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Engineering Soluble Human Paraoxonase 2 for Quorum Quenching

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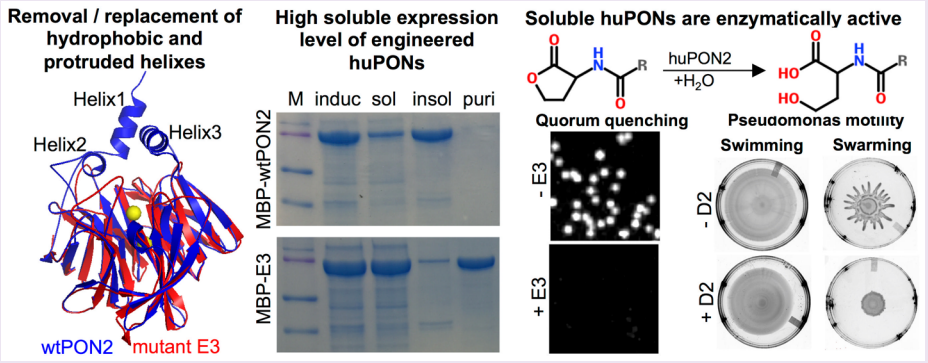
Supporting Information



ABSTRACT: Many pathogenic bacteria utilize quorum sensing (QS) systems to regulate the expression of their virulence genes and promote the formation of biofilm, which renders pathogens with extreme resistance to conventional antibiotic treatments. As a novel approach for attenuating antibiotic resistance and in turn fighting chronic infections, enzymatic inactivation of QS signaling molecules, such as N-acyl homoserine lactones (AHLs), holds great promises. Instead of using bacterial lactonases that can evoke immune response when administered, we focus on the human paraoxonase 2 (huPON2). However, insolubility when heterologously overexpressed hinders its application as anti-infection therapeutics. In this study, huPON2 was engineered for soluble expression with minimal introduction of foreign sequences. On the basis of structure modeling, degenerate linkers were exploited for the removal of hydrophobic helices of huPON2 without disrupting its folding structure and thus retaining its enzymatic function. High soluble expression levels were achieved with a yield of 76 mg of fully human PON2 variants per liter of culture media. Particularly, two clones, D2 and E3, showed significant quorum quenching (QQ) bioactivities and effectively impeded *Pseudomonas aeruginosa* swimming and swarming motilities, signs of an early stage of biofilm formation. In addition, by correlating QQ with luminescence signal readouts, quantitative analysis of QQ toward natural or non-natural AHL-regulator combinations suggested that D2 and E3 exhibited strong lactone hydrolysis activities toward five AHLs of different side chain lengths and modifications widely utilized by a variety of biomedically important pathogens.

Besides living as unicellular organisms, bacteria also interact socially by utilizing chemical signaling compounds to communicate within and between species.[1−3] Particularly, to survive in a host and to attack the host defense mechanisms, many bacterial pathogens have evolved multicellular organization, which regulates numerous virulence gene expressions and promotes biofilm development.[4−6] Once biofilm generates, infections such as cystic fibrosis, chronic wounds, and diabetic foot ulcers raise resistance to antibiotics and other conventional antimicrobial agents.[5] Bacteria activate these protective traits only after attaining a particular population density to launch a concerted attack and produce ample virulence factors to overwhelm the host defenses.[7] To sense their population density, bacteria employ quorum sensing (QS), a process in which bacteria secrete and recognize small signaling molecules called autoinducers or quorum sensing molecules (QSMs).[8] The concentration of these QSMs correlates with the abundance of secreting microorganisms in the vicinity. Because biofilm development and QS are closely connected, QSMs have been recognized as attractive pharmaceutical targets for fighting infections.[9] In fact, most high-priority category A, B, and C pathogenic bacteria identified by NIAID involve QS and therefore are potentially targetable by quorum quenching (QQ).

One approach to interfering with intercellular communication, thus disrupting biofilm formation and inhibiting the expression of virulence factors, is through the usage of synthetic QSM analogues, e.g., brominated furanone C-30, to act as agonists or antagonists.[10,11] This strategy makes pathogens more sensitive to the host immune system and to some antimicrobials.[12] Its potential for treating infections such as *Pseudomonas aeruginosa* in cystic fibrosis and *Enterobacter cloacae* in chronic wounds has been demonstrated.[13,14] In general, disturbing QS communication does not directly cause

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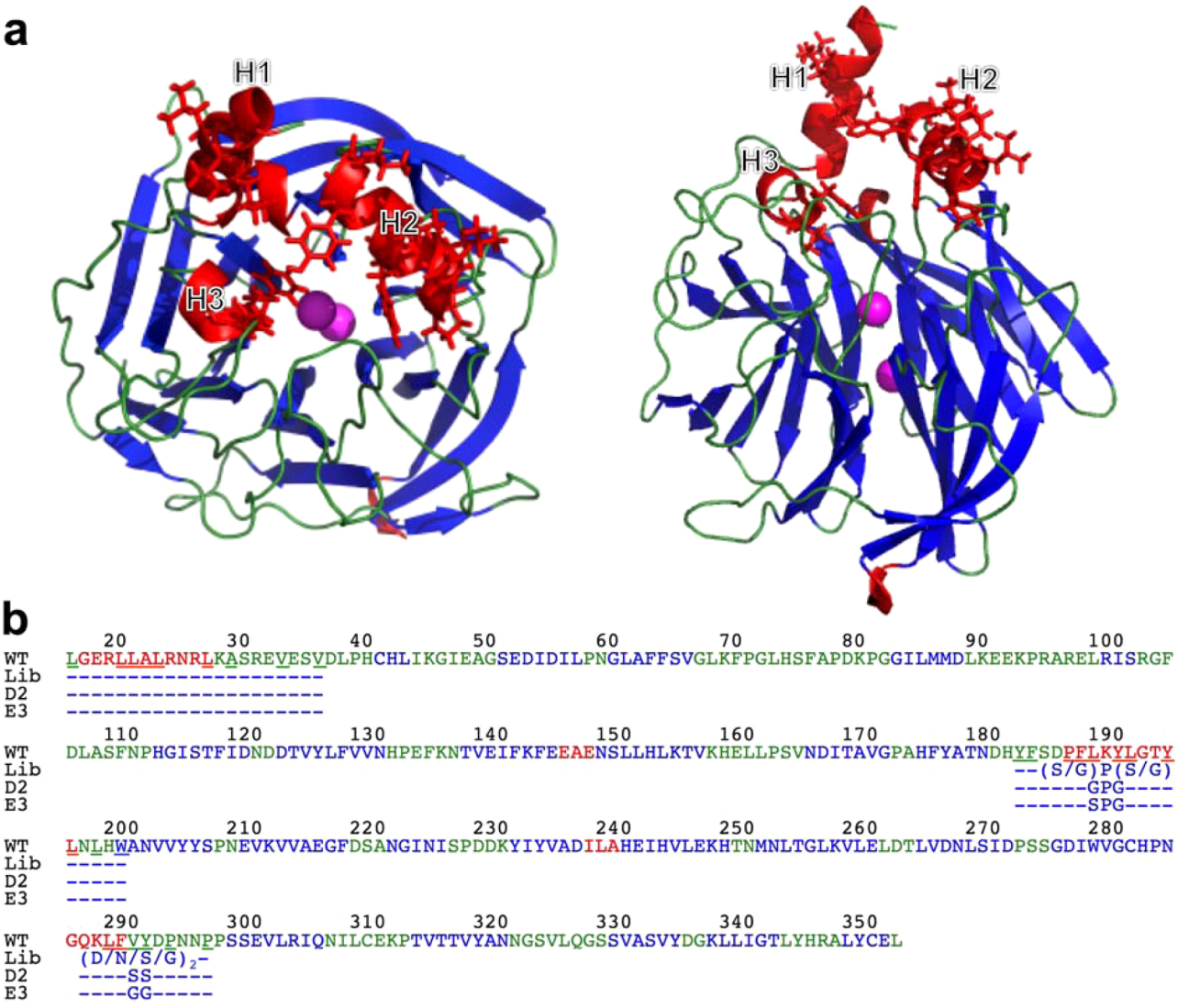

Figure 1. Engineering human PON2 for soluble expression by removal of hydrophobic and protruded helixes. (a) Top and side views of huPON2 structure, which is characterized as a six-bladed β propeller fold (blue) with three protruded helixes (red, hydrophobic residues within the helices were shown as sticks) and loop regions (green). The reactive Ca^{2+} ions (magenta) are in the center of the propeller fold. Structure mode was generated by Robetta based on a mammalian PON1 (PDB ID 1V04). (b) Amino acid sequences of wild type huPON2, library design with 64 possibilities, and selected variants D2 and E3. Colors match with the structure in a. The hydrophobic residues removed or replaced by linkers are represented as dash lines.

cell death; therefore applying less selective pressure compared to antibiotics.[15] However, it has also been found that in some cases pathogens tend to evolve resistance such as by increasing efflux of QQ agents.[16] As an alternative approach for disrupting QS, the use of enzymes to abolish the biological activity of QSMs can therefore be an encouraging method of QQ.[17]

At least three important criteria are required for the development of an enzymatic antibiofilm QQ treatment: high catalytic efficiency, minimal immunogenicity, and soluble expression with low production cost. Currently, there is no such enzyme meeting all the requirements. Studies of a broad range of bacterial lactonases[18,19] and acylases[20,21] suggest they are highly active toward QSMs, *i.e.*, N-acyl homoserine lactones (AHLs), easy to express, and amenable to engineering.[22,23] However, the prokaryotic origin of these enzymes could very likely evoke adverse immune responses when administrated to humans. On the other hand, mammalian paraoxonases (PONs), named after their capability to detoxify organophosphate compounds,[24] can hydrolyze lactones of various modifications and carbon chain lengths.[25] Three members of the human PON family (huPON1−3) sharing ~60% sequence homology with highly similar secondary structures have been identified.[26] Among these, huPON2 exhibits the highest AHLs hydrolysis activities,[25] which was suggested as the native function of huPONs.[27,28] Mounting evidence suggests that mammalian PONs play an important role in quenching of bacterial QS.[4,17,29] For example, paraoxonase-1 transgenic flies were protected from *P. aeruginosa* lethality;[30] expression of PONs inversely correlated with *P. aeruginosa* infection in cystic fibrosis;[31] and PON2 deficiency enhanced *P. aeruginosa* QS in murine tracheal epithelia cells.[32]

