# Engineering acyl-homoserine lactone-interfering enzymes toward bacterial control

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**Enzymes able to degrade or modify acyl-homoserine lactones (AHLs) have drawn considerable interest for their ability to interfere with the bacterial communication process referred to as quorum sensing. 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 a view to developing potent candidates for bacterial control.**

Over the last 2 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 (AIs) (Fig. 1*A*) (2, 3). Thanks to this cell-to-cell communication process, bacteria can collectively adapt their behavior as AIs 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 noneffective and may be deleterious at low cell density. A wide variety of chemical molecules have been integrated into bacterial communication, with Gram-negative bacteria mainly using acyl-homoserine lactones (AHLs) (4, 5). AHL chemical structure includes a homoserine lactone ring with an acyl chain that can vary in length or functionalization (Fig. 1*B*). 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*, and *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 (16, 17). This strategy, also known as quorum quenching (QQ), can be achieved using various compounds, including natural or synthetic QS inhibitors (QSIs) able to compete with AHL, sequestering antibodies or degrading enzymes (18). AHL-interfering enzymes have been extensively investigated as they do not need direct contact with bacteria conversely to QSIs. Indeed, they can catalytically degrade AHL without the need for entering cells, being less invasive than QSIs, and may further show bactericidal effects (19–21). The 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, whereas acylases remove the acyl chain from the homoserine lactone moiety (Fig. 1*B*) (22, 23). AHL-degrading lactonases from different protein families were reported, including metallo-$\beta$-lactamase, phosphotriesterase-like lactonase (PLL), or paraoxonase. Conversely, acylases active toward AHL mainly belong to the Ntn-hydrolase family (21). 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 (19, 21). 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 (21).

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 compared with native enzymes, and the potential applications of these biocatalysts are further examined.

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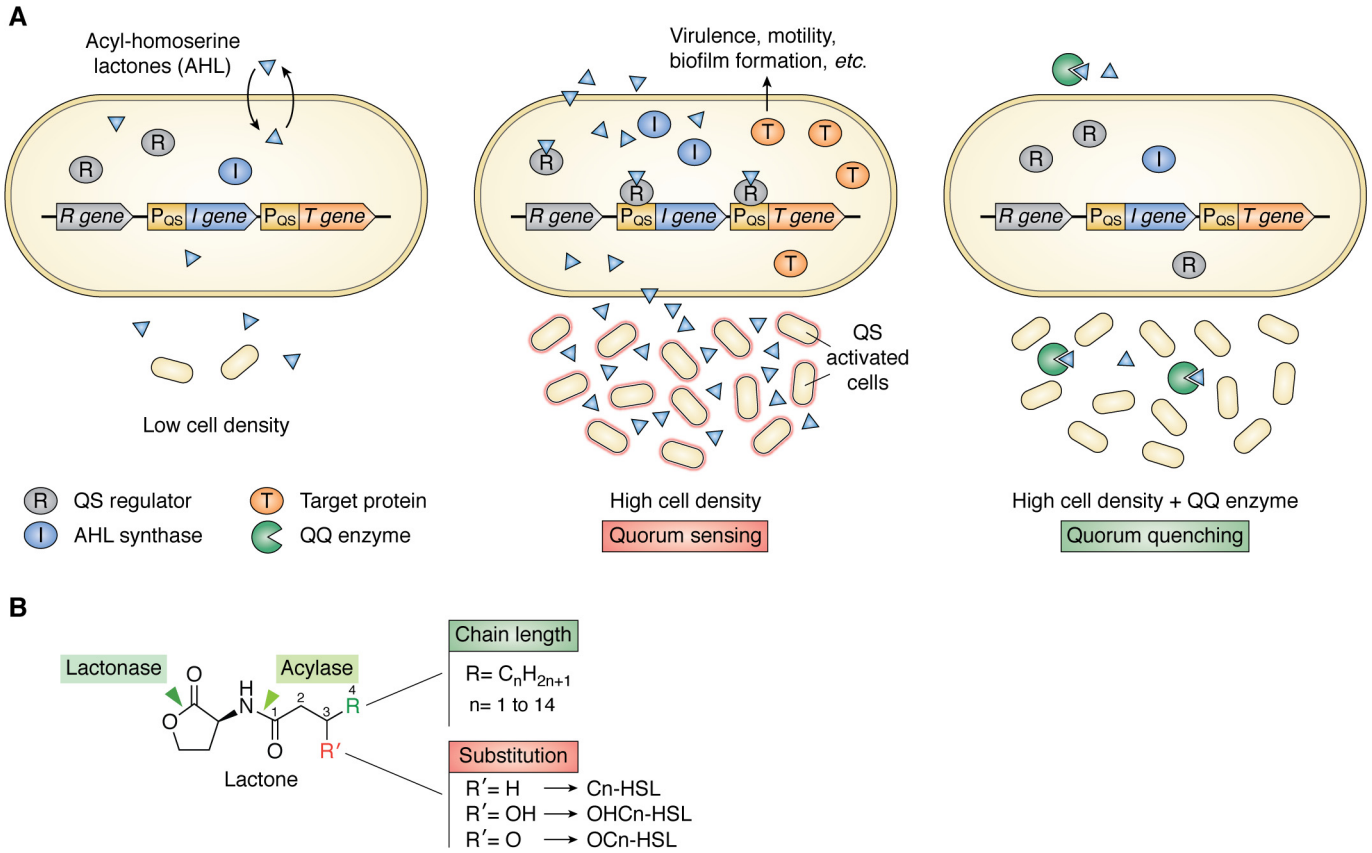



