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Review Article

Engineering quorum quenching enzymes: progress and perspectives

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Quorum sensing is a key contributor to the virulence of many important plant, animal and human pathogens. The disruption of this signalling—a process referred to as 'quorum quenching'—is a promising new approach for controlling microbial pathogens. In this mini-review, we have focused on efforts to engineer enzymes that disrupt quorum sensing by inactivating acyl-homoserine lactone signalling molecules. We review different approaches for protein engineering and provide examples of how these engineering approaches have been used to tailor the stability, specificity and activities of quorum quenching enzymes. Finally, we grapple with some of the issues around these approaches—including the disconnect between *in vitro* biochemistry and potential *in vivo* applications.

Introduction

Enzymes that can degrade quorum sensing molecules are being increasingly explored as potential antimicrobials to target pathogenic bacteria. Quorum sensing is a communication process that allows bacteria to co-ordinate group behaviours, such as virulence factor secretion and biofilm formation [1]. Quorum sensing involves the synthesis, release, and subsequent detection of chemical signal molecules called autoinducers. Bacteria use these chemical signals to assess their local population density. Once the bacterial population is sufficiently high (i.e. a quorum is reached), bacteria may begin to co-ordinate expression of virulence genes and regulate processes such as biofilm formation [2–4].

Targeted disruption of this signalling is referred to as 'quorum quenching'. Quorum quenching approaches typically involve either: use of small molecules to inhibit production, transport, or detection of quorum sensing signals; or use of enzymes to degrade quorum sensing signalling molecules (Figure 1). Quorum quenching is a rapidly growing field of research, and there are several excellent reviews on the topic [5–7]. In this mini-review, we focus on the efforts to engineer enzymes with improved quorum quenching abilities. This is an emerging field that will expand our understanding of quorum quenching enzymes and their potential applications.

Quorum quenching enzymes

Arguably the two most widely studied types of quorum quenching enzymes are lactonases and acylases. Enzymes from both families target acyl-homoserine lactones (AHLs). AHLs are the predominant class of quorum sensing signals produced by Gram-negative bacteria (including many pathogens). AHLs vary in the length of the acyl side chain (usually 4–18 carbons) and substituents (e.g. 3-oxo or hydroxyl group) [8] (Figure 2A). These variations dictate the specificity of the signal, although inter-species cross-talk can also occur.

Lactonases exhibit a range of different specificities, inactivating both short- and long-chain AHLs by hydrolysing the ester bond of the lactone ring to yield acyl-homoserine (Figure 2B). In contrast, acylases are generally most effective against AHLs with side chains longer than 10 carbon

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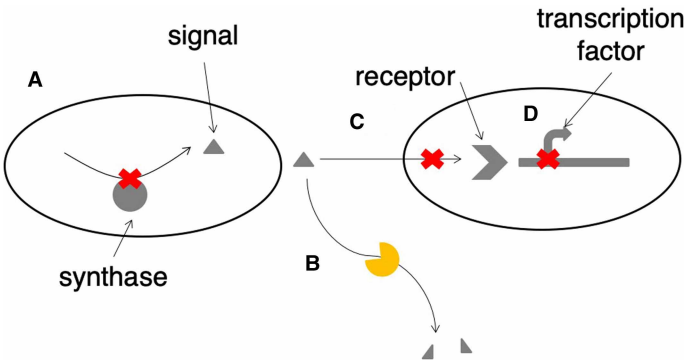

Figure 1. Examples of quorum quenching strategies.

(**A**) Small-molecule inhibition of signal molecule biosynthesis. (**B**) Enzymatic degradation of signal molecules. (**C**) Small-molecule inhibition of signal reception. (**D**) Small-molecule inhibition of signal transduction.

atoms. Acylases inactivate AHLs by hydrolysing the amide bond that joins the lactone moiety and the acyl side chain to yield homoserine lactone and fatty acid (Figure 2C). Both types of enzymes render AHL signal molecules inactive. However, only the acylase reaction is irreversible; the product of the lactonase reaction can spontaneously undergo ring formation in acidic environments, reverting back to an active form [9].