However, mammalian paraoxonases are cell-membrane or high-density-lipoprotein (HDL)-associated enzymes[33−35] and thus are difficult to express in soluble forms.[33,36] Active huPON1 has been successfully produced by fermentation in *E. coli* with considerable yield.[37] Aiming to produce a more

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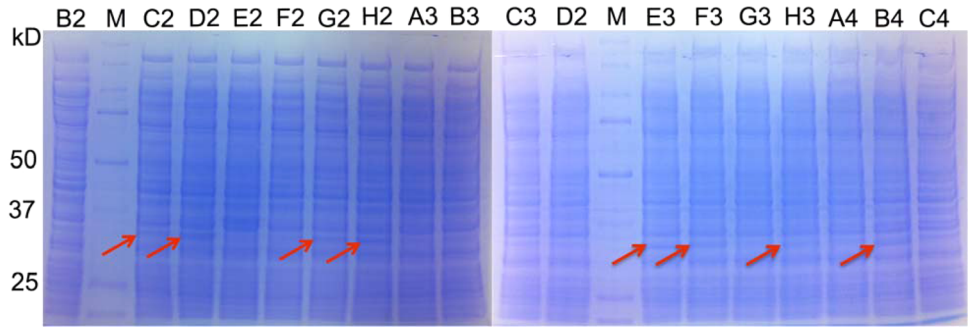

Figure 2. Identification of soluble expressed helix-free huPON2 variants. Whole cell lysates without purification were analyzed by SDS-PAGE to identify soluble expressed PON2 variants (arrowed) at the expected MW of 34.7 kDa. Totally, 96 colonies were tested. Results of remaining clones are shown in Supporting Figure 1.

soluble version of PON1, directed evolution was applied to shuffle human, rabbit, mouse, and rat PON1 genes, for the generation of soluble and functional chimeric PON1 recombinants,[38] one of which (G2E6) was crystallized for structure determination.[39] However, sequences of the resulting PON1 variants were more rabbit than human—91% identity to rabbit PON1 and dozens of amino acids different from human PON1. Further, since the locations of these mutations are scattered throughout the sequence, mainly on the surface of the enzyme, deimmunization of the hybrid PON1 variants, e.g., by B-cell or T-cell epitope removal,[40,41] would be significantly challenging. On the basis of structure analysis, huPON1 has been further engineered by substituting the hydrophobic residues of the HDL binding site with hydrophilic residues derived from the chimeric PON1s. This humanized PON1 variant demonstrated an improved solubility but still differed in a significant number of positions from huPON1.[42]

To avoid immunological complications, it would be desirable to have a protein therapeutic that is as close as possible to the native human sequence with minimum introduction of foreign or immuno-stimulating segments. In this study, we replaced the protruding hydrophobic helices of huPON2 with degenerate short peptide linkers. From the constructed linker library of limited design diversity, we isolated fully human PON2 variants exhibiting a high level of soluble expression. In addition to lactone hydrolysis activities toward a spectrum of QSMs found in clinically important pathogens, these engineered huPON2 variants were also biologically functional in *P. aeruginosa* swimming and swarming motility tests.

RESULTS AND DISCUSSION

Generation of Soluble Expressed huPON2 Variants. Because of the high similarity in secondary structures among mammalian PONs,[26] huPON2 was modeled[43] based on the crystal structure of rabbit-human hybrid PON1 G2E6 (PDB # 1V04),[39] which has 63.1% amino acid sequence identity to wild type huPON2. The modeling demonstrated that huPON2 is a six-bladed β-propeller with catalytic Ca^{2+} ions at the center and three helices (H1−H3) on the top of its propeller (Figure 1a). These helices are distanced from the reaction center and highly enriched with hydrophobic residues (shown as sticks in Figure 1a). Likely, these hydrophobic residues on protruding helices form a putative interface involved with HDL particle and cell membrane anchoring.[33,35] As a QQ agent to hydrolyze extracellular AHLs, engineered huPON2 will be presumably administered as a topical medication (i.e., for chronic wounds) or lung inhalant (i.e., for cystic fibrosis). Therefore, it is not necessary to localize therapeutic huPON2 on cell membranes or HDLs. Moreover, it is well accepted that extended hydrophobic patches on the protein surface promote aggregation and result in insolubility of recombinant proteins.[44] Notably, dozens of enzymes with propeller topology have been crystallized, and their structures suggest that most of propeller fold proteins do not possess extra helices outside of their main architecture.[45] Also, many of these proteins can be expressed in soluble form at high yields, *e.g.*, 200−300 mg/L of squid phosphotriesterases in *E. coli*.[46] On the basis of these considerations, we hypothesize that H1−H3 of huPON2 are not essential for its catalytic activity, and removal of these hydrophobic helices can substantially improve its solubility, a well documented protein engineering method for solubility improvement.[44,47]

To test this hypothesis, we modified huPON2 by removing its N-terminal H1 region (residue 17−37, LGERLLALRNRLKASREVESV), replacing the H2 region (residue 184−201, YFSDPFLKYLGTYLNLHW) with a degenerate tripeptide linker carrying a proline turn flanked by one flexible residue at both sides Gly/Ser-Pro-Gly/Ser, and replacing the H3 region (residue 288−298, QKLFVYDPNNP) with a flexible and hydrophilic degenerate dipeptide linker Asp/Asn/Ser/Gly-Asp/Asn/Ser/Gly (Figure 1b). The proline turn and the lengths of linkers replacing H2 and H3 were determined according to huPON2 structure modeling (Figure 1a). The total design diversity of the linker library was 64, which was encoded by associated degenerate codons (Supporting Table 1).

The helix-free huPON2 genes with degenerate linkers were constructed by DNA assembly, cloned in pET28b vector, and expressed for SDS-PAGE analysis (Figure 2). Given the small design diversity, 96 clones were randomly picked, and their cell lysates (clarified by centrifugation at 10 000*g* for 30 min) were analyzed without purification. Soluble expressed helix-free huPON2 variants were identified as bands with an expected MW of 34.7 kDa. Results indicated that 34 of 96 picked clones carried soluble expressed huPON2 variants (Supporting Figure 1). Particularly, clones D2 and E3 displayed strong bands associated with the helix-free huPON2, suggesting a high expression level (Figure 2). DNA sequencing revealed that the linkers of D2 and E3 were Gly-Pro-Gly and Ser-Pro-Gly for their H2 regions and Ser-Ser and Gly-Gly for their H3 regions, respectively. As polyhistidine-tag fusion proteins, D2 and E3 were soluble expressed with yields of 6.2 mg and 3.2 mg per liter of culture medium after purification (Supporting Figure 2).

To increase their soluble expression, D2, E3, and wild-type huPON2 were subcloned into the C-terminal of maltose

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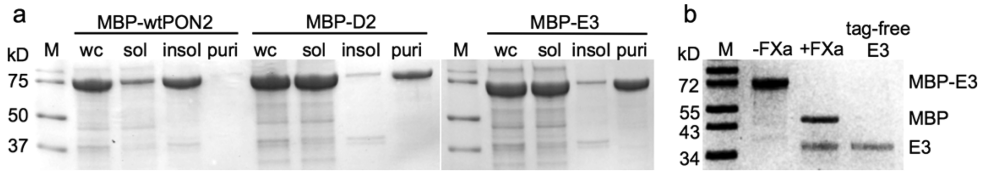

Figure 3. Production of soluble huPON2 variants D2 and E3. (a) The MBP fusion proteins were expressed in *E. coli* and purified by amylose resin chromatography. Samples of whole cell (wc), soluble cell lysate (sol), insoluble pellet (insol), and purified proteins (puri) were normalized based on cell density and loaded to gels. After purification, 320 mg of MBP-D2 and 200 mg of MBP-E3 were typically yielded per liter of culture media. (b) Production of tag-free E3. Samples of purified MBP-E3 fusion, cleaved by protease Factor Xa, and separated MBP-free E3 were analyzed by SDS-PAGE. A total of 76 mg of tag-free E3 was produced per liter of culture media.

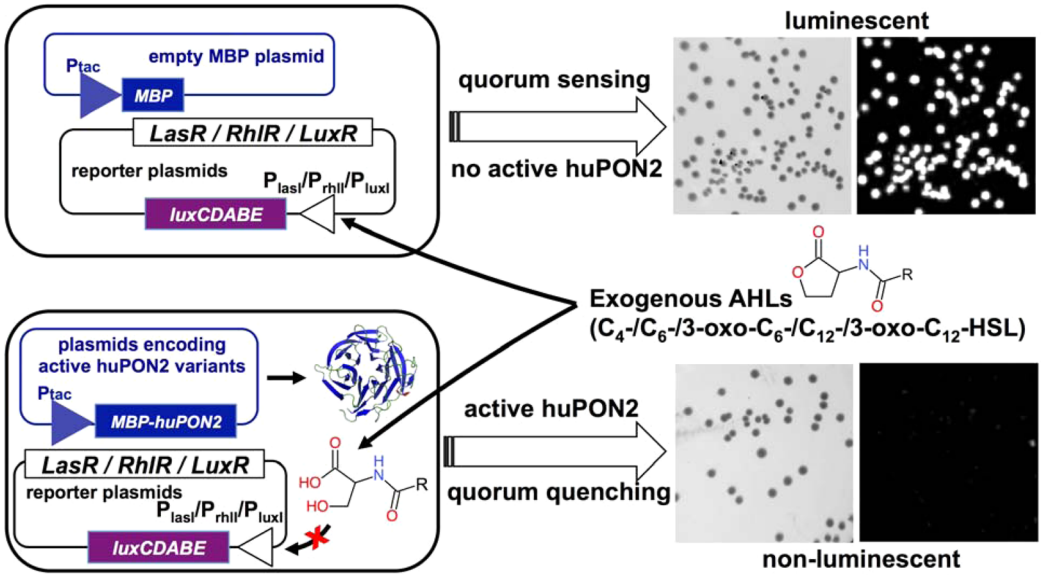

Figure 4. Characterization of lactone hydrolysis activity of D2 and E3 by bioluminescence assays. (Above) Exogenously added AHLs bind to regulator LasR/RhlR/LuxR and induce the expression of the luxCDABE cassette through and promoter $P_{lasI}/P_{rhlI}/P_{luxI}$, resulting in strong bioluminescence signals on agar plates. (Bottom) With the expression of active huPON2 variants, hydrolysis of AHLs leads to quorum quenching and low bioluminescence signals. Five lactones, N-butyryl-/N-hexanoyl-/N-(β-ketocaproyl)-/N-dodecanoyl-/N-3-oxo-dodecanoyl-L-homoserine, were tested in the assays.

