategies. Although targeting AHL-based QS using enzymes is appealing for mitigating the virulence of Gram-negative bacteria, similar strategies must be considered for targeting the wide natural diversity of AI. In Gram-positive bacteria, autoinducing cyclic peptides were largely described (128) while their enzymatic quenching was poorly considered to date. In Gram-negative bacteria, although AHL are widespread, other molecules including epinephrine (AI-3) (129) or quinolones (130) are found in various pathogens and could constitute relevant target to broaden QQ range of action. Moreover, the furanosyl diester AI-2, is found in both Gram-positive and Gram-negative bacteria and may be involved in inter-kingdom signalling (131). AI-2 could thus also constitute a promising target and enzymes able to interfere with this compound need to be further investigated (132). Once potential QQ enzymes are identified for these AI, rational engineering and random mutagenesis strategies will allow to enhance their activity and enhance their anti-virulence effect. In any case, the isolation of enzyme variants efficiently targeting bacterial pathogenicity will require novel screening methodologies not only to determine their ability to degrade AI but also to evaluate their capacity to directly compete with bacterial phenotypes (e.g. biofilm formation, production of virulence factors) in conditions that may be realistic as regard to a defined final application.

In the short term, expanding and diversifying the repertoire of QQ enzymes will be necessary to finely control bacterial communications. Particularly, the role of QS in polymicrobial infections or dysbiosis, is still poorly understood and developing a wide variety of enzymatic quenchers will provide miscellaneous tools to interfere with complex multicellular process. Moreover, the complementarity of QQ enzymes with classical antimicrobial treatments was demonstrated (85) and has to be further considered as it would constitute a promising strategy to strengthen the therapeutic arsenal and potentially limit the doses of antibiotics in Human and Animal health or biocides in environmental applications. Finally, enzymes need to meet industrial and

regulation requirements to reach concrete applications. Toxicity or immunogenicity for example will have to be considered for healthcare applications and strategies such as nanoencapsulation may have to be envisaged to limit immune system response. For large-scale environmental applications including antifouling or agriculture, production costs or stability may constitute an economical bottleneck and need to be carefully considered. Protein, metabolic and process engineering will have to be combined to allow for the high-level microbial production of highly active enzymes to turn the enzymatic QQ into an economically attractive solution.

Conflict of Interest

ME and EC have a patent WO2014167140 A1 licensed to Gene&GreenTK. RB, LP, PJ, DD and EC report personal fees from Gene&GreenTK.

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Table 1. QQ enzyme applications, assayed bacteria and related phenotypic changes

Applications	QQ enzymes	Quenched bacteria	Measured phenotypes	Ref.
Human health	GKL-E101G/R230C	*A. baumanii*	Reduction of biofilm	(92, 93)
	huPON D2	*P. aeruginosa* PAO1	Diminution of swarming and swimming motilities	(69)
	huPON E3	*P. aeruginosa* PAO1	Diminution of swarming and swimming motilities	(69)
	MBP-AiiM	*S. marcescens* AS-1	Decrease of QS dependent pigment	(94)
	*Sso*Pox-W263I	*P. aeruginosa*	Reduction of biofilm/Decrease of QS dependent pigment/ Diminution of proteases/ Reduction of CRISPR-cas gene expression/ Decreased mortality in rats	(83–86, 91)
	PvdQ-F24Y/L146W	*B. cenocepacia*	Decreased mortality in moth larvae	(38)
Aquaculture	AiiA from *B. licheniformis* DAHB1	*V. parahaemolyticus*	Reduction of biofilm/ Decreased mortality in shrimps	(102)
	AiiA from *B. thuringiensis*	*V. harveyi*	Diminution of bioluminescence	(101)
	AiiA from *Bacillus* sp. AI96	*A. hydrophila*	Decreased mortality in zebrafishes	(104)
	AiiA from *Bacillus* sp. B546	*A. hydrophila*	Decreased mortality in carps	(103)
Agriculture	AiiA from *B. amyloliquefaciens*	*P. carotovorum* subsp. *carotovorum*	Reduction of infection on carrots	(119)
	AiiA from *B. subtilis* BS-1	*P. carotovorum* subsp. *carotovorum*	Reduction of infection on potatoes	(118)
	MomL-I144V	*P. carotovorum* subsp. *carotovorum*	Reduction of infection on cabbages	(43)
	MomL-V149A	*P. carotovorum* subsp. *carotovorum*	Reduction of infection on cabbages	(43)
Biofouling	Acylase from porcine kidney	Complex communities/ *P. aeruginosa*	Reduction of biofilm	(122, 123)
Biocorrosion	*Sso*Pox-W263I	Complex communities	Reduction of biofilm/ Diminution of biocorrosion/ Changes in bacterial population proportions	(124, 125)