While naturally occurring quorum quenching enzymes have shown promising effects both *in vitro* and *in vivo* [10,11], protein engineering approaches are being explored to further improve the stability, catalytic activity and/or substrate specificity of these enzymes for downstream applications.

Protein engineering approaches

Broadly speaking, there are two main approaches for engineering enzymes: directed evolution and rational design [12]. Directed evolution is a powerful and well-established method for improving and tailoring enzymes [13]. It is often referred to as a 'blind approach' [14] because it does not require prior knowledge of protein structure. In general terms, directed evolution involves the accumulation of beneficial mutations during iterative cycles of mutagenesis and screening or selection.

In contrast, the rational design is heavily reliant on prior knowledge of protein sequence, structure, and functional data in order to design specific mutations. Although it can be extremely difficult to predict what mutations will be required for the desired outcome, advances in computational protein design are continually improving [15–19]. Even so, in most studies, constructs based on computational design must undergo several rounds of directed evolution to reach acceptable levels of catalytic efficiency and stability [20–22].

For both approaches (and combinations thereof), enzymes with a good yield, promiscuous activities, and/or thermostability are ideal starting templates for engineering [23,24]. For example, thermostability can be an

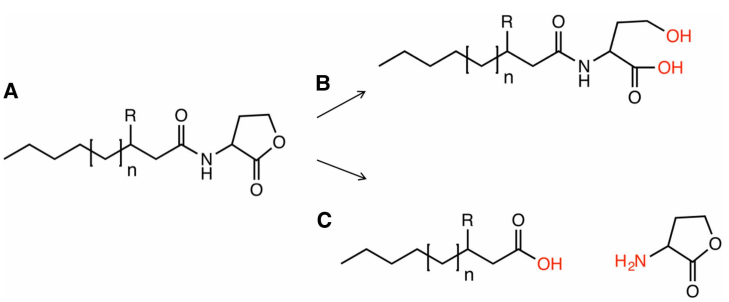

Figure 2. AHL signalling molecules and main routes of enzymatic degradation.

(**A**) An AHL consists of a homoserine lactone moiety and an acyl chain; the length and substitutions of acyl side chains vary. (**B**) Lactonases inactivate AHLs by hydrolysing the ester bond of the lactone ring to yield acyl-homoserine; (**C**) Acylases cleave the amide bond that joins the lactone moiety and the acyl side chain.

important consideration because mutations that enhance activity may also destabilise the enzyme [25]. Similarly, high protein yields can be important for later production scale-up.

Quorum quenching lactonase engineering

There are three structurally distinct families of quorum quenching lactonases: phosphotriesterase-like lactonases (PLLs), metallo-β-lactamase-like lactonases, and serum paraoxonases (PONs) [26] (Figure 3A–C). There are extensive sequence, structural and functional data available for representatives of each family, and numerous studies have used lactonases as templates for engineering. Some key highlights are described below.

An early engineering effort focused on increasing the catalytic efficiency and substrate range of a quorum quenching lactonase from *Mycobacterium avium* [31]. This lactonase is mesophilic and a member of the PLL family. It has a $(\beta/\alpha)_8$-barrel (or TIM barrel) fold (Figure 3A) and was chosen as a template for engineering due to its high solubility and ease of purification. A directed evolution approach (i.e. random mutagenesis, coupled with a bioluminescence-based screen) identified a single point mutation (Asn266Tyr) that had both increased catalytic efficiency and a broadened substrate specificity [31]. The increased catalytic efficiencies (4- to 32-fold) towards different AHL substrates are a result of both increased k_{cat} values and decreased K_M values. The single Asn266Tyr mutation also conferred new activities towards C4-HSL and 3-oxo-C6-HSL. Unfortunately, this variant enzyme was too unstable for use in potential downstream biomedical applications. Thus, for the next attempt at engineering, a thermophilic lactonase from *Geobacillus kaustophilus* was used as the template [32]. In this study, both rational design and directed evolution approaches were attempted. The rational designs explored both site-directed mutagenesis and loop-grafted variants, though both approaches were ultimately unsuccessful. In contrast, random mutagenesis was used to successfully identify a double mutant (Glu101Asn/Arg230Ile) with increased AHL reactivity and broadened substrate specificity.