binding protein (MBP). SDS-PAGE analysis of expression and purification profiles after amylose resin-based chromatography indicated that while the majority (90%) of MBP-wtPON2 was expressed in its insoluble form, almost all helix-free variants MBP-D2 and MBP-E3 were present in soluble fractions (Figure 3a). The typical after purification yields for MBP-D2 and MBP-E3 variants were 320 and 200 mg of protein per liter of culture medium, respectively. The enzymatic activity of purified MBP-E3 was measured using N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) as the substrate. The consumption of substrate and the production of hydroxycarboxylic acid N-(3-oxodecanoyl)-L-homoserine were monitored using HPLC (Supporting Figure 3). Results indicated that when 80 μM 3-oxo-C_{12}-HSL was used, MBP-E3 exhibited a specific activity of 0.16 μmol/min/mg of protein. We further measured the kinetics of MBP-D2 and MBP-E3 on AHL hydrolysis using Phenol Red as the pH indicator. The results showed that MBP-D2/E3 hydrolyzed a panel of AHLs having side chains ranging from C_4 to C_{12} in length with or without 3-oxo substituents, including C_4-, C_6-, 3-oxo-C_6-, and 3-oxo-C_{12}-HSLs (Supporting Table 2). Among tested AHLs, 3-oxo-C_{12}-HSL was the most efficient substrate for both MBP-D2 and MBP-E3 mainly due to its low K_m values. In addition, turnover rates of MBP-E3 in general were 1 or 2 orders of magnitude higher than these of MBP-D2, but considerably lower than G2E6.[28]

To attenuate chronic bacterial infections, repeated administrations are needed, therefore it is expected that a tag-free enzyme without the MBP component would be preferred over the fusion protein to avoid an adverse immune response. In order to produce MBP-free huPON2, purified MBP-E3 fusion was digested with protease Factor Xa, and E3 was separated from MBP by amylose resin chromatography. Per liter of culture medium, 76 mg of tag-free E3 was typically obtained (Figure 3b). Size exclusion chromatography analysis demonstrated that purified tag-free E3 was mainly a soluble monomer with smaller amounts of dimer and trimer (Supporting Figure 4), an observation consistent with the wild type huPONs recombinantly produced in mammalian cells.[25] Furthermore, CD spectra of purified tag-free E3 showed a broad minimum centered around 210 nm with a crossover point of 205 nm (Supporting Figure 5), in a good agreement with known β-fold proteins such as diisopropylfluorophosphatase.[46,48] And compared with wild type huPON1,[49] E3 has the negative peak shifted from 217 to 210 nm, indicating less composition of α-helices. The enzymatic kinetics of tag-free E3 was also measured toward C_4-HSL and C_6-HSL. The results (k_{cat} of 1.54 min^{-1} and 1.94 min^{-1} and K_m of 4.7 mM and 0.46 mM, respectively) suggested a similar specific activity to that of MBP-E3 (Supporting Table 2).

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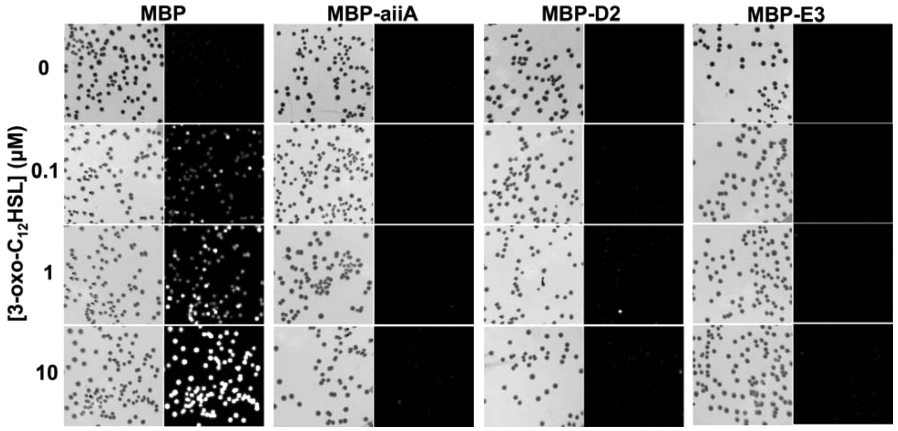

Figure 5. Quorum quenching activities of D2 and E3. LasR/P_{lasI} receptor system and its associated QSM 3-oxo-C_{12}HSL (0 to 10 μM) were used for the assays. Without active enzymes, bioluminescence signals increased at higher concentrations of AHL. With expression of D2 or E3, QS signals were completely quenched even at 10 μM of AHL. A highly active bacterial lactonase aiiA was included as the positive control. Locations of colonies are shown in the left columns.

Quorum Quenching Activity of Helix-free Soluble huPON 2s. QQ activities of soluble huPON2 variants D2 and E3 were examined by bioluminescence bioassay using reporter strains, which mimic the luxR-luxI type QS system of natural Gram-negative bacteria (Figure 4). Particularly, the host contained two plasmids—an enzyme plasmid encoding MBP-huPON2 fusion and a reporter plasmid carrying a QS regulator gene LasR/RhlR/LuxR and the complete luciferase operon luxCDABE under the control of the associated QS promoter $P_{lasI}/P_{rhlI}/P_{luxI}$.[50] When exogenously added, AHLs such as 3-oxo-C_{12}-HSL will freely transport into cells and bind with QS regulators and thus induce the expression of luxCDABE, which generates bioluminescence signals on agar plates. In the presence of active huPON2, AHLs will be hydrolyzed, resulting in no or low bioluminescence signals known as QQ.

As shown in Figure 5, helix-free huPON2 mutants D2 and E3 exhibited strong lactonase activity toward 3-oxo-C_{12}-HSL in the QQ bioassay. In the absence of AHLs, the background luminescence of all the clones was undetectable. Increasing the concentration (0.1−10 μM) of 3-oxo-C_{12}HSL generated a stronger luminescence signal as a result of the higher expression level of the luxCDABE gene cassette. However, when D2 or E3 was expressed (either with or without IPTG induction), the luminescence signal intensity was quenched to the background level even at 10 μM 3-oxo-C_{12}HSL. QQ activity was also confirmed with the positive control aiiA, a bacteria lactonase possessing a high activity toward a broad range of AHLs.[51]

Inhibition of *P. aeruginosa* Swimming and Swarming Motilities. At the early stage of biofilm formation, bacteria use their flagella to sense the environment and swim as a group.[52] It is well-known that syntheses of flagella, polysaccharides, and rhamnolipids are effected by QS, through both the direct regulation by the *rhl* system and the indirect regulation by the *las* system.[53] As a model strain for motility study in an aqueous environment, *P. aeruginosa* is commonly used for swimming assays, in which cells migrate away from the point of inoculation and form a concentric chemotactic ring. To test the effects of soluble huPON2 on swimming motility of *P. aeruginosa*, D2, E3, and aiiA genes were subcloned to a shuttle plasmid, and transformed into *P. aeruginosa* PAO1. After 24 h of culture at 37 °C, *P. aeruginosa* carrying the empty vector quickly spread out with an area of 14.1 ± 1.8 cm^2 and at 48 h covered the entire plates with a spreading zone area of 41.3 ± 3.3 cm^2 (Figure 6). However, when huPON2 mutants D2 or E3 were expressed, swimming motility was significantly attenuated

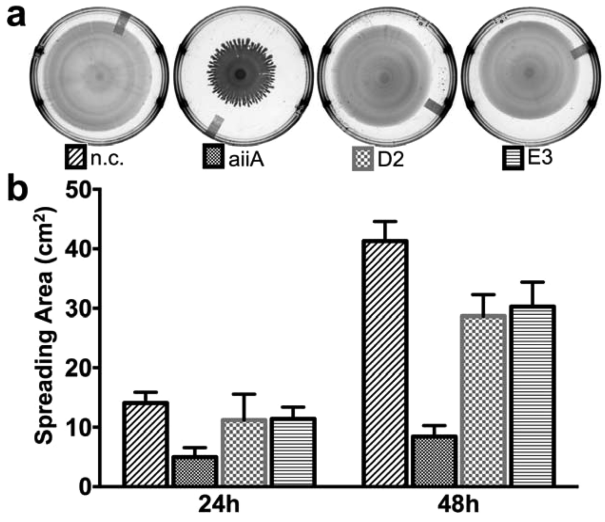

Figure 6. Inhibition effects of D2 and E3 on *Pseudonomas aeruginosa* PAO1 swimming motilities. Representative agar plates showing PAO1 spreading zones 48 h after inoculation. PAO1 cells were transformed with D2, E3, aiiA, or the vector plasmid (n.c.) (b) Quantitative measurements of spreading zone areas.

with spreading region areas, respectively, of 11.2 ± 4.4 cm^2 and 11.4 ± 2.0 cm^2 at 24 h and 28.7 ± 3.6 cm^2 and 30.2 ± 4.1 cm^2 at 48 h, which account for a ∼30% average decrease. As a positive control, *P. aeruginosa* expressing aiiA was tested in parallel and showed a ∼70% decrease of the spreading zone area, suggesting a stronger QQ bioactivity compared with engineered huPON2 variants. We further tested swimming motilities when 100 nM C_4-HSL and/or 3-oxo-C_{12}-HSL were present in agar to mimic the social behavior of *P. aeruginosa* colonies. Results showed that with exogenous AHLs, *P. aeruginosa* exhibited increased motility as expected. However, transformants with aiiA or E3 genes significantly reduced swimming motilities compared to the strain carrying the empty vector (Supporting Figure 6).