Figure 1

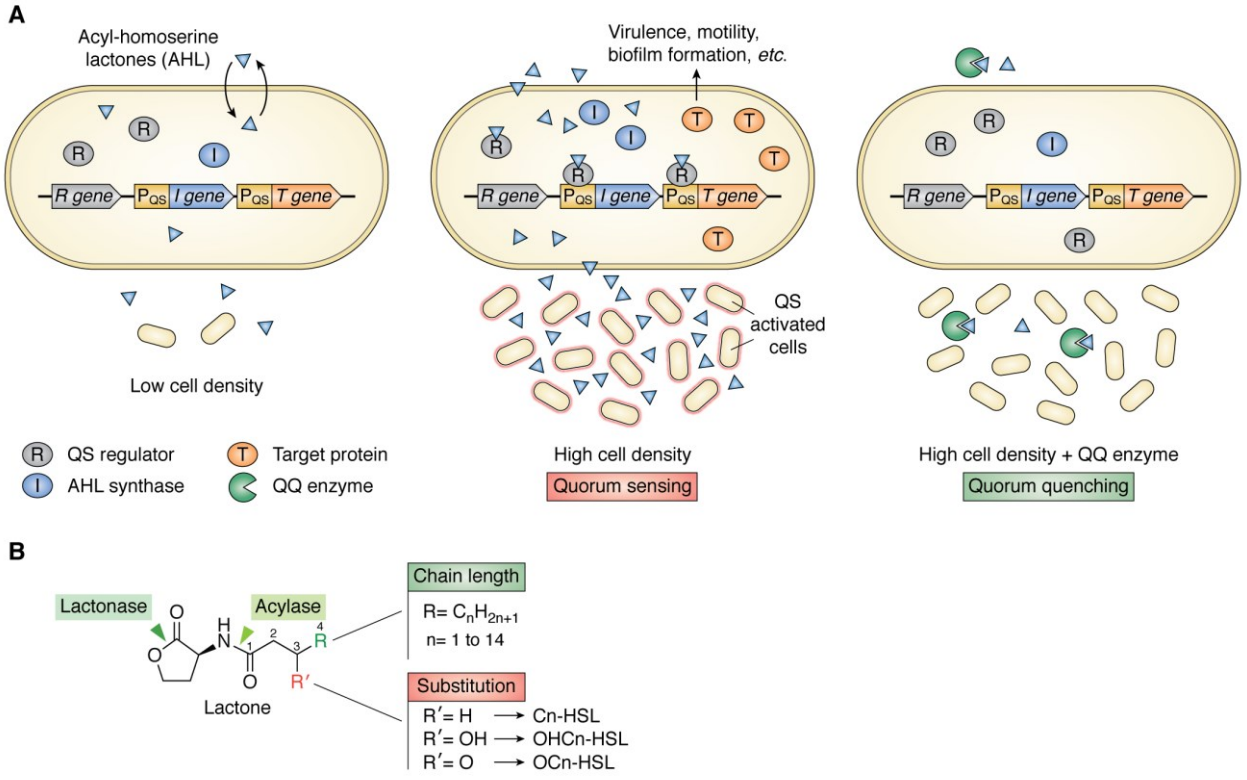

Figure 1. Canonical Quorum Sensing in Gram-Negative bacteria and Quorum Quenching. (**A**) AHL (blue triangles) are produced by cells and diffuse freely in and out cells. AHL concentration increases with cell concentration. Above a certain threshold AHL bind and activate the QS regulator which in turn can bind QS promoter sequences and induce the expression of QS genes such as the AHL synthase gene (I) and other target genes (T). QQ enzymes degrade extracellular AHL, the QS regulator is not activated and QS genes are not expressed. Strings, arrows and boxes represent genetic arrangements. (**B**) AHL consist of a homoserine lactone ring with an acyl chain that can vary in length (in green) or functionalization (in red). AHL can be differentially targeted by lactonase and acylase enzymes.

