Enzyme and Microbial Technology 160 (2022) 110092

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Applying molecular and phenotypic screening assays to identify efficient quorum quenching lactonases

Raphaël Billot [a,b], Laure Plener [a], Damien Grizard [a,c], Mikael H. Elias [d], Éric Chabrière [b], David Daudé [a,*]

[a] Gene&GreenTK, 19-21 Boulevard Jean Moulin, 13005 Marseille, France
[b] Aix Marseille Univ, IRD, APHM, MEPHI, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France
[c] Proxis Développement, Levallois-Perret, France
[d] Department of Biochemistry, Molecular Biology and Biophysics – BioTechnology Institute, University of Minnesota, St. Paul, MN, United States

ARTICLE INFO

Keywords:
Quorum sensing
Quorum quenching
Lactonase
Acyl homoserine lactones
Enzyme, engineering
Enzyme screening

ABSTRACT

Quorum sensing (QS) is a molecular communication system used by microorganisms to adopt behaviors in a cell density-dependent manner. Lactonase enzymes, able to hydrolyze the signal molecules acyl-homoserine lactones (AHL) can counteract QS-mediated virulence in Gram-negative bacteria. Optimizing lactonases activity or specificity for AHL through enzyme engineering approaches is thus highly attractive to increase protective effect. However, only a limited number of screening methods have been developed to handle and evaluate AHL-degrading enzyme libraries. Here, a series of screening procedures were developed to identify improved lactonases using two previously reported enzymes as benchmarks, namely *Sso*Pox and *Gc*L. Specifically, molecular screenings using six different AHL and based on two reporter strains; i.e., *Chromobacterium violaceum* CV026 and *Pseudomonas putida* KS35, are reported. In addition, three phenotype-based screenings aiming to evaluate the ability of enzymes to quench a particular QS-related behavior are reported, using *C. violaceum*, *Pseudomonas aeruginosa* and *Vibrio harveyi* as pathogenic type strains. These assays were used to screen a small-sized library and allowed for the identification of various improved variants. To confirm that these variants were real "hits", four of them were produced and purified. Their kinetic parameters against AHL substrates were found to be increased by 2–44.5 -fold as compared to the starting enzyme. Moreover, their increased activity was confirmed by measuring their ability to quench QS in different bacterial systems. These new assays will facilitate the screening of enzyme libraries and will pave the way for the development of proficient engineered QS-disrupting enzymes.

1. Introduction

Quorum sensing (QS) is a cell-to-cell communication process used by bacteria to regulate gene expression in a cell density-dependent manner. QS relies on the release, detection and response to small diffusible signal molecules, referred to as autoinducers (AI). Once the concentration of AI in the environment increases, the expression of numerous genes is activated, leading to various phenotypes, including production of virulence factors and antibiotics, formation of biofilm, or resistance to antimicrobials [1]. In Gram-negative bacteria, acyl-homoserine lactones (AHL) are commonly used as AI [2]. Various AHL molecules, differing in their acyl chain lengths and/or substitutions, may be used alone or in combination, depending on the bacteria [3,4].

Considering the crucial involvement of QS in bacterial virulence, strategies aiming at interfering with this communication process have gained considerable interest, particularly in regard to the global emergence of antibiotic resistance across bacterial species. New sustainable strategies against bacteria are urgently needed, and quorum quenching (QQ) approaches, aiming at disrupting QS by interfering with AI signals while not challenging bacterial survival, are highly appealing. Different QQ approaches were developed based on chemical inhibitors that compete with AI, AI-sequestering antibodies or AI-degrading enzymes [5]. The latter are particularly promising, considering their tremendous catalytic potential that allows efficiently inactivating signal molecules.

AHL-inactivating enzymes have been widely studied, mainly lactonases (EC 3.1.1.81), phosphotriesterase-like-lactonase (PLL, EC 3.1.8.1),

* Corresponding author.
E-mail address: david.daude@gene-greentk.com (D. Daudé).

https://doi.org/10.1016/j.enzmictec.2022.110092
Received 1 March 2022; Received in revised form 27 June 2022; Accepted 28 June 2022
Available online 1 July 2022

Table 1
Kinetic parameters of reference enzymes: k_{cat}/K_M ($M^{-1}.s^{-1}$). * Data taken from *Hiblot* et al.[14]. • Data taken from *Bergonzi* et al.[12]. ⁘ Data taken from *Rémy* et al.[9]. All other data are from this study. ND: activity not detected.