Bacteria exhibiting biofilm formation also display the feature of swarming, which is a specialized flagella-driven surface

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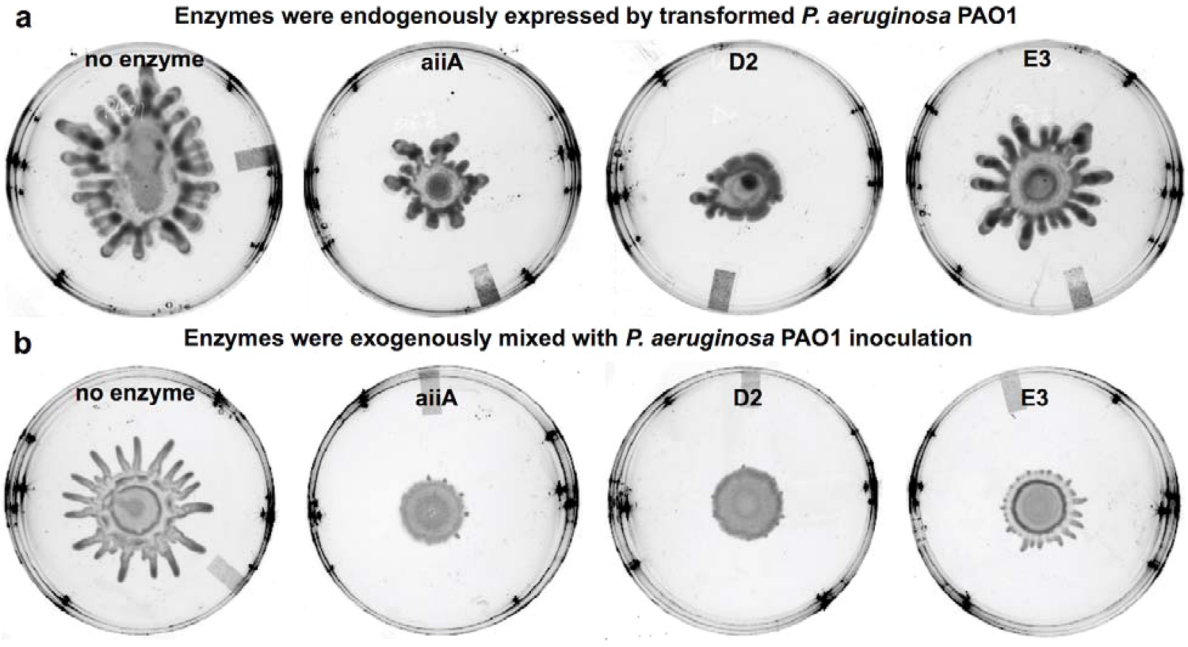

Figure 7. Reduction of *P. aeruginosa* PAO1 swarming motility with D2 or E3. Enzymes were either endogenously expressed by transformed POA1 (a) or exogenously added on agar plates (b).

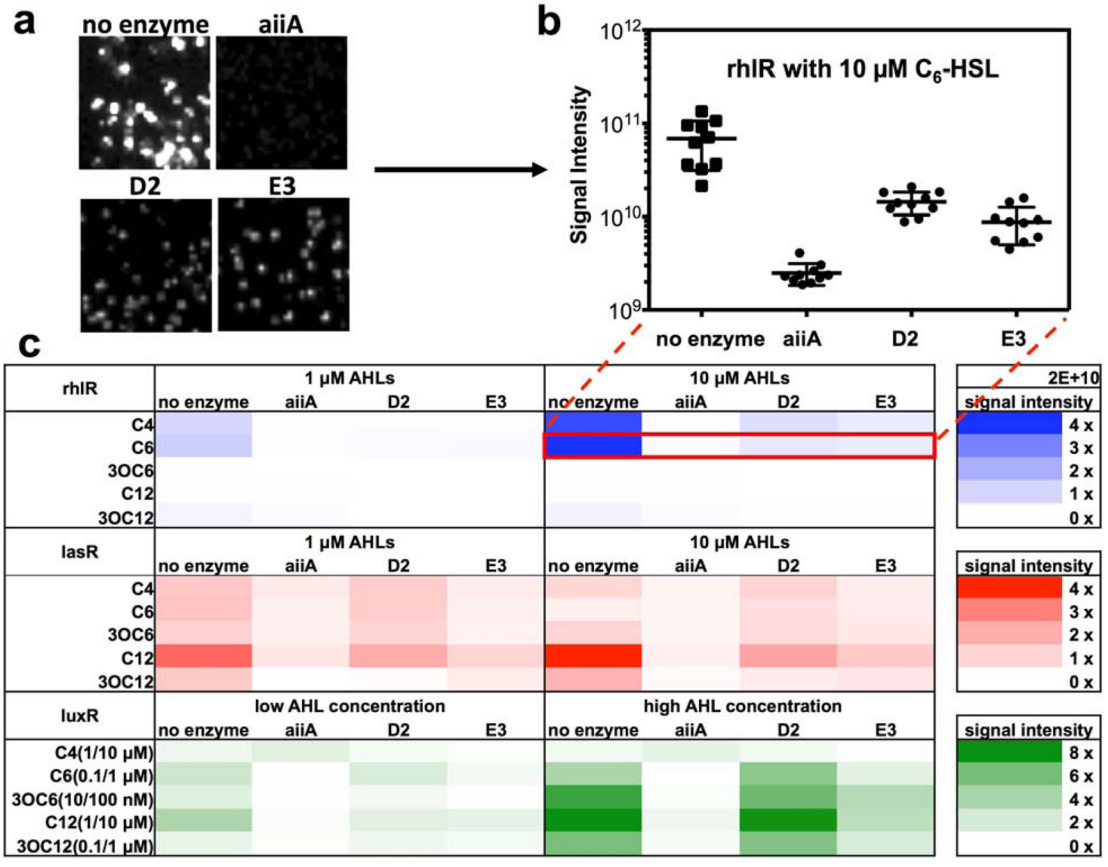

Figure 8. Quorum quenching analysis of D2 and E3 on different AHL-regulator combinations. Three quorum sensing signal receptors, rhlR, lasR, and luxR, and five AHLs at various concentrations were utilized for the analysis. D2, E3, and aiiA were under the control of a weak β-lactamase promoter to reduce their expression levels. (a) Colony bioluminescence images of agar plates for rhlR with 10 μM C_6-HSL are shown as an example. (b) Bioluminescence signal intensities of 10 representative colonies for each sample were randomly selected for quantitative analysis. (c) Heat maps of quorum quenching analysis on huPON2 variants with different AHL-regulator combinations.

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motility that bacteria employ on semisolid surface under nitrogen limiting conditions.[54] Swarming cells express two polar flagella, which can facilitate bacteria movement by providing more propulsion,[55] and thus gain a competitive advantage in searching for nutrient-rich environments. In addition, production of surfactant rhamnolipid is fundamental for the swarming motility to facilitate insoluble hydrocarbon biodegradation and reducing surface tension.[56] Because both flagella and rhamnolipid synthesis are regulated by the *rhl* system, swarming motility is closely related to QS as well.[57] We evaluated the effect of endogenous D2 or E3 expression on *P. aeruginosa* swarming motility. After 48 h of incubation at 37 °C, surface areas covered by swarming were measured. Results indicated that cells transformed with empty plasmid (as a negative control) freely spread across the media surface exhibiting typical dendritic-like patterns (Figure 7a). In contrast, the swarming motility was inhibited in *P. aeruginosa* clones carrying huPON2 variants, with 76% and 42% reduction of spreading zones for D2 and E3, respectively. As expected, *P. aeruginosa* transformed with aiiA also reduced *P. aeruginosa* swarming motility by 70%, a similar efficacy compared with D2. In therapeutic applications, the engineered AHL hydrolyzing enzymes will likely be topically administrated. Therefore, it will be more relevant to test motilities when D2 or E3 was exogenously added to culture medium rather than endogenously expressed. For this test, a PAO1 strain that pronounces significant branch formations was used.[58] Results demonstrated that when 720 μg of MBP-D2 or 520 μg of MBP-E3 were applied on agar plates during inoculation, *P. aeruginosa* swarming motilities were significantly obstructed or completely abolished (Figure 7b).