**Figure 1. Canonical quorum sensing in Gram-negative bacteria and quorum quenching.** *A*, AHLs (*blue triangles*) are produced by cells and diffuse freely in and out cells. AHL concentration increases with cell concentration. Above a certain threshold, AHLs bind and activate the QS regulator, which 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 AHLs, the QS regulator is not activated, and QS genes are not expressed. *Strings*, *arrows*, and *boxes* represent genetic arrangements. *B*, AHLs consist of a homoserine lactone ring with an acyl chain that can vary in length (*green*) or functionalization (*red*). AHLs can be differentially targeted by lactonase and acylase enzymes.

| | Lactones | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HSL | | | | | | | | OH-HSL | | | | | | | Oxo-HSL | | | | |
| Bacteria | C4-HSL | C5-HSL | C6-HSL | C7-HSL | C8-HSL | C10-HSL | C12-HSL | C14-HSL | OC6-HSL | OC7-HSL | OC8-HSL | OC9-HSL | OC10-HSL | OC12-HSL | OC4-HSL | OHC4-HSL | OHC6-HSL | OHC8-HSL | OHC10-HSL | OHC12-HSL | Associated diseases |
| Priority pathogens | | | | | | | | | | | | | | | | | | | | | |
| *Acinetobacter baumannii* | | | ■ | | | | | | ■ | | | | | | | | | | | | Chronic infections, Iraqibacter infections |
| *Klebsiella pneumoniae* | | | ■ | | ■ | | ■ | | | | | | | | | | | | | | Pulmonary infections |
| *Pseudomonas aeruginosa* | ■ | | ■ | | ■ | ■ | ■ | | ■ | | ■ | | ■ | ■ | ■ | | | | | | Burn, pulmonary and chronic infections |
| *Serratia marcescens* | ■ | | ■ | ■ | ■ | | | | ■ | | | | | | | | | | | | Hospital-acquired infections, particularly catheter-associated bacteremia, urinary tract infections, and wound infections |
| Other major pathogens | | | | | | | | | | | | | | | | | | | | | |
| *Acinetobacter nosocomialis* | | | | | | | | | | | | | | | | | | | | ■ | Hospital-acquired infections |
| *Brucella melitensis* | | | | | | | ■ | | | | | | | | | | | | | | Brucellosis |
| *Burkholderia cenocepacia* | | | ■ | | ■ | | | | | | | | | | | | | | | | Infections in cystic fibrosis patients |
| *Burkholderia cepacia* | | | ■ | | ■ | | | | | | | | | | | | | | | | Chronic infections, pulmonary infections |
| *Burkholderia glumae* | | | ■ | | ■ | | | | | | | | | | | | | | | | Pulmonary infections |
| *Burkholderia mallei* | | | | | ■ | ■ | | | | | | | | | | | | ■ | ■ | | Glanders |
| *Burkholderia pseudomallei* | | | | | ■ | ■ | | | | | | | | | ■ | | | ■ | ■ | ■ | Melioidosis |
| *Burkholderia thailandensis* | | | ■ | | ■ | ■ | | | | | | | | | | | | | | | Infections comparable to *B. pseudomallei* |
| *Burkholderia vietnamensis* | | | ■ | | ■ | ■ | ■ | ■ | | | | | ■ | | | | | | | | Infections in cystic fibrosis and immunocompromised patients |
| *Cronobacter sakazakii* | | | | | | | | | ■ | | ■ | | | | | | | | | | Bacteremia, meningitis and necrotizing enterocolitis in infants |
| *Vibrio harveyi* | ■ | | | | | | | | | | | | | | | ■ | | | | | Zoonosis |
| *Yersinia enterocolitica* | | | ■ | | | | | | ■ | | | | ■ | ■ | ■ | | | | | | Yersiniosis |
| *Yersinia pestis* | | | ■ | | ■ | | | | ■ | | ■ | | ■ | | | | | | | | Plague |
| *Yersinia pseudotuberculosis* | | | ■ | | ■ | | | | ■ | ■ | ■ | ■ | ■ | | | | | | | | Pseudotuberculosis |