Figure 2

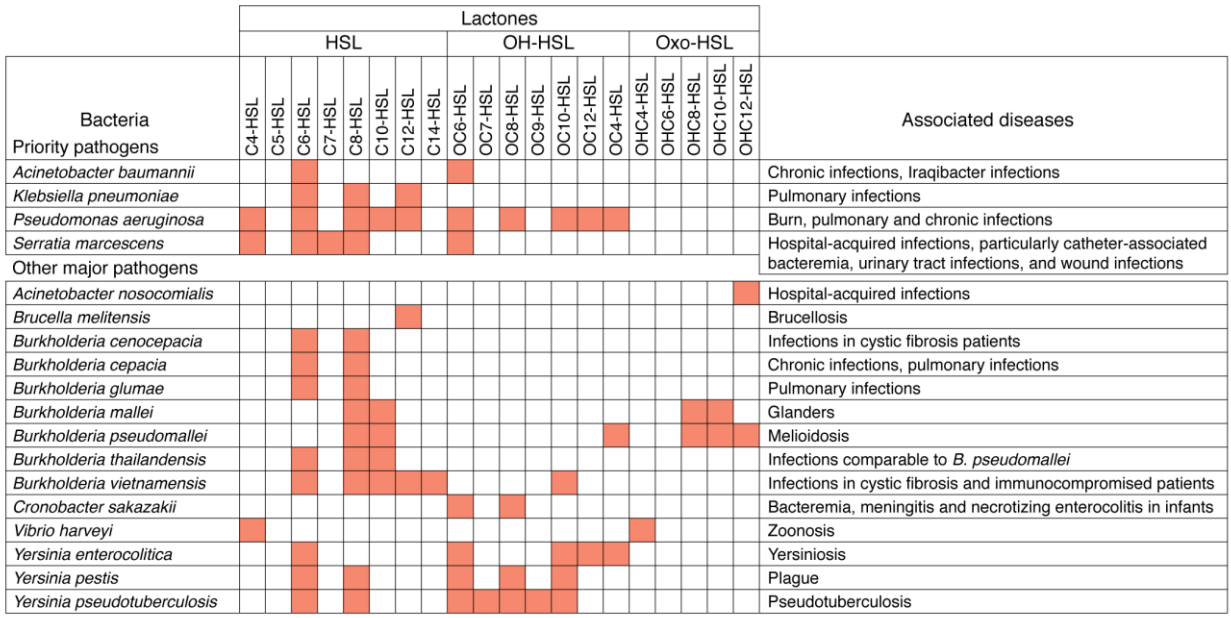

Figure 2. Various Gram-negative bacteria that use AHL-based sensing to control pathogenicity. AHL reported for each bacterium are highlighted in red (23).