Promiscuous Hydrolysis Activities of Soluble huPON2. Because QS systems play important roles in numerous pathogenic bacteria for their survival from host immune responses, many NIAID category A, B, and C priority pathogens are associated with QS. As one of the major signaling molecules for Gram-negative bacteria, AHLs thus serve as an excellent group of targets to attenuate chronic infections. However, a broad range of AHLs with different 3′ modifications and side chain lengths are utilized in a variety of pathogens. For example, in *Yersinia pestis* (plague) the predominant AHLs are 3-oxo-C_8-HSL and 3-oxo-C_6-HSL synthesized by gene YspI,[59] and in *Burkholderia pseudomallei* (melioidosis) at least three luxI and five luxR homologues are involved in its pathogenicity, and several signaling molecules including C_8-/C_{10}-/OH-C_8-/OH-C_{10}-/3-oxo-C_{14}-HSLs are generated.[60]

To evaluate the effects of engineered soluble huPON2 variants D2 and E3 on different AHLs and the associated QS receptors, we tested five AHLs including C_4-HSL, C_6-HSL, 3-oxo-C_6-HSL, C_{12}-HSL, and 3-oxo-C_{12}-HSL with a combination of three receptors *P. aeruginosa* rhlR (C_4-HSL as its natural QSM), *P. aeruginosa* lasR (3-oxo-C_{12}-HSL as its natural QSM), and *Vibrio fischeri* luxR (3-oxo-C_6-HSL as its natural QSM; Figure 4).[50] *E. coli* JLD271 carrying one of the reporter plasmids was transformed with an enzyme plasmid encoding MBP-D2, MBP-E3, or MBP-aiiA (included as the positive control). The empty vector encoding the MBP tag alone was also transformed into the QS reporter cells as the negative controls. These double transformants were cultured on LB-Amp-Tet plates supplemented with different AHLs at variable concentrations. After 16 h of incubation, bioluminescence signal images were taken (Figure 8a), and 10 colonies from each sample were randomly selected for quantitative analysis. As demonstrated in Figure 8b, high luminescence signals represent strong QS and low luminescence signals represent QQ and thus high lactone hydrolysis activities. The collected data for all AHL-regulator combinations were then compiled to generate heat maps. Initially, when MBP fusion proteins were cloned following a strong promoter P_{tac}, all the QQ effects of D2, E3, and aiiA were significantly strong, and signals were indistinguishable from each other even at high concentrations of AHLs, i.e. 10 μM of 3-oxo-C_{12}-HSL (Supporting Figure 7). To better characterize huPON2 mutants by increasing the response range, P_{tac} was replaced by a weak and constitutive β-lactamase promoter P_{bla}. SDS-PAGE verified an approximately 20-fold reduced expression level for MBP-D2 under P_{bla} (Supporting Figure 8) and thus making it more suitable to evaluate huPON2 mutants.

The overall results of QQ analysis of D2 and E3 toward different AHL-regulator combinations are shown as heat maps in Figure 8c. Briefly, in the absence of lactonase, receptor rhlR strongly responded to its natural QSM C_4-HSL and non-natural QSM C_6-HSL at the same level, but C_{12}-HSL and 3-oxo-C_{12}-HSL were not able to stimulate the rhlR-mediated QS and resulted in low luminescence signals. In contrast, lasR and luxR exhibited AHL preferences to C_{12}-HSL with moderate response to 3-oxo-C_6-HSL and 3-oxo-C_{12}-HSL and low response to C_4-HSL and C_6-HSL. Also as expected, the higher AHL concentrations produced the stronger luminescence signals. In the presence of huPON2 variants D2 or E3, luminescence signals were potently quenched in general. Importantly, D2 and E3 exhibited lactone hydrolysis activities toward all five AHLs tested. Their catalytic promiscuity is not surprising given that wild type huPON1−3s can hydrolyze a broad range of substrates including lactones, organophosphates, aryl esters, etc.[28] Notably, E3 was more effective than D2 on QQ for most AHL-receptor combinations tested. Since D2 was usually expressed ∼40% more than E3, these results suggested E3 exhibited a higher specific activity than D2, consistent with kinetics measurements (Supporting Table 2). As a positive control, expression of bacterial lactonase aiiA gave very low luminescence signals and effectively eliminated QS for all five AHLs, in excellent agreement with a previous report.[18] Collectively, the bioluminescence systems demonstrated here can be used for evaluation of lactonases and pave the way for further selection of huPON2 variants with high AHL hydrolysis activities targeting QSM-regulator combinations of biomedical importance.

Conclusion. In this study, huPON2 was engineered for soluble expression with minimal introduction of foreign sequences. Both a rational approach based on structure modeling and a combinatory approach of degenerate linker design are exploited for removing the hydrophobic helices without disruption of the folding structure and thus retaining the function of huPON2. High soluble expression levels were achieved with yields of 76 mg of fully human PON2 variants per liter of culture media. Particularly, two clones, D2 and E3 were characterized for their quorum quenching bioactivities. Results demonstrated that D2 and E3 effectively inhibited *P. aeruginosa* swimming and swarming motilities. In addition, using the reporter strains to correlate QQ with luminescence signal readouts, quantitative analysis of QQ toward natural or unnatural AHL-regulator combinations was performed, which allowed us to better evaluate huPON2 variants for the desired lactonase activities toward pathogens of interest. Compared

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with bacterial lactonase aiiA, the engineered soluble huPON2 mutants D2 and E3 exhibited relatively moderate catalytic activities of lactone hydrolysis. For the generation of therapeutic QQ human enzymes with required high turnover rates, directed evolution by random and site-directed mutagenesis followed by bioluminescence-based screening is currently undertaken. Moreover, the technique of engineering highly soluble huPON2 described in this paper can be readily applied for huPON1, which exhibits organophosphate hydrolysis activities, for use in environmental bioremediation of pesticides and detoxication of nerve agents.

METHODS

Design and Construction of the huPON2 Library. Wild type human paraoxonases 2 (huPON2) sequence (GenBank AAC41995.1) and the crystal structure of a hybrid mammalian PON1 derived from directed evolution (PDB 1V04)[39] were used for the design of the huPON2 library of this study. Twenty-five primers (Supporting Table 1) encoding the huPON2 library were designed using DNAWorks and chemically synthesized. Three segments of huPON2 genes were PCR assembled to construct full-length huPON2 by overlapping PCR. The resulting fragment (904 bp) was gel purified and cloned between NheI and XhoI sites on pET28b to encode N-terminal His-tag fused huPON2 variants. The ligated vectors were transformed into *E. coli.* Jude-1 (DH10B harboring the "F" factor derived from XL1-blue) cells and the quality of the constructed library were verified by DNA sequencing.

Selection of Soluble Expressed huPON2 Variants. BL21 (DE3) cells were transformed with huPON2 library plasmids, and 96 colonies were randomly picked and cultured. A total of 200 μL of Terrific Broth supplemented with 50 μg/mL kanamycin was inoculated with 2 μL of overnight seed cultures, and protein expression was induced with 0.2 mM IPTG at RT for 16 h. Cells were harvested by centrifugation at 4000*g* for 15 min at 4 °C and resuspended in 200 μL of PBS. After sonication and centrifugation, 20 μL cell lysates of all 96 picked clones were analyzed using 12% SDS-PAGE gels to examine soluble huPON2 bands at the expected 34.7 kDa.

Cloning, Expression, and Purification of MBP Fused huPON2 Variants. Assembled genes of wild type huPON2 and selected mutants D2 and E3 were PCR amplified with primers xl39 and xl40 and cloned between *Bam*HI and NcoI sites on pMAL-c5x (NEB). MBP-PON2 fusion proteins were expressed in *E. coli* BL21 (DE3) using LB media supplemented with 1 mM $CaCl_2$. Expression was induced with 0.2 mM IPTG at OD_{600} of 0.5−0.6. After culturing at RT for 16 h, cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$), and lysed by sonication. Cell lysates were clarified by centrifugation at 10000*g* for 30 min. MBP-huPON2 fusion proteins were captured by an amylose resin column (NEB) and washed with column buffer, and MBP-huPON2 proteins were eluted with column buffer supplemented with 10 mM maltose. The eluted fractions containing fusion proteins were dialyzed overnight at 4 °C against 20 mM Tris-HCl at pH 7.4, 5 mM NaCl, and 1 mM $CaCl_2$. Purified protein samples were aliquoted in 20% glycerol and stored at −80 °C. The gene of lactonase *aiiA* was amplified from genomic DNA of *B. thuringiensis* 4A3 (Bacillus Genetic Stock Center) with primers xl28 and xl29 and cloned into pMAL-c5x. The MBP-aiiA expression and purification procedure was similar to that of MBP-huPON 2s, except 0.5 mM $CoCl_2$ was used in all buffers instead of 1 mM $CaCl_2$.

Lactonase Activity Assays. Hydrolysis of acyl-homoserine lactones was detected using HPLC by measuring the amount of substrates consumed. Then, 0.4 μM purified MBP-huPON2 variants were preinoculated in 5 mM Tris-HCl (pH 7.4) and 1 mM $CaCl_2$ at 37 °C for 1 min, and reactions were initiated by adding a 1% volume of the 8 mM 3-oxo-C_{12}HSL solution (in methanol) and incubated at 37 °C for various times. Reactions were stopped with an equal volume of acetonitrile, and the mixtures were vortex mixed and centrifuged to pellet the precipitated proteins. Then, 100 μL supernatants were injected to a HPLC system equipped with a ZORBAX Eclipse XDB-C18 column (Agilent Technologies, 150 × 4.6 mm, 5 μm particles) and a UV/visible detector set at 205 nm. Samples were eluted isocratically with water/acetonitrile/formic acid (32:68:0.03 [vol/vol/vol]) at 1 mL/min. The retention time for 3-oxo-C_{12}HSL was around 1.9 min. Assay buffer without enzymes and purified bacterial lactonase aiiA MBP fusion protein (MBP-aiiA) were used as negative and positive controls, respectively, in the enzyme assays. A pH-sensitive colorimetric assay was performed using an Epoch microplate reader. The reactions (200 μL final volume) contained 1 mM HEPES at pH 8.0, 25 mM NaCl, 1 mM $CaCl_2$, 0.002% (w/v) Phenol Red, and 10 μL of purified enzymes. Reactions were initiated with 2 μL of substrates stock solution in methanol, and absorbance decreases at 558 nm were monitored for 3−10 min.