**Figure 2. Various Gram-negative bacteria that use AHL-based sensing to control pathogenicity.** AHLs reported for each bacterium are highlighted in *red* (15).

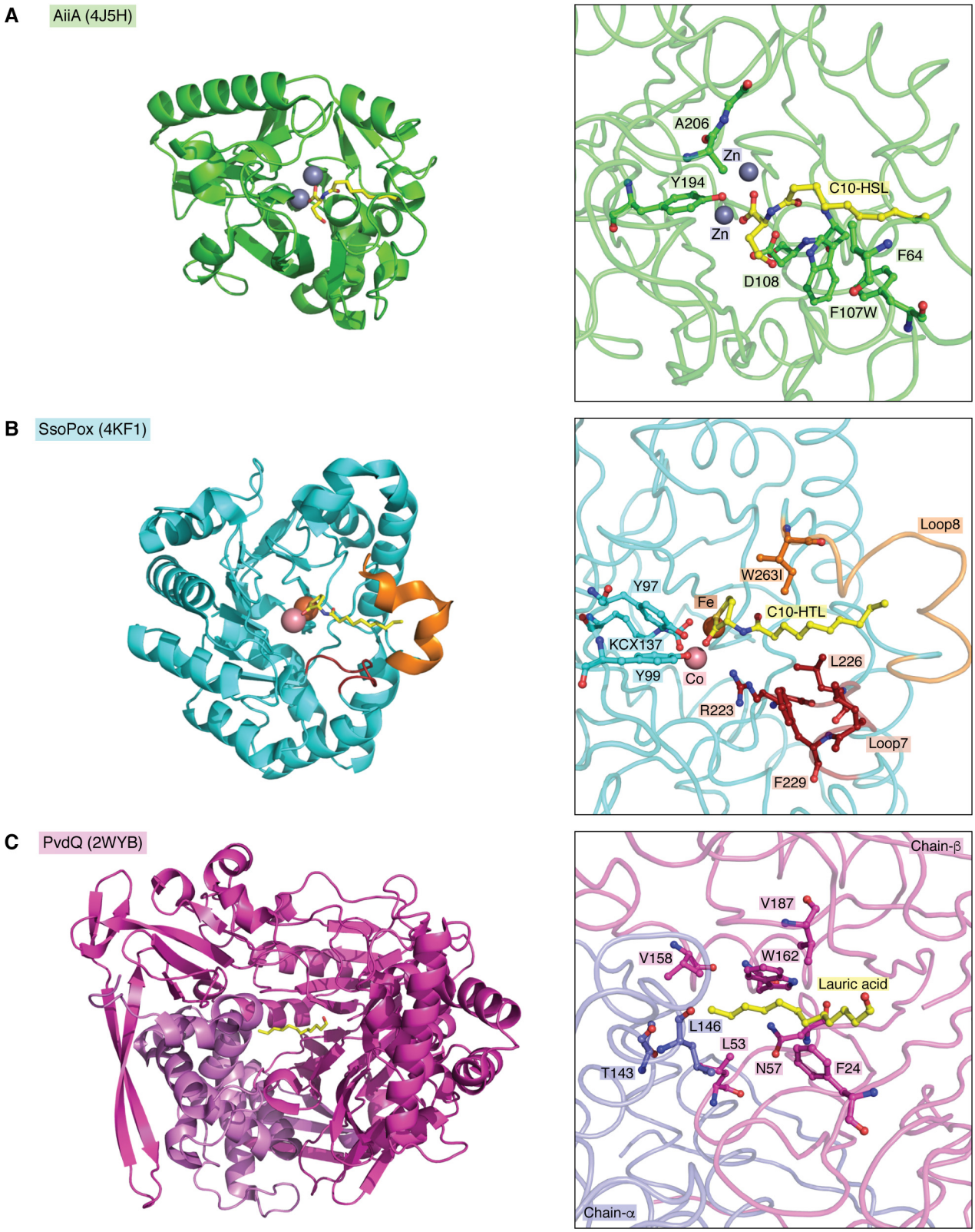



**Figure 3. Structural overview of AHL-interfering enzymes.** AiiA and *Sso*Pox lactonases and the acylase PvdQ are presented using the same *scale*. *A*, crystal structure of the 28-kDa metalloenzyme lactonase (EC 3.1.1.81) AiiA mutant F107W from *Bacillus thuringiensis* with *N*-decanoyl-L-homoserine bound at the active site (PDB entry 4J5H). AiiA belongs to the metallo-$\beta$-lactamase superfamily and harbors two Zn(II) ions bound at the active site essential to catalytic activity. *B*, crystal structure of the 35-kDa metalloenzyme, PLL *Sso*Pox W263I (EC 3.1.8.1) in complex with $C_{10}$-HTL (PDB entry 4KF1). *Sso*Pox belongs to the