Figure 3

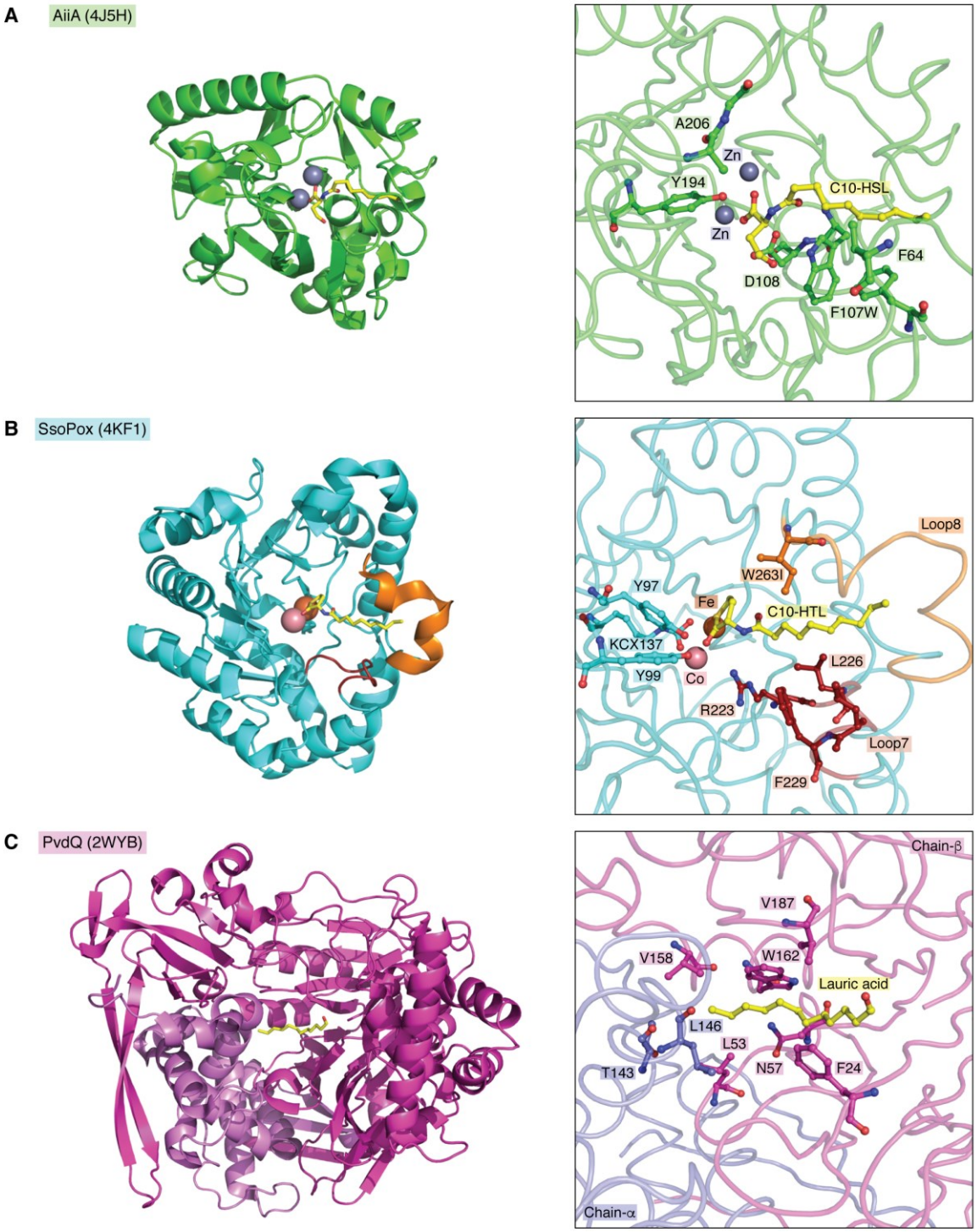

Figure 3. Structural overview of AHL-interfering enzymes. AiiA and *Sso*Pox lactonases and the acylase PvdQ are presented using the same scale. (**A**) Crystal structure of the 28 kDa metalloenzyme lactonase (EC 3.1.1.81) AiiA mutant F107W from *Bacillus thuringiensis* with *N*-decanoyl-*L*-homoserine bound at the active site (PDB ID 4J5H). AiiA belongs to the metallo-β-lactamase superfamily and harbors two zinc (II) ions bound at the active site essential to catalytic activity. (**B**) Crystal structure of the 35 kDa metalloenzyme, phosphotriesterase-like lactonase (PLL) *Sso*Pox W263I (EC 3.1.8.1) in complex with C_{10}-HTL (PDB ID 4KF1). *Sso*Pox belongs to the amidohydrolase superfamily and exhibits a (α/β)8-barrel fold (the so-called TIM-barrel) and

harbors a bi-cobalt active site. Loops 7 (in red) and 8 (in orange) play key roles in substrate recognition and protein flexibility. (**C**) Crystal structure of the acylase PvdQ (EC 3.5.1.97) with a covalently bound dodecanoic acid (PDB ID 2WYB). PvdQ is a member of the Ntn-hydrolase superfamily and is formed by an 18 kDa α-chain (purple) and a 60 kDa β-chain (pink).