Preparation and Characterizations of Tag-Free E3. MBP-E3 was cleaved by incubating with Factor Xa (1 mg per 50 mg of fusion protein) at RT for 12 h. The cleaved mixture was dialyzed against 20 mM Tris-HCl and 25 mM NaCl at pH 7.4, then loaded to amylose resin. The flowthrough was saved for SDS-PAGE and following analysis. Size exclusion chromatography experiments were performed on an ÄKTAprime plus system using 20 mM Tris-HCl at pH 7.4, 100 mM NaCl, and 1 mM $CaCl_2$ as the column buffer. Newly digested and purified tag-free E3 was load onto a Superdex 75 10/300 GL column (GE Healthcare) for gel filtration at a flow rate of 0.5 mL/min. The CD spectra of purified E3 were recorded at 25 °C using a Jasco J-815 CD spectrophotometer with a 0.1 mm path length cell. The bandwidth was set at 1 nm, and scans were obtained between 190 and 260 nm with wavelength increments of 1 nm. A total of 1 mg mL^{-1} of tag-free E3 in 20 mM Tris-HCl at pH 7.4, 25 mM NaCl, and 1 mM $CaCl_2$ was measured. Secondary structure content was analyzed with the online CAPITO server.[61] The data used for graphical presentation and analyses were an average of eight different scans.

Bioluminescence Quorum Sensing Bioassay. The promoter regions on plasmids pMAL-D2/-E3/-aiiA were replaced with a weak β-lactamase promoter P_{bla} by Gibson assembly. The P_{bla} gene was amplified with primer xl50, xl51, xl52, and xl63. Constructed pMAL-bla-huPONs plasmids were transformed to an sdiA-deficient *E. coli.* strain JLD271 carrying one of the cognate receptors: rhlR (C_4HSL), lasR (3-oxo-C_{12}HSL), or luxR (3-oxo-C_6HSL).[50] Single-colony transformants were cultured overnight at 37 °C, in LB media supplemented with 100 μg/mL ampicillin and 20 μg/mL tetracycline. Inoculations were diluted and plated on LB/Amp/Tet Agar plates with 0.1−10 μM C_4HSL, C_6HSL, 3-oxo-C_6HSL, C_{12}HSL, or 3-oxo-C_{12}HSL. After incubation at 37 °C overnight, bioluminescence signals were detected using a ChemiDoc imager (Bio-Rad) with justified exposure times based on signal intensity. Luminescence signals of individual colonies were then quantified using Fiji software.[62] More specifically, each colony region was selected as the region of interest (ROI), and the integrated density values were measured for at least 10 colonies per sample, for calculating mean values of luminescence signals.

***P. aeruginosa* PAO1 Swarming and Swimming Motility Tests.** Genes of huPON2 mutants D2 and E3 and *aiiA* were subcloned to a broad-host vector pBBR1MCS4 at XhoI and XbaI sites.[63] After confirmation by DNA sequencing, *P. aeruginosa* PAO1 competent cells were transformed with the plasmids[64] and cultured at 37 °C overnight in LB media supplemented with 200 μg/mL carbenicillin. Swimming motility was tested on 10 g/L tryptone, 5 g/L NaCl, and 0.3% (w/v) agar plates with 5 μL inoculation of overnight cultures and incubation at 37 °C for 24−48 h. The mean areas of the swimming motility zones were measured from three replicates, and the error bars represent the standard deviations. In swarming motility tests, 0.5% (w/v) agar plates containing 8 g/L nutrient broth (Becton, Dicknson and Company) and 5 g/L D-glucose were dried for 1 h and then inoculated with 2.5 μL overnight cultures on the center of the agar surface. The areas of the swarming motility zones were measured after incubation at 37 °C for 48 h.

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■ ASSOCIATED CONTENT

Supporting Information

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Supporting Tables 1 and 2 and supporting Figures 1−8 (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Engineering Soluble Human Paraoxonase 2 for Quorum Quenching

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Supporting Table 1. Oligonuceotides used in this paper

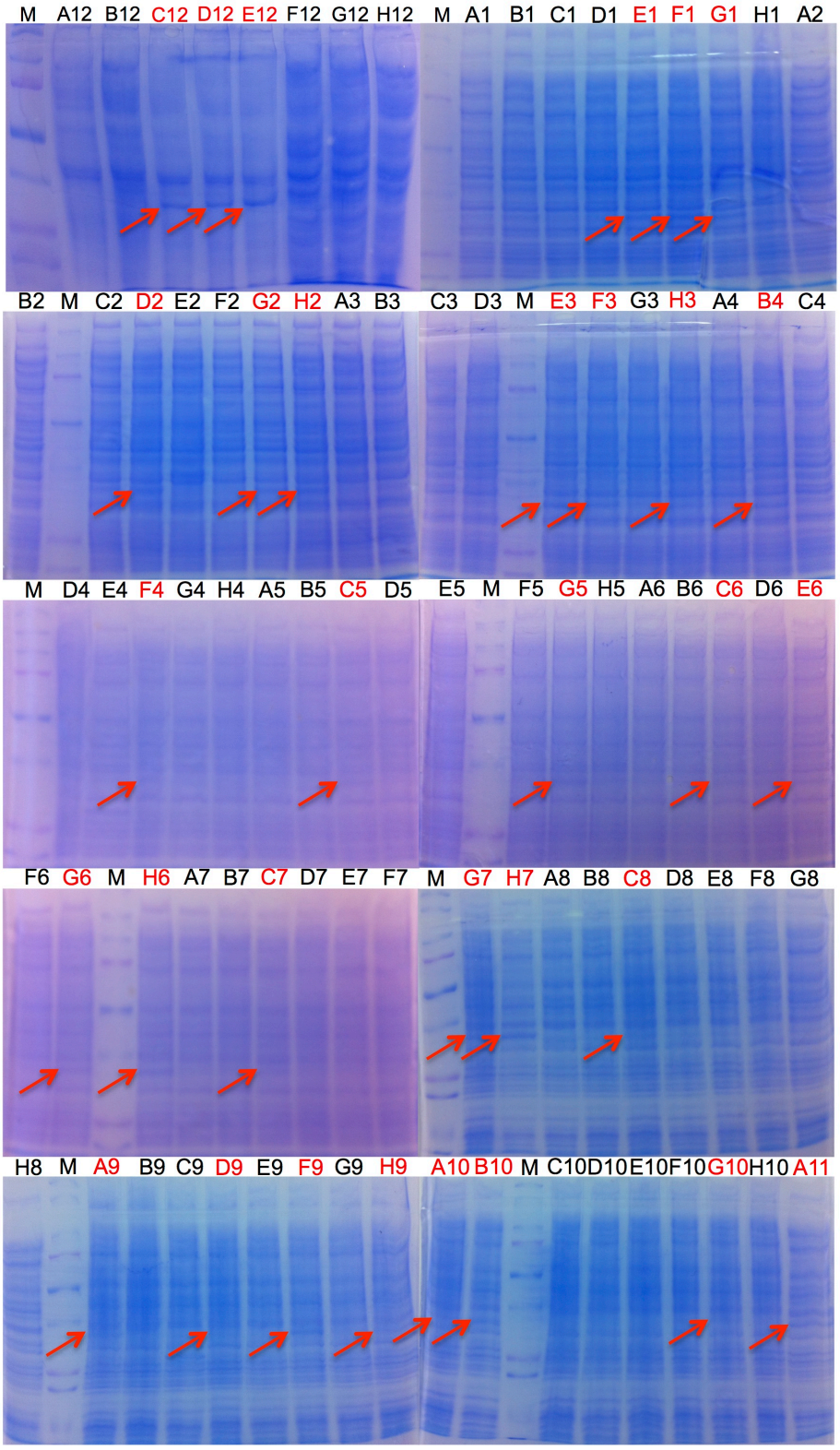
Name	Sequence
PON2a1[1]	CGTACGCTAGCGACCTGCCGCACTGCCATCTGATCAAAGGTA
PON2a2[1,2]	CGTTCGGCAGAATGTCGATGTCTTCAGAACCCGCTTCGATACCTTTGATCAGATGGCAGT
PON2a3[1,2]	ATCGACATTCTGCCGAACGGTCTGGCGTTCTTCTCTGTTGGTCTGAAATTCCCGGGTCTG
PON2a4[1,2]	TCATCAGGATACCACCCGGTTTGTCCGGTGCGAAAGAGTGCAGACCCGGGAATTTCAGAC
PON2a5[1,2]	CCGGGTGGTATCCTGATGATGGACCTGAAGGAAGAAAAACCGCGTGCGCGTGAACTCCGT
PON2a6[1,2]	ATACCGTGAGGATTGAAAGACGCCAGGTCGAAACCACGAGAGATACGGAGTTCACGCGCA
PON2a7[1,2]	CGTCTTTCAATCCTCACGGTATCTCCACCTTCATCGATAACGACGACACCGTTTACCTGT
PON2a8[1,2]	GATTTCAACCGTGTTCTTAAATTCAGGGTGGTTAACAACGAACAGGTAAACGGTGTCGTC
PON2a9[1,2]	TGAATTTAAGAACACGGTTGAAATCTTCAAATTCGAAGAAGCGGAAAACTCTCTGCTGCA
PON2a10[1,2]	CGTTAACAGACGGCAGCAGTTCGTGTTTAACGGTTTTCAGGTGCAGCAGAGAGTTTTCCG
PON2a11[1,2]	TGCTGCCGTCTGTTAACGACATCACCGCAGTTGGTCCGGCGCACTTCTACGCGACCAACG
PON2a12[1]	GGAGAGTAGTAAACAACGTTCGCGCYCGGGCYGTGGTCGTTGGTCGCGTAGAAGTG
PON2b1[1]	GCGAACGTTGTTTACTACTCTCCGAACGAAGTTAAAGTTGTAGCGGAAGGTTTCG
PON2b2[1,2]	TATTTGTCGTCCGGAGAGATGTTGATGCCGTTCGCAGAGTCGAAACCTTCCGCTACAACT
PON2b3[1,2]	CATCTCTCCGGACGACAAATACATCTACGTTGCGGACATCCTGGCGCACGAAATCCACGT
PON2b4[1,2]	CACTTTCAGCTGGGTCAGGTTCATGTTGGTGTGTTTTTCCAGAACGTGGATTTCGTGCGC
PON2b5[1,2]	CCTGACCCAGCTGAAAGTGCTGGAACTGGACACCCTGGTTGACAACCTGTCTATCGACCC
PON2b6[1]	ACCGTTCGGGTGGCAACCAACCCAGATGTCACCAGAAGACGGGTCGATAGACAGGTTGTC
PON2c1[1]	CCGTCTTCTGAAGTTCTGCGTATCCAGAACATCCTGTGCGAAAAACCGA
PON2c2[1,2]	CTGCAGAACAGAACCGTTGTTCGCGTAAACGGTGGTAACGGTCGGTTTTTCGCACAGGAT
PON2c3[1,2]	ACAACGGTTCTGTTCTGCAGGGTTCTTCTGTTGCGTCTGTTTACGACGGTAAACTGCTGA
PON2c4[1,2]	CAGTTCGCAGTACAGCGCACGATGGTACAGGGTACCGATCAGCAGTTTACCGTCGTAAA
PON2bc[1]	GGTTGCCACCCGAACGGTRRCRRCCCGTCTTCTGAAGTTCTGCG
PON2c5[1]	GCGCTGTACTGCGAACTGTAACTCGAGCATGC
PON2c6[1]	GCATGCTCGAGTTACAGTTCGCAGTACAGCGCACG
xl28[3]	GTACCTCGGGGAAAACCTGTATTTTCAGGGAATGACAGTAAAGAAGCTTTATTTCATCCC
xl29[3]	GGACTGAATTCTATATATTCCGGGAACACTCTACAACTC
PON1H1[2]	CTGCCCATGGGTGCGTGGGTTGGTTGTGGCCTGGCTGGTGACCGTGCCGGCTTTTTGGG
PON1H2[2]	GATTACGAAGGGCGAGGAGACGTTCTCCCAAAAAGCCGGCACGG
PON1H3[2]	CTCCTCGCCCTTCGTAATCGTCTTAAAGCGAGCCGCG
PON1H4[2]	GGCAGGTCTACGCTTTCAACTTCGCGGCTCGCTTTAAGAC
PON1H5[2]	GTTGAAAGCGTAGACCTGCCTCACTGCCACCTGATCAAAGGTAT
PON2A12RE[2]	CAGATAGGTACCCAGGTACTTCAGGAACGGATCGCTGAAGTAGTGGTCGTTGGTCGCGTA
PON2H1[2]	GTACCTGGGTACCTATCTGAACTTACACTGGGCGAACGTT
PON2H2[2]	CTTCGTTCGGAGAGTAGTAAACAACGTTCGCCCAGTGTAAG
PON2H3[2]	GTTTACTACTCTCCGAACGAAGTTAAAGTTGTTGCGGAAGGTTT
PON3H1[2]	GTTGCCACCCGAACGGTCAGAAATTATTTGTCTATGACCCGAATAACCCG
PON3H2[2]	CGCAGAACTTCAGAAGACGGCGGGTTATTCGGGTCATAG
PON2CZ[2]	GCGCTGTACTGCGAACTGTAAGGATCCCTAG
PON2CZ_NEW[2]	CTAGGGATCCTTACAGTTCGCAGTACAGCGCACG
xl39[4]	CAGTCCATGGACCTGCCGCACTGCCATC
xl40[4]	CTAGGGATCCTTACAGTTCGCAGTACAGCGCAC
xl65[5]	ACGTCTCGAGGATCCATATGACAGTAAAGAAGCTTTATTTCATCCC
xl66[5]	GGACTTCTAGATTATATATATTCCGGGAACACTCTACAACTC
xl50[6]	GGACCCAACGCTGCCCGAAATTCCTGCGTTTCTACAAACTCTTTCGGTCCGTTG
xl51[6]	GAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAACGGACCGAAAGAGTTTGTAG
xl52[6]	CTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATA
xl63[6]	TTCTTCGATTTTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTG

[1] For gene assembly of huPON2 mutants.
[2] For gene assembly of wild type huPON2.
[3] For gene amplification of lactonase aiiA from genomic DNA of *Bacillus thuringiensis* 4A3.
[4] For gene cloning of D2 and E3 to pMAL-c5x.
[5] For gene cloning of aiiA to pBBR1MCS4.
[6] For gene cloning of D2 and E3 to pBBR1MCS4.

Supporting Table 2. Specific lactonase activities of MBP-D2 and MBP-E3.

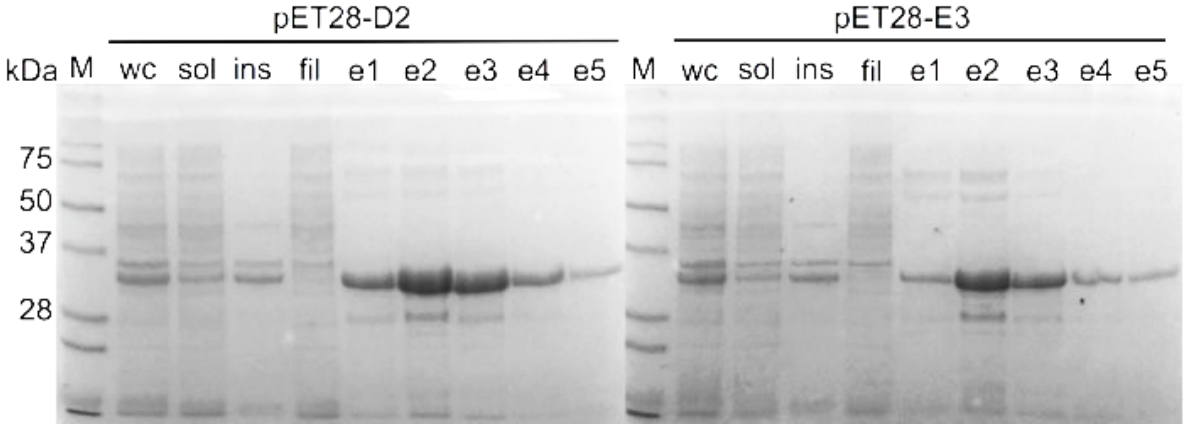
	Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}-min^{-1})
MBP-E3	C_4HSL	1.28	2.45	1.91
	C_6HSL	0.49	2.24	4.57
	3-oxo-C_6HSL	1.29	1.63	1.26
	3-oxo-C_{12}HSL	0.15	1.22	8.13
MBP-D2	C_4HSL	2.53	0.30	0.12
	C_6HSL	2.70	0.22	0.08
	3-oxo-C_6HSL	4.57	0.20	0.04
	3-oxo-C_{12}HSL	0.12	0.23	1.95

Supporting Figure 1:



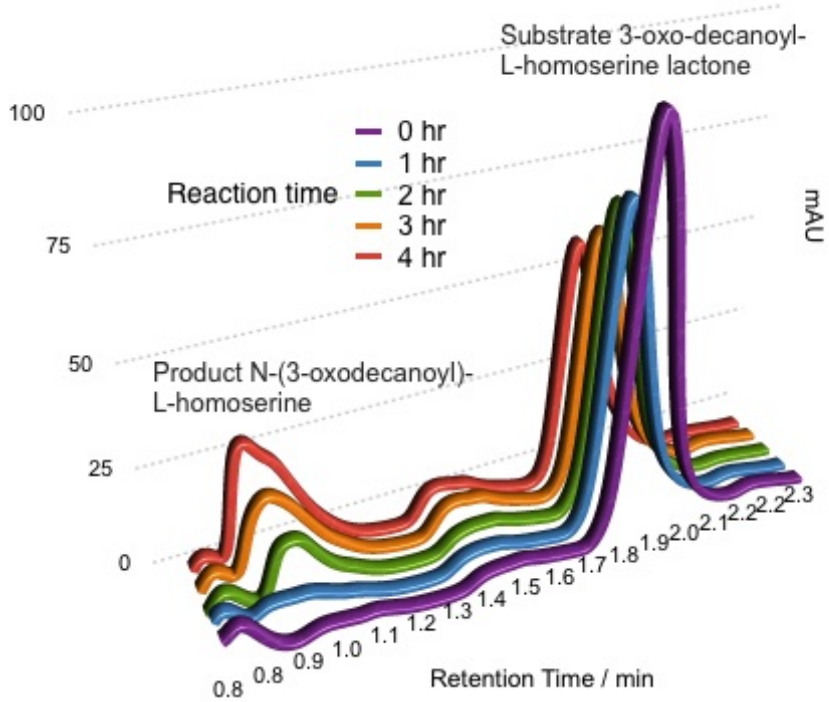
Supporting Figure 1. Identification of soluble huPON2 variants by SDS-PAGE. Supplementary to the results shown in **Figure 2**, totally 34 out of 96 randomly picked clones carried soluble expressed huPON2 variants.

Supporting Figure 2:



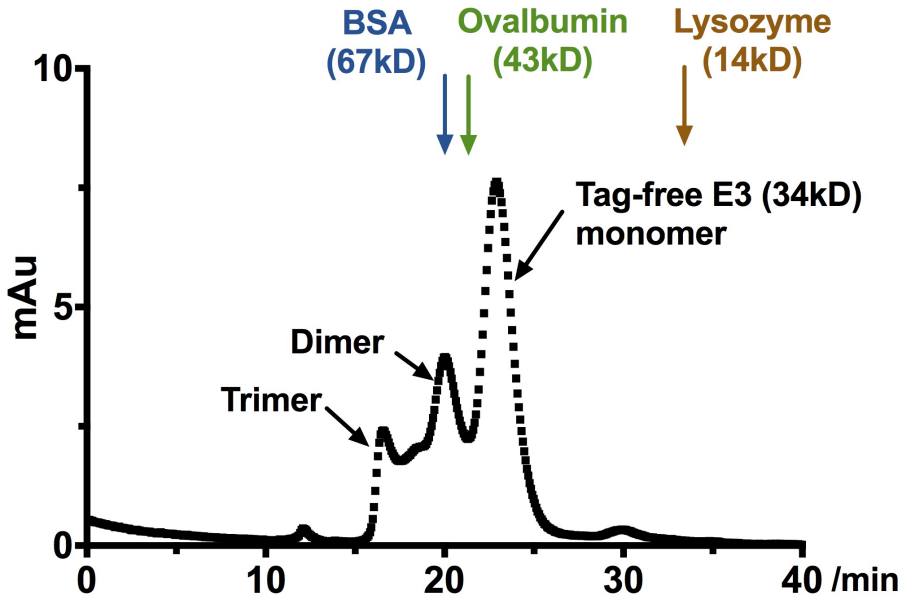
Supporting Figure 2. Expression and purification of soluble huPON2 variants D2 and E3. Samples included whole cell (wc), soluble fraction (sol), insoluble fraction (ins), filtrate through His-tag resin columns (fil), and elution fractions from the columns (e1-e5).