amidohydrolase superfamily and exhibits a ($\alpha$/$\beta$)8-barrel fold (the so-called TIM-barrel) and harbors a bicobalt active site. Loops 7 (*red*) and 8 (*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 entry 2WYB). PvdQ is a member of the Ntn-hydrolase superfamily and is formed by an 18-kDa $\alpha$-chain (*purple*) and a 60-kDa $\beta$-chain (*pink*).

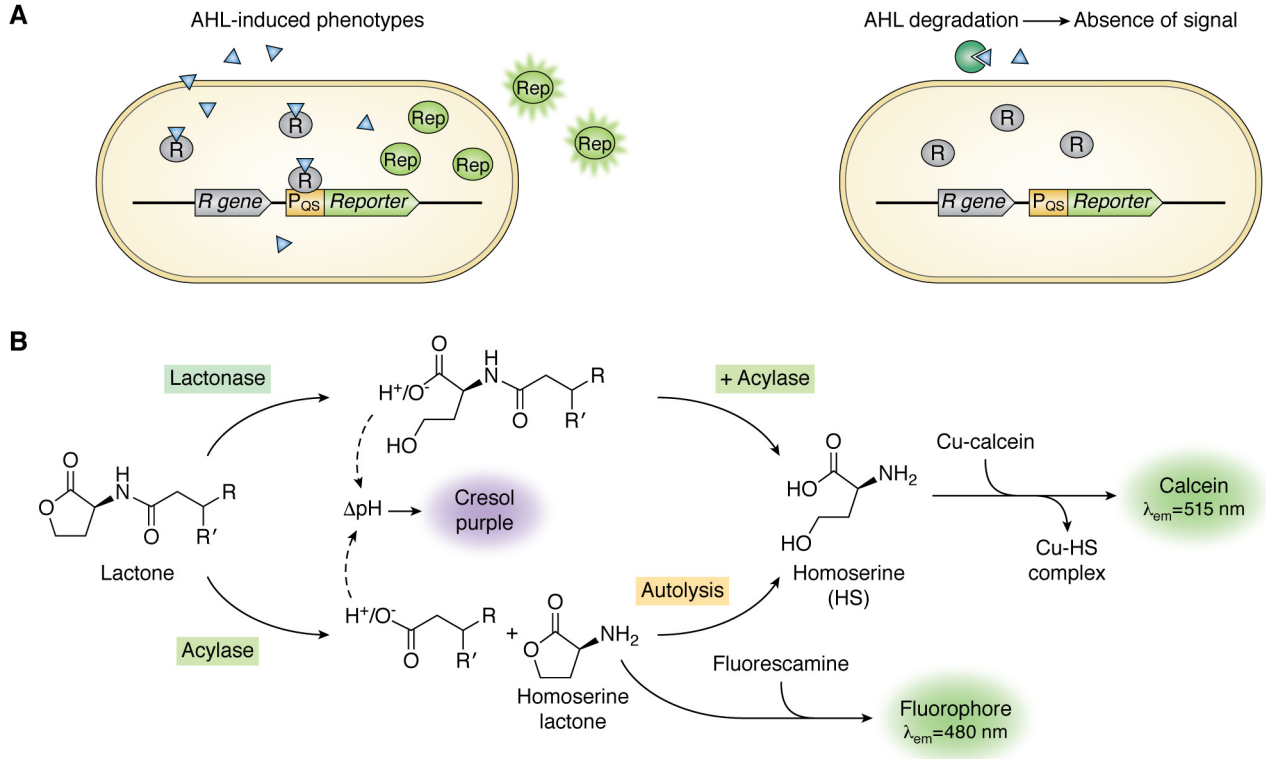



**Figure 4. Screening approaches for identifying novel or improved AHL-interfering enzymes.** *A*, *in vivo* assays based on natural QS systems. AHLs are perceived by biosensor cells that consist of a regulator that is activated upon AHL binding and in turn induces the expression of a reporter gene (luminescence, violacein, fluorescence, or β-gal). In the presence of active QQ enzymes, AHLs 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).