Other PLLs have also been explored as scaffolds for engineering. *Sso*Pox is a hyperthermostable PLL isolated from the archaeon *Sulfolobus solfataricus* [33]. Structural analysis of *Sso*Pox identified a residue (Trp263) in the active site loop that was predicted to play a key role in the substrate specificity of this enzyme. Site-saturation

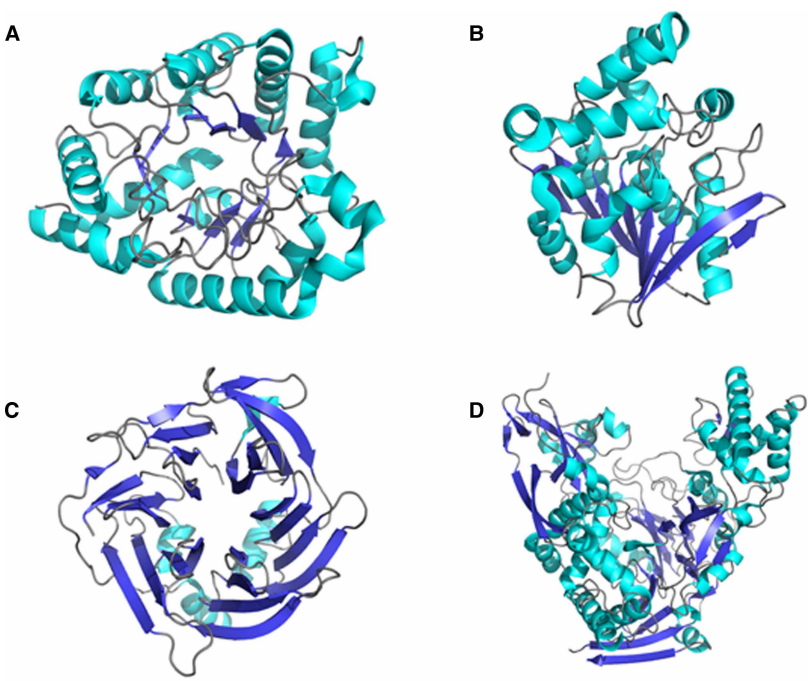

Figure 3. Diversity of quorum quenching enzyme families.

(**A**) PLLs, e.g. *Sulfolobus solfataricus* lactonase (PDB 2VC5) [27]. (**B**) metallo-β-lactamase-like lactonases, e.g. Est816 (PDB 5EGN) [28]. (**C**) PONs, e.g. PON1 G2E6 (PDB 1V04) [29]. (**D**) Ntn-hydrolase AHL acylases, e.g. PvdQ (PDB 2WYE) [30]. Loops shown in grey, sheets shown in blue, helices shown in cyan.

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mutagenesis coupled with activity screening identified a Trp263Ile variant that is 45-fold more active against 3-*oxo*-C12-HSL [34]. This variant was later shown to have exceptional resistance to conditions such as heat resistance, contact with organic solvents, sterilisation, storage, and immobilisation — all properties useful for industrial applications [35]. The variant also decreased virulence factor production and biofilm formation in clinical isolates of *P. aeruginosa* [36].

Interestingly, in all of these examples of successful engineering of PLLs, the mutations conferring improved AHL lactonase activity occurred in loops at the end of β strands, where substrate specificity is determined [37].