Supporting Figure 3:



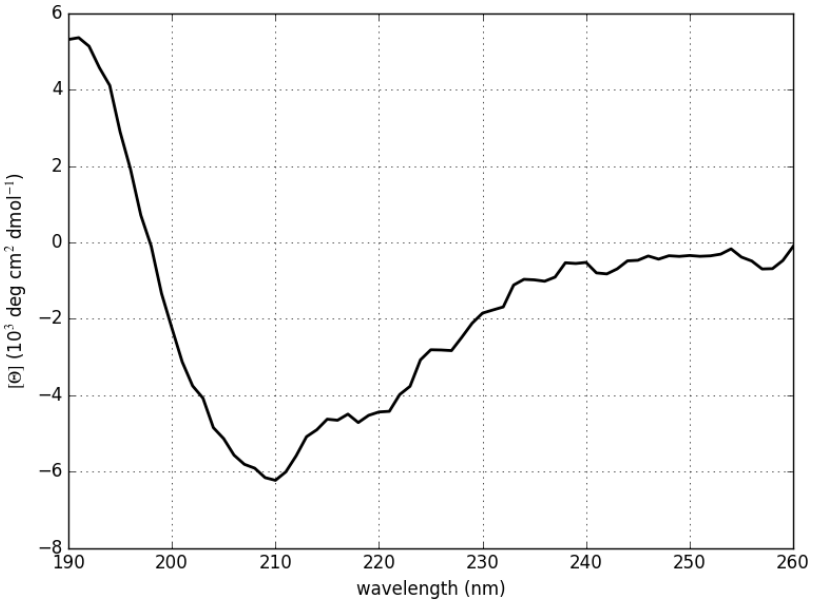
Supporting Figure 3. AHL hydrolysis activity of MBP-E3 measured by HPLC. 3-oxo-C_{12}-HSL was used as the substrate (with a retention time of 1.9 min) and the associated hydroxycarboxylic acid N-(3-oxodecanoyl)-L-homoserine (with a retention time of 0.9 min) was produced. Background auto-hydrolysis of AHLs without presence of enzymes was subtracted for specific activity calculations.

Supporting Figure 4:



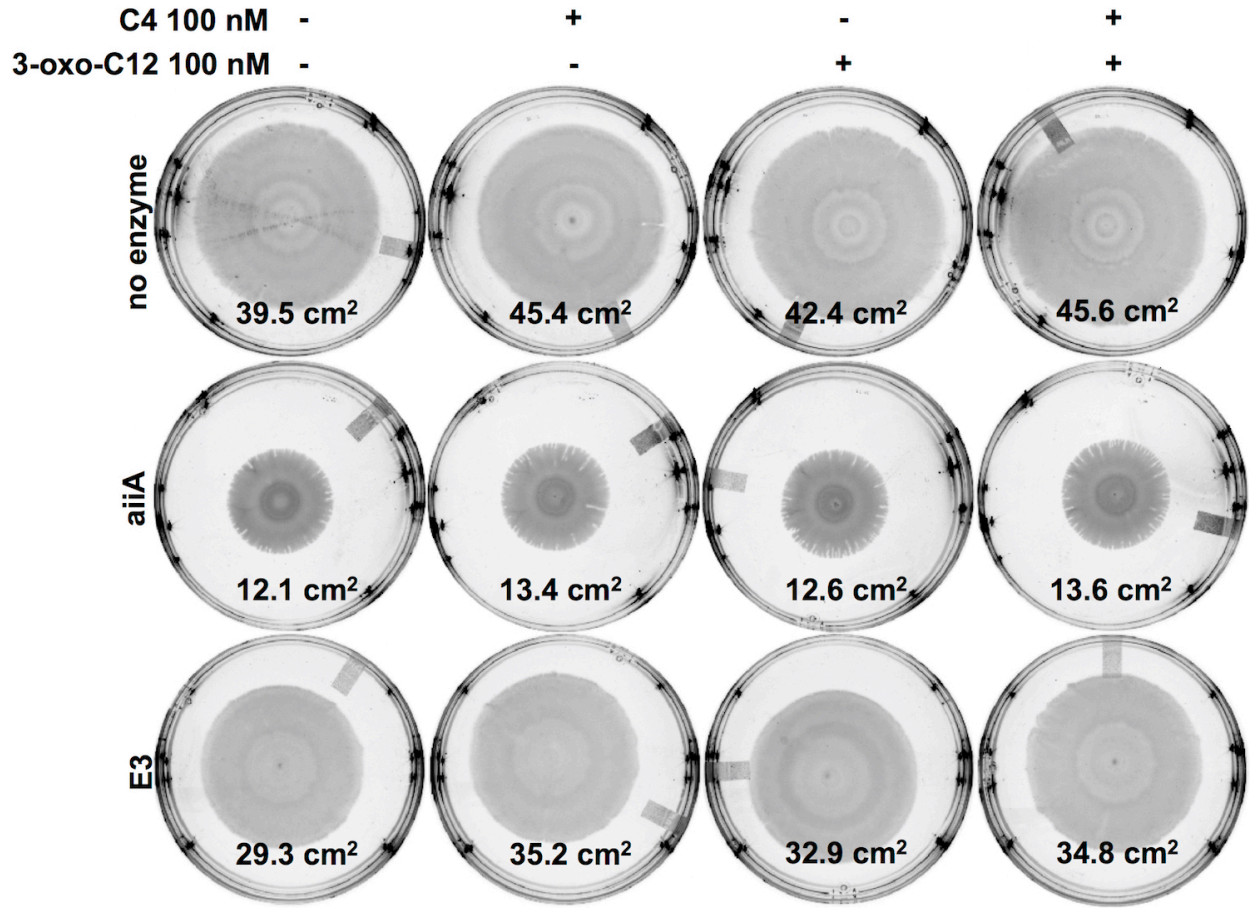
Supporting Figure 4. Size exclusion chromatography of purified tag-free E3 (MW= 34kDa). 103 μg of E3 in column buffer (20 mM Tris-HCl pH7.4, 200 mM NaCl, 1 mM $CaCl_2$) was loaded to Superdex 75 10/300 GL column at a flow rate of 0.5 mL/min. The retention times of 22.5 min (monomer) and 19.5 min (dimer) indicate tag-free E3 is a soluble protein without aggregation. E3 trimmer was also observed, consistent with the results of wt huPONs produced by recombinant mammalian cells.[25] Standard proteins and their retention times are also shown.

Supporting Figure 5:



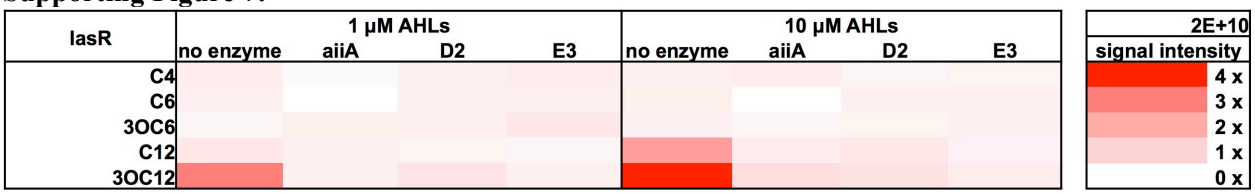
Supporting Figure 5. CD spectra of purified tag-free E3. A broad negative peak at 210 nm with a crossover point of 205 nm is in a good agreement with known β-fold proteins such as diisopropylfluorophosphatase.[46, 48]

Supporting Figure 6:



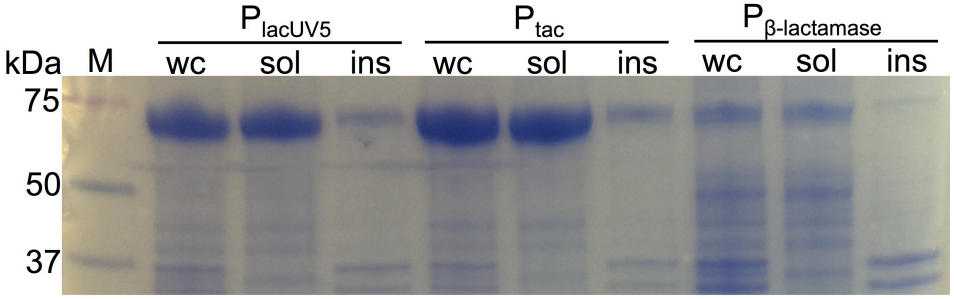
Supporting Figure 6. E3 mediated swarming motility reduction in the presence of exogenous AHLs. Enzymes were endogenously expressed by transformed *P. aeruginosa* PAO1. Adding 100 nM C4 or/and 100 nM 3-oxo-C12 in agar plates promoted swimming motilities by 13-20%. However, transformants with aiiA or E3 genes significantly reduced swimming motilities compared to the strain carrying an empty vector. Colony spreading areas were measured and the values were shown for each agar plate in the pictures.

Supporting Figure 7:



Supporting Figure 7. Quorum quenching analysis of MBP-huPON2 variants when expressed at the downstream of a strong promoter P_{tac}.

Supporting Figure 8:



Supporting Figure 8. SDS-PAGE analysis of MBP-D2 expression levels under the control of promoters P_{lacUV5}, P_{tac} or P_{bla}. P_{lacUV5} and P_{bla} resulted in approximately 3- and 20-folds lower expression than P_{tac}.