Figure 4

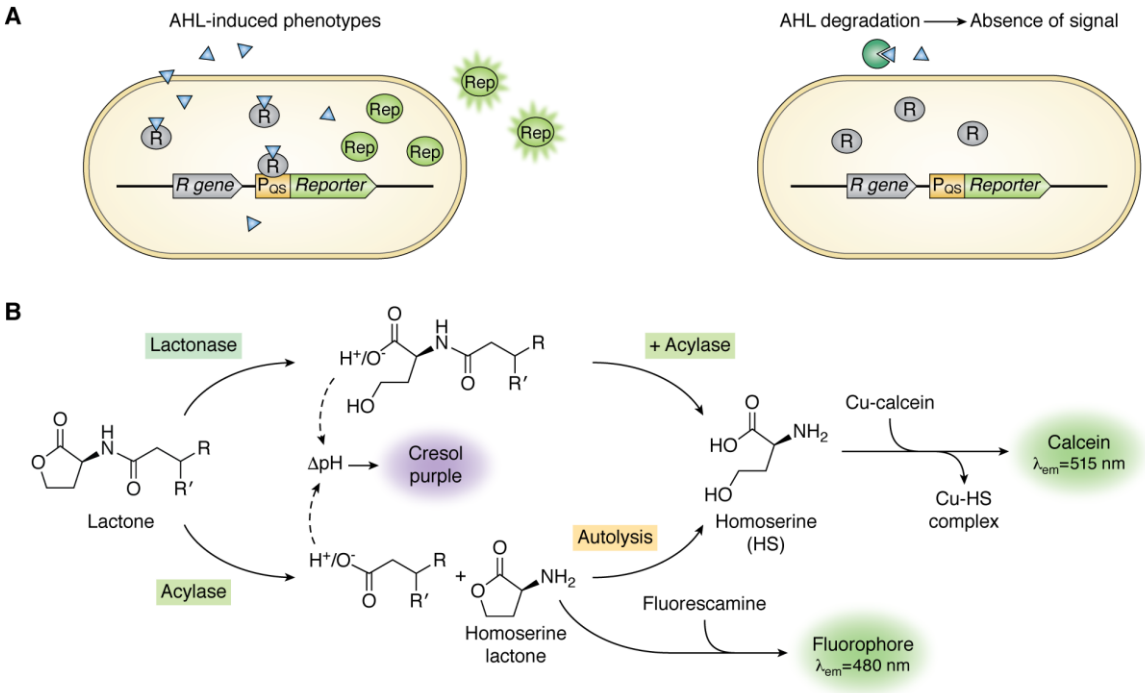

Figure 4. Screening approaches for identifying novel or improved AHL-interfering enzymes (A) *In vivo* assays based on natural QS systems. AHL are perceived by biosensor cells that consist of a regulator which is activated upon AHL binding and in turn induces the expression of a reporter gene (luminescence, violacein, fluorescence or β-galactosidase). In the presence of active QQ enzymes, AHL are degraded and no signal is induced. **(B)** *In vitro* assays. AHL degradation can be measured *in vitro* by colorimetric assays (Cresol Purple) or by fluorescent probes that recognize AHL-degradation products (fluorescamine) or react with them through copper competition (calcein).