## QQ enzyme 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: AHLs diffuse freely through cell membranes and bind and activate a specific response regulator, which in turn binds to its target promoters activating QS gene expression (Fig. 4*A*). 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-gal (35, 36), or fluorescent marker (*e.g.* GFP) (37, 38) (Fig. 4*A*). 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 AHLs. Reporter strains mostly differ by their ability to respond to a variety of structurally different AHLs, as a function of the chosen regulator, and in the type of the reporter gene used. Luminescence, fluorescence, and β-gal activity are convenient for miniaturized screening (*e.g.* in microplate format). However, choosing β-gal requires a biochemical assay to obtain quantitative results, whereas luminescence and fluorescence reporters can be directly measured (39).

One of the most common reporter strains 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, which acts as an antibiotic at high cell density. Therefore, it can be used for screening purposes and responds best to exogenous AHLs 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/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 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 AHLs 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 the presence of the β-lactam antibiotics. Upon selection of an effective QQ enzyme, AHLs 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 $5 \times 10^5$ clones (45).

Nevertheless, all of 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 of QQ enzymes. To limit AHL hydrolysis, specific buffers have been established using *Agrobacterium tumefaciens* A136 β-gal reporter strain (46). A second origin of false positive clones is the inverse proportionality between the efficiency of QQ and the readout of the reporter of these turn-off assays. Indeed, the higher the activity of the QQ enzyme is, the more hydrolyzed AHLs 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 $4.1 \times 10^5$ 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, which also degrade AHLs, 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.

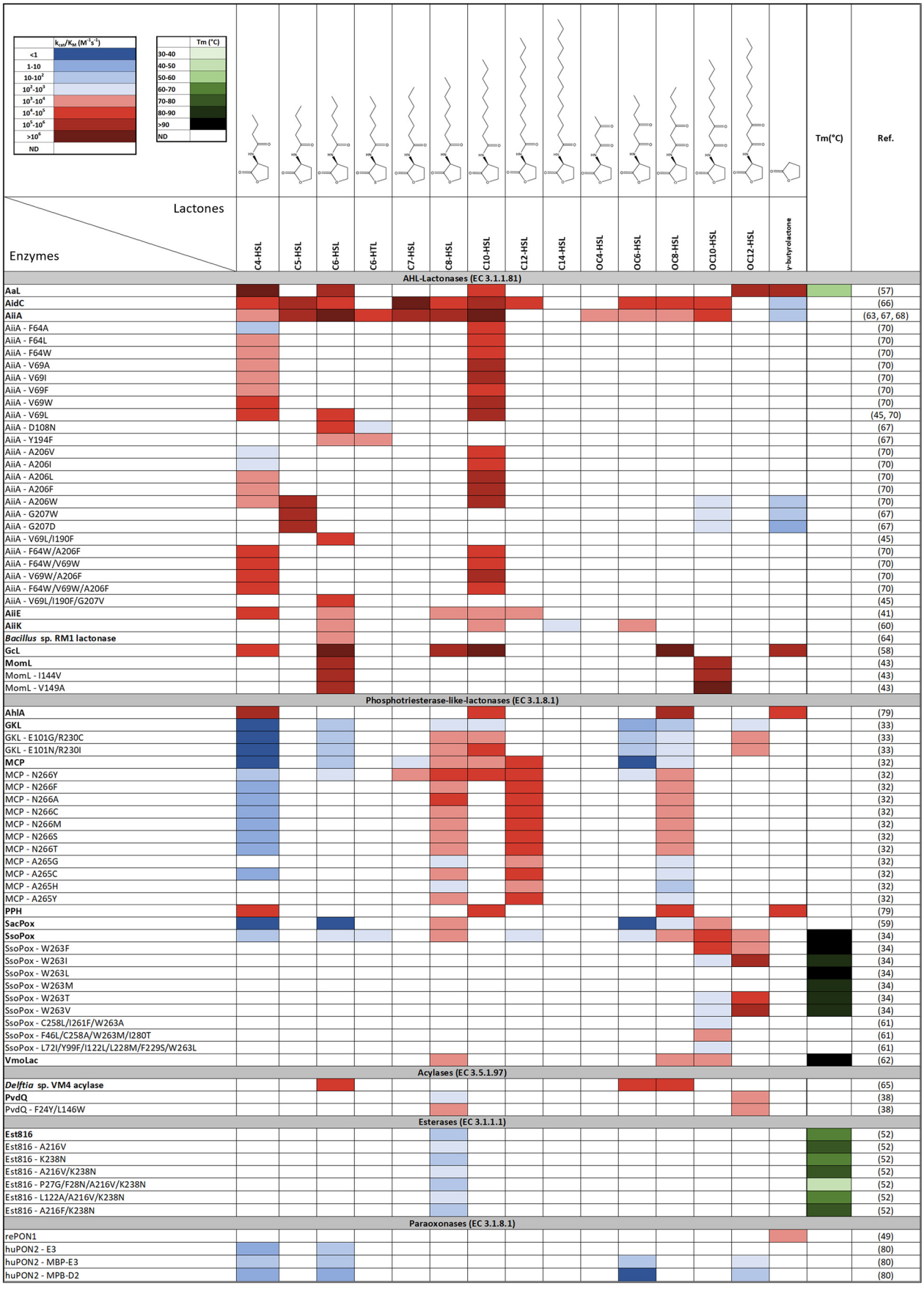
In the absence of representative chromogenic substrate, other *in vitro* methods have been developed for the identification of acylases or lactonases. Using *A. tumefaciens* β-gal biosensors, cell free lysates were prepared bulk and stored at −80 °C (53). These lysates can be used with two substrates, 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. 4*B*). 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) 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. 3*B*).