AHL	*Sso*Pox 5A8	*Sso*Pox V82I	*Sso*Pox W263I	*Gc*L
C4	ND	1.02 (± 0.17) x 10^2	4.61 (± 1.22) x 10^1	8.31 (± 2.19) x 10^4 •
C6	ND	2.15 (± 0.24) x 10^3	4.58 (± 0.48) x 10^2	1.12 (± 0.27) x 10^6 •
3-oxo-C6	ND	2.35 (± 0.25) x 10^2	4.34 (± 1.79) x 10^1	1.64 × 10^4
3-oxo-C8	ND	1.39 (± 0.19) x 10^3	9.69 (± 1.35) x 10^2	4.31 (± 0.76) x 10^6 •
3-oxo-C10	ND	2.91 (± 0.81) x 10^4	3.74 (± 1.17) x 10^2 *	3.7 × 10^6
3-oxo-C12	ND	5.01 (± 1.31) x 10^3	1.01 (± 0.28) x 10^5 *	1.1 (± 0.1) x 10^5 ⁘

acylases (EC 3.5.1.97), oxidoreductases (EC 1) and esterases (EC 3.1.1.1). These enzymes vary in their ability to interact with AHL, albeit enzymes belonging to esterases, lactonases and PLL families share esterase activity that catalyzes the opening of the lactone moiety through hydrolysis. These lactone hydrolases have been investigated in the past two decades and have been shown to disrupt QS of various Gram-negative bacteria, including human, animal and plant pathogens [6–8]. Moreover, recent studies have underlined that their specificity to AHL (i.e., their capacity to interact preferentially with one AHL or another) may drive different QQ effects in bacteria that can use more than one AHL-based signal [9,10]. Tailoring the specificity of AHL-degrading enzymes is thus challenging in controlling their anti-virulence effect. Moreover, increasing the activity of enzymes for the degradation of AHL is of utmost interest in developing cost-effective solutions that could constitute sustainable and environmentally-friendly alternatives to conventional antimicrobials such as antibiotics and biocides.

Engineering strategies have been used to improve the various properties of lactone-degrading enzymes, including their range of activity, catalytic efficiencies, or solubility, with the goal of enhancing their biotechnological potential [7]. To further harness the potential of QQ enzymes for specific applications, the combination of enzyme engineering approaches with efficient screening procedures suitable for large libraries is required [11]. In this work, we optimized several molecular and phenotypic screening assays that can be implemented to detect proficient lactonases in a rapid, simple, and effective way. Two approaches were combined: (i) molecular screening, involving AHL-reporter strains, which can evaluate exogenous synthetic AHL degradation by the tested enzymes and (ii) phenotypic screening, directly reporting on the impact of the enzymes on an AHL-based, QS-dependent phenotype. Two previously described lactonases belonging to distinct families and exhibiting different AHL substrate specificities were used as benchmarks to develop these screenings. On the one hand, three variants from the lactonase *Sso*Pox, isolated from the extremophilic archaeon *Sacharrolobus solfataricus* and belonging to the phosphotriesterase-like lactonase (PLL) family were selected: *Sso*Pox 5A8 is an inactive mutant used as a negative control in our assays; *Sso*Pox V82I has average catalytic efficiency values ranging from 10^2 to 10^4 $M^{-1}.s^{-1}$ against the tested AHL molecules, and *Sso*Pox W263I exhibits improved activity on 3-oxo-C12 HSL. On the other hand, *Gc*L, isolated from another thermophilic bacterium, *Parageobacillus caldoxylosilyticus*, and belonging to the metallo-β-lactamase (MBL) family, was chosen for its broad substrate specificity and catalytic efficiencies against AHL molecules (10^4 to 10^6 $M^{-1}.s^{-1}$) [12]. The measured kinetic parameters for these enzymes towards six AHL with different acyl chain length and functionalization, representative of a large number of QS system, are displayed in Table 1.

These screening assays were further utilized to evaluate a small-sized library of variants generated by mutating the *Sso*Pox enzyme. This robust enzyme was previously shown to have considerable biotechnological potential, in regards to its extreme resistance to various harsh conditions and processes [13]. Increasing *Sso*Pox efficacy to AHL while maintaining its stability is thus highly appealing for developing concrete QQ-based applications. Through an alanine-scanning approach, 15 residues located on loop 8, a loop involved in substrate recognition [14], were mutated, and their impact on AHL specificity and QQ performance was evaluated. (Fig. 1). Improved variants with 2–45-fold increase in catalytic efficiency were obtained, confirming the sensitivity of the screening procedures. The ability of the screened mutants was further evaluated in vitro to confirm their enhanced QQ activity. This strategy can be used to develop proficient QQ enzymes and may pave the way for the development of enhanced biocatalysts able to replace or strengthen the use of antimicrobials against infections.

2. Materials and methods

2.1. Chemicals

All enantiomerically pure acyl-homoserine lactones were purchased from COGER and resuspended in dimethyl sulfoxide (DMSO) at a concentration of 200 mM.