Figure 5

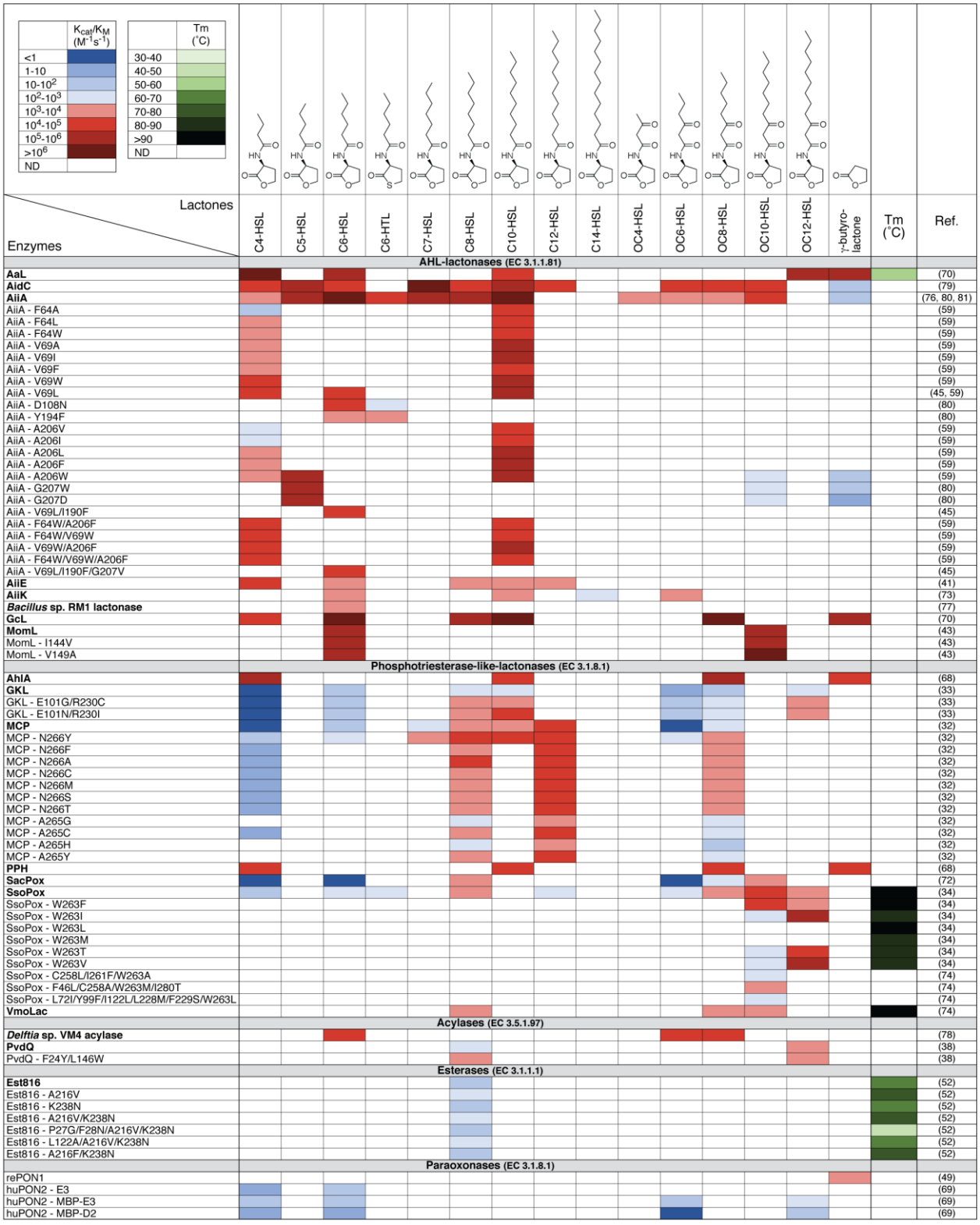

Figure 5. **Catalytic performances and stability of native (70–81) and engineered AHL-interfering enzymes.** Only enzymes with described k_{cat}/K_M values are represented in this figure. Enzymes are classified by their EC number. Catalytic efficiency (k_{cat}/K_M) on various lactones, corresponding to the highest values reported in the literature are presented using color gradients

from blue to red diverging scale. Lactone names and structures are presented at the top of the figure. Melting temperatures (Tm) values, are presented with shades of green from light green to dark green. Colors and their respective values are detailed in the top left corner.

Engineering Acyl-Homoserine Lactone-Interfering Enzymes Towards Bacterial Control

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