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 PCR (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 a 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 toward various AHLs. Random mutagenesis first led to identification of two-point mutations (A216V and K238N) exhibiting increased $k_{cat}/K_m$ values toward 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 with WT enzyme. Conversely, K238N mutation increased $k_{cat}$ value by 8-fold while decreasing affinity. These mutations have been further combined, leading to an A216V/K238N variant with 3-fold enhancement in $k_{cat}/K_m$ with C8-HSL compared with WT Est816 (52). Whereas Ala-216 is close to the lactone ring and has a direct impact on ligand-substrate interactions, Lys-238 is located on the enzyme surface, and its impact on activity is yet 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, whereas no activity was detected on these substrates for the WT enzyme (32). The same approach was also applied to a thermostable PLL GKL from *Geobacillus kaustophilus*, revealing a quadruple mutant exhibiting better QQ ability than WT enzyme. Retro-engineering allowed identification of the double mutant E101G/R230C with a global increase in catalytic performance toward AHLs, with a 1.2- and 32-fold increase, respectively, for C6-HSL and OC12-HSL and, compared with 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 two- or three-dimensional 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, key amino acids can be identified to aid design (69).

Docking analysis and molecular dynamics simulations have been used to enhance activity of AiiA in favor of substrates having a short acyl chain and to the detriment of those with a long acyl chain. Computational docking of various AHLs 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 a long acyl chain was reduced. To this end, 15 mutants with hydrophobic residue substitutions were constructed. Eight mutants showed increased $k_{cat}/K_m$ values with C4-HSL, whereas mutant V69W showed >6-fold increase. Combining the best mutations, double and triple mutants were created, leading to a >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 (70).

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 (71, 72). 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 (73). *Sso*Pox active-site structure analysis identified a residue (Trp-263) at the beginning of loop 8 (Fig. 3*B*) 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 improved OC12-HSL degradation by 45- and 54-fold, respectively,

**Figure 5. Catalytic performances and stability of native** (57–68) **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*. Melting temperature ($T_m$) values are presented with *shades of green* from *light* to *dark*. *Colors* and their respective values are detailed in the *top left*.

whereas all mutations decreased OC10-HSL activity. Interestingly, all mutations also increased lactonase activity toward δ- and γ-lactones, some of them, such as γ-butyrolactone, being potentially involved in *Streptomyces* sp. signaling (74, 75). Mutations of the Trp-263 residue were thus demonstrated to strongly alter *Sso*Pox specificity and activity, whereas mutants conserved great stability (as measured by melting temperature), allowing the W263I variant to resist harsh industrial conditions (71). Among the diverse bacterial AHLs, *Burkholderia cenocepacia* mainly uses C8-HSL. To specifically target this pathogen, an acylase from *P. aeruginosa,* PvdQ, was engineered (38). This enzyme, originating from *P. aeruginosa*, specifically degraded AHLs from C11-HSL to OHC14-HSL and was thus an ideal candidate for enzyme engineering toward the degradation of C8-HSL (23). 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 increased C8-HSL activity 4.3-fold, whereas OC12-HSL degradation was reduced 3.8-fold. This shifted preference suggests an accommodation of the active site toward the targeted substrate and implies that PvdQ would not be an ideal candidate to engineer a broad-range degrading enzyme.