Lactonases from the metallo-β-lactamase-like family have also been engineered for use as quorum quenching enzymes. Metallo-β-lactamase-like lactonases have an αβ/βα sandwich fold, with a compact core made up of β sheets, surrounded by helices that form the outer solvent exposed layer [38] (Figure 3B). One target for engineering was the thermostable metallo-β-lactamase-like lactonase Est816. This lactonase degrades a wide range of AHLs (C4-C12) and is highly thermostable, with a half-life of ∼5 months at room temperature [39]. This enzyme was engineered for improved AHL-degrading activity using a combination of directed evolution and rational design [28]. First, a small random mutagenesis library (∼26 000 variants) was screened for improved C8-AHL activity. From this, a variant with two point mutations (Ala216Val/Lys238Asn) was identified with a three-fold improvement in overall catalytic efficiency towards C8-HSL. The crystal structure of the variant was determined next, and this information was used to rationally design an additional mutation (Leu122Ala). The k_{cat}/K_M value of the triple mutant (Ala216Val/Lys238Asn/Leu122A) is ∼22-fold higher than that of the wild-type enzyme [28]. This improvement is largely due to a decrease in K_M, so additional gains may be possible with further engineering to increase k_{cat} [28].

All of the studies described above-used lactonases from prokaryotic origins as the starting template for engineering. However, there are concerns about potential immune responses if prokaryotic enzymes are used as human therapeutics. As an alternative, the use of human PON enzymes as quorum quenchers has also been explored. PONs adopt a six-bladed β-propeller fold [29] and thus are structurally distinct from PLL or metallo-β-lactamase-like lactonases (Figure 3C). Although the name of this enzyme family refers to its paraoxon-hydrolysing activity, PONs are in fact lactonases, capable of hydrolysing AHLs. The low potential immunogenicity of PONs makes them attractive targets as quorum quenching protein-based therapeutics. Of the characterised human PONs, PON2 has the highest activity towards AHLs [40]. However, it is largely insoluble when heterologously overexpressed in *Escherichia coli* [41]. This has limited its potential as a therapeutic, as protein yields can be an important factor for industrial-scale production [23,24]. To address the low solubility of PON2, a combination of rational design and targeted degenerate sequences were used to replace the protruding hydrophobic helices of PON2 [41]. Target helices were identified using structural modelling, then small libraries of hydrophilic linkers containing randomised residues were constructed. The best variants identified from solubility screening of the crude lysates were then re-cloned with an additional solubility tag (maltose-binding protein). Ultimately, this strategy achieved high levels of soluble expression, with yields of ∼76 mg of fully human PON2 variants per litre of culture media. The two most soluble variants maintained their activity towards AHLs (C4-C12) and effectively inhibit quorum sensing-associated phenotypes (e.g. swimming and swarming motilities) of *Pseudomonas aeruginosa* [41]. This study demonstrates the utility of engineering for traits other than catalytic efficiency, such as solubility or immunogenicity, and also highlights the need for appropriate engineering scaffolds according to the downstream application.

Engineering quorum quenching acylases

AHL acylases are the other major class of quorum quenching enzymes [42]. Unlike lactonases, the majority of AHL acylases are members of a single protein family: the N-terminal nucleophile (Ntn) family. To date, relatively few (∼12) AHL acylases have been characterised, and structural information is only available for two representatives [30,43]. However, these structural data have been critical for rational protein engineering of AHL acylase specificity.

The first structure of an AHL acylase (PvdQ, from *P. aeruginosa*) was solved in 2010 [30] (Figure 3D). PvdQ has a distinct and unusually large hydrophobic binding pocket, which reflects the specificity of this enzyme for long-chain acyl-HSLs (i.e. AHLs with side chains of 11–14 carbons) [44].

Using the crystal structure of PvdQ, a rational engineering approach has been used to alter the substrate range of PvdQ toward shorter AHLs. The ability to tune the specificity of quorum quenching enzymes is

important, as it potentially allows the targeting of pathogenic microbes, while limiting deleterious side effects on the beneficial host microbiota. Koch et al. used available structural data combined with molecular docking and *in silico* mutagenesis to identify potential variants with increased C8-HSL activity [45]. C8-HSL is an important signalling molecule for *Burkholderia*, a pathogen commonly isolated together with *P. aeruginosa* from the lungs of cystic fibrosis patients. Two rounds of computational analyses combined with *in vitro* testing of site-directed mutants identified a double mutant (α-Leu146Trp, β-Phe24Tyr) with substantially higher hydrolytic activity towards C8-HSL. These mutations also resulted in reduced activity towards C12-HSL, resulting in a 16-fold overall difference in catalytic efficiency [45]. This study demonstrates the power of rational/computational design approaches to tune enzyme specificity and highlights the potential of acylases as future antimicrobial therapies.

Biotechnological advances and applications

Many biotechnological advances in quorum quenching enzyme engineering have focused on 'upstream' methodologies such as improved screening tools [46,47]. In addition, 'downstream' methodologies such as enzyme formulation and/or delivery are providing key advances. For example, both acylases and lactonases have been successfully formulated as dry powders suitable for pulmonary delivery [48,49]. Another clinical application is coating medical implants with quorum quenching enzymes. For example, coating urinary catheters with the AHL-degrading *Aspergillus melleus* aminoacylase inhibits *P. aeruginosa* PAO1 biofilm formation both *in vitro* and *in vivo* [50,51]. Importantly, the deposited acylase is not toxic to human skin cells [51]. Immobilisation is also important for applications to prevent biofouling. Immobilisation using magnetic particles has allowed recovery and reuse of quorum quenching enzymes. For example, AiiA, the first discovered AHL lactonase [52] can be immobilised and retain up to 60% of its activity after four cycles of use and recovery [53]. Similarly, adsorbing porcine kidney aminoacylase I onto magnetically separable silica and cross-linked the enzymes increases acylase stability [54]. Testing of this immobilised acylase in a wastewater treatment model system incubated with *P. aeruginosa* PAO1 showed that the filtration membrane retains its permeability for up to 14 days in the presence of this immobilised acylase, and *P. aeruginosa* PAO1 does not form mature biofilms on the membrane. Overall, improved formulation and immobilisation strategies will facilitate the use of quorum quenching enzymes in both clinical and industrial applications.

Concluding remarks

Both engineered and native quorum quenching enzymes can reduce bacterial pathogenicity in various model systems. However, it remains unclear how effective these agents will be in clinical and/or field settings. For example, the AHL lactonase MomL shows promising anti-biofilm effects in single-species biofilms, but no effects are observed with a dual-species biofilm and/or a more complex wound model [55]. In addition, the impact of quorum quenching approaches on beneficial host-associated microbes remains to be explored. It is often assumed that highly specific quorum quenching enzymes will have fewer effects on non-target organisms, but this has not yet been proven.

Another potential issue is the development of resistance. It has been frequently hypothesised that because quorum quenching approaches do not kill pathogens, they impart less selective pressures — therefore, resistance is less likely to develop [56,57]. However, resistance towards small-molecule quorum quenching inhibitors has already been shown to arise from laboratory evolution [58] and exist in clinical settings [59]. It is possible that targeting extracellular quorum sensing signals may exert less pressure as compared with intracellular targets [60]. Therefore, the type of quorum quenching approach used (i.e. small-molecule inhibitors versus signal-degrading enzymes) may affect how likely resistance is to arise [61].

There are still significant gaps in our knowledge of quorum quenching enzymes (and how to best use them). This includes gaining a better understanding of the threat and potential mechanisms of resistance. Quorum quenching enzymes should also be evaluated in contexts that match the environment in which they will be used as closely as possible. Furthermore, quorum quenching enzymes may be better for prevention rather than treatment, as biofilms are more difficult to eradicate once they are established.

Overall, while quorum quenching enzymes hold great promise as antimicrobials across a wide range of potential applications, more research is needed before these approaches can be used in the clinic or field.

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Perspectives

- Quorum quenching enzymes are promising next-generation antimicrobials.
- Protein engineering can be used to further improve the stability, specificity and activities of quorum quenching enzymes.
- However, enzyme activity *in vitro* does not always translate to efficacy *in vivo*. Furthermore, the evolution of resistance to quorum quenching enzymes is largely unexplored. More research into these (and other) issues will be required before quorum quenching enzymes reach their potential as therapeutics.

Abbreviations

AHL, acyl-homoserine lactone; PLLs, phosphotriesterase-like lactonases; PONs, paraoxonases.

Author Contribution

All authors contributed to the drafting of the manuscript and approved the final submission.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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