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 Leu-122 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 toward C8-HSL compared with WT Est816 (52). Furthermore, these three improving mutations did not impact Est816's 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 a 2-fold increase compared with E101G/R230C, for 72-fold total improvement toward this lactone as compared with WT enzyme (33). Mutations of the Glu-101 residue alter the lactone ring positioning by enhancing a critical loop flexibility, whereas mutations of Arg-230 modulate the position of the attacking hydroxide nucleophile, resulting in a more efficient nucleophile attack angle (76).

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. PLLs are well-known to have latent phosphotriesterase activity, and it has been demonstrated that phosphotriesterase (PTE) quickly diverged from PLL (77, 78). 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 $10^4\ M^{-1}\ s^{-1}$ (79). 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/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/liter of culture for MBP-D2 and MBP-E3, respectively, whereas MBP-huPON2 has only been expressed in its insoluble form (80).

Rational design appears as a useful tool to directly target enzyme hotspots to enhance lactonase activity. Both random mutagenesis using epPCR and rational design experiments resulted in new enzyme variants with strongly enhanced activities (Fig. 5). Whereas random mutagenesis improved activities by an order of magnitude, rational design led to close to 100-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, whereas stability and solubility of the protein are rarely considered. Only a few studies have 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 Table 1.

### *Human health*

QS plays an important role in pathogenicity of numerous invasive bacteria such as *P. aeruginosa* or *A. baumannii* (13, 18, 19). Due to rising antibiotic resistance, alternative and/or

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

| Applications | QQ enzymes | Quenched bacteria | Measured phenotypes | Reference |
|---|---|---|---|---|
| Human health | GKL-E101G/R230C | *Acinetobacter baumanii* | Reduction of biofilm | 91, 92 |
| | huPON D2 | *P. aeruginosa* PAO1 | Diminution of swarming and swimming motilities | 80 |
| | huPON E3 | *P. aeruginosa* PAO1 | Diminution of swarming and swimming motilities | 80 |
| | MBP-AiiM | *S. marcescens* AS-1 | Decrease of QS-dependent pigment | 93 |
| | *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 | 82–85, 90 |
| | 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 | 94 |
| | AiiA from *B. thuringiensis* | *V. harveyi* | Diminution of bioluminescence | 95 |
| | AiiA from *Bacillus* sp. AI96 | *A. hydrophila* | Decreased mortality in zebrafishes | 96 |
| | AiiA from *Bacillus* sp. B546 | *A. hydrophila* | Decreased mortality in carps | 97 |
| Agriculture | AiiA from *B. amyloliquefaciens* | *P. carotovorum* subsp. *carotovorum* | Reduction of infection on carrots | 98 |
| | AiiA from *B. subtilis* BS-1 | *P. carotovorum* subsp. *carotovorum* | Reduction of infection on potatoes | 99 |
| | 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 | 100, 101 |
| Biocorrosion | *Sso*Pox-W263I | Complex communities | Reduction of biofilm/diminution of biocorrosion/changes in bacterial population proportions | 102, 103 |

complementary therapeutic strategies are required (81). 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 toward 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) (82, 83). *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* (84) 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 (85–87). Finally, this variant also reduced biofilm formation and 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 (85, 88, 89). *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, whereas mortality was reduced to 20% in treated animals (90). 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 (91, 92).

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 preincubating the bacteria and the variant before injection in *Galleria mellonella* larvae, drastic enhancement in survival was obtained as compared with larvae treated with WT enzyme; nearly 100% of larvae survived with PvdQ F24Y/L146W and around 20% of larvae survived with PvdQ WT (38). One of the limitations of the therapeutic use of QQ enzymes is their potential to trigger an immune response (80). To that end, the huPON2, as described previously, has been engineered for QQ purposes to benefit from 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 (80).

Engineered enzymes have also been immobilized for antivirulence purposes. *Sso*Pox W263I was immobilized in polyurethane coating and was able to decrease virulence factors of *P. aeruginosa* PAO1 (82). AiiM, a WT lactonase isolated from *Microbacterium testaceum* StLB037, was overexpressed in *E. coli* DH5$\alpha$ as a recombinant protein with a maltose-binding protein tag (MBP-AiiM) for its purification process and was then successfully incorporated in polyvinyl alcohol fibers by electrospinning. MBP-AiiM was capable of quenching QS-dependent prodigiosin production in *Serratia marcescens* AS-1 (93, 104).

### *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 multibillion US dollar annual losses (105). Gram-negative bacteria, including *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila*, are responsible for numerous diseases in a large variety of marine animals (106–108). 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 (109). Thus, native and engineered QQ enzymes constitute an appealing strategy for aquaculture (18, 19).

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% (95). Another AiiA, from

*Bacillus licheniformis* DAHB1, was able to decrease the 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 into the abdominal cavity (94). 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 with 79% without enzyme (97). Furthermore, another AiiA, from *Bacillus* sp. AI96, decreased mortality rate in zebrafish by oral administration down to 20% as compared with 60% without enzyme (96).

These promising results, based on WT lactonases, show that the use of engineered enzymes that offer better stability, efficiency, or immobilization may 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 humans and 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 (110, 111), *Erwinia amylovora* responsible for fire blight (112, 113), or *A. tumefaciens* causing crown gall disease (114, 115). These and other diseases have a significant economic impact on agriculture and on the quantity and quality of food (116, 117), 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 (18). 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 (118, 119). Conversely, soil bacteria are known to produce QQ enzymes, like AiiA from *Bacillus* sp., AttM from *A. tumefaciens*, or QsdA from *Rhodococcus erythropolis* (120–122). 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 (98, 99). Similarly, the variants MomL I144V and MomL V149A obtained by random engineering were able to decrease infection of Chinese cabbage by *Pcc*. Cabbage leaves have been infected with *Pcc,* in the presence of MomL variants or controls, on a cut surface of the leaf; thus, MomL variants greatly reduced 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 (MBRs) from biofilm formation using whole bacteria (123, 124) or QQ enzymes. Mainly, the porcine kidney acylase was used to reduce biofilm formation in MBR systems. For instance, acylase WT immobilized in sodium alginate capsules reduced biofouling formation and improved filterability of MBR systems (100). 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 (101). 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 a filtration cartridge containing bacteria expressing *Sso*Pox W263I in silica capsules was developed to assess the impact of QQ on complex bacterial communities. The presence of *Sso*Pox variant in capsules led to changes in bacterial communities and to biofilm inhibition (102). *Sso*Pox W263I immobilized in a silica gel coating painted on steel plates was also able to reduce corrosion tubercles by 50% compared with controls after 8 weeks of immersion in Duluth-Superior Harbor (Minnesota, USA) (103). The use of enzymes instead of or as a complementation to biocides in paint is attractive, due to use restriction of some biocides because of their negative impact on the environment (125). 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 of these promising applications need further development, such as a larger range of improved enzymes, upscaling 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 advances achieved thanks to enzymatic engineering approaches. Numerous efforts have been dedicated to isolating 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 the development of 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, revealing that lactonases with distinct efficacy toward AHLs yielded drastically different quenching effects at both molecular and phenotypic levels, the broadly active enzyme being not necessarily the most efficient (83, 126). These results underscore the determinant role of enzyme specificity on QQ at a single-species level and demonstrate that catalytic performance may not be used as the sole selection criterion and that specific screening has 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 AIs. In Gram-positive bacteria, autoinducing cyclic peptides have largely been described (127), whereas their enzymatic quenching has been poorly considered to date. In Gram-negative bacteria, although AHLs are widespread, other molecules, including epinephrine (AI-3) (128) or quinolones (129), 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 interkingdom signaling (130). AI-2 could thus also constitute a promising target, and enzymes able to interfere with this compound need to be further investigated (131). Once potential QQ enzymes are identified for these AIs, rational engineering and random mutagenesis strategies will allow the enhancement of their activity and of their antivirulence 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 AIs but also to evaluate their capacity to directly compete with bacterial phenotypes (*e.g.* biofilm formation and production of virulence factors) in conditions that may be realistic with 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 processes. Moreover, the complementarity of QQ enzymes with classical antimicrobial treatments has been demonstrated (84) 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 economic 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.

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*Abbreviations*—The abbreviations used are: QS, quorum sensing; AI, autoinducer; AHL, acyl-homoserine lactone; QQ, quorum quenching; PLL, phosphotriesterase-like lactonase; HTS, high-throughput screening; epPCR, error-prone PCR; PTE, phosphotriesterase; MBP, maltose-binding protein; MBR, membrane bioreactor; PDB, Protein Data Bank.

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# Engineering acyl-homoserine lactone-interfering enzymes toward bacterial control

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