2.2. Alanine scanning

An alanine scanning library targeting 15 of 17 residues of *Sso*Pox

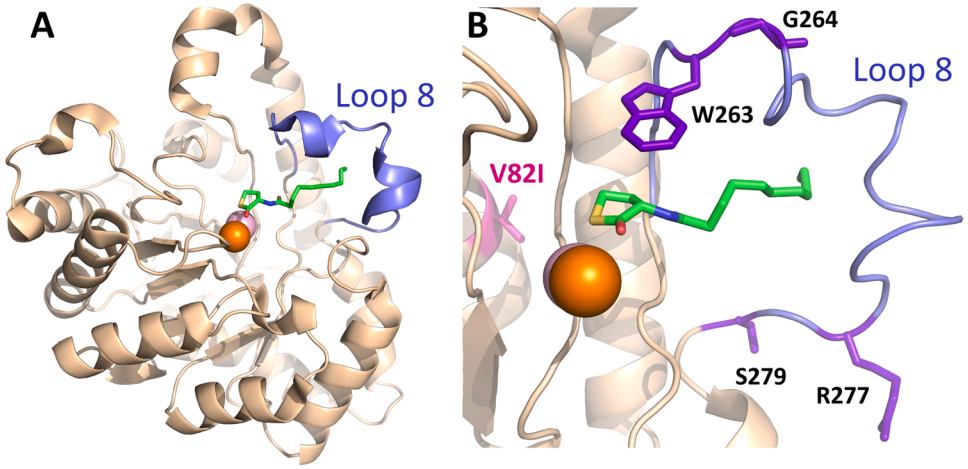

Fig. 1. *Sso*Pox crystal structure (PDB 2VC7 [15]). **(A)** Crystal structure of *Sso*Pox WT bound to N-Decanoyl-L-homocysteine thiolactone (C10-HTL). **(B)** The binding cleft of the *Sso*Pox lactonase enzyme. Loop 8 is shown in blue cartoon mode (A) or loop mode (B). The bound substrate mimic is shown as green (for carbon atoms) sticks. Heterobinuclear cobalt/iron is shown as orange and pink spheres, respectively. The key discussed positions, W263, G264, R277 and S279 are shown in purpleblue sticks. The position for V82I mutation is shown as pink sticks.

loop 8, namely W263, G264, T265, K267, P268, E269, Y270, K271, P272, K273, L274, P276, R277, W278 and S279, was synthesized by GenScript, using site directed-mutagenesis onto a pET-22b plasmid containing the *Sso*Pox V82I gene.

2.3. Enzyme lysate production

Plasmids were transformed into *Escherichia coli* strain BL21(DE3)-pGro7/GroEL (TaKaRa). Cells were grown on a 96-well plate in 1 mL of ZYP5052 medium as previously described [14]. Production of proteins and chaperones was induced after five hours of culture at 37 °C by reducing the temperature to 25 °C and adding $CoCl_2$ (0.2 mM) and arabinose (0.2 % (w/v)). After overnight growth, cells were pelleted by centrifugation (3500 rpm, 20 min) and resuspended in 250 μL of lysis buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 0.2 mM $CoCl_2$, 0.25 mg.mL^{-1} lysozyme, 0.1 mM PMSF and 10 μg.mL^{-1} DN*Ase*I) and were stored at − 80 °C. Cells were thawed and debris was removed by centrifugation (3500 rpm, 20 min). The supernatant was then recovered, transferred to a 96-well plate, and stored at − 20 °C.

2.4. Molecular screenings

2.4.1. C. violaceum CV026

CV026 was pre-cultivated in LB for 8 h at 30 °C under agitation at 350 rpm. The pre-culture was diluted 1/1000 in LB medium, supplemented with C4, C6 or 3-oxo-C6 HSL at concentrations ranging from 10 to 100 μM, and the latter was split into a 96-well plate. An enzymatic lysate plate was thawed prior to the experiment and lysates were added to the wells in determined concentrations. The final volume was 200 μL per well. A screening plate was incubated at 30 °C and violacein production was monitored by scanning the screening plate after 16 h growth.

2.4.2. P. putida KS35

P. putida KS35 was pre-cultivated in LB supplemented with 50 μg.mL^{-1} kanamycin for 16 h at 30 °C under 350 rpm agitation [16]. The pre-culture was then diluted 1/100 in LB medium supplemented with the chosen concentration of 3-oxo-C8, 3-oxo-C10 or 3-oxo-C12 HSL, as well as kanamycin (50 μg.mL^{-1}). A homogenized mix was then split into a 96-well plate. An enzymatic lysate plate was thawed prior to the experiment and lysates were added to the wells in determined concentrations. The final volume was 200 μL per well. A screening plate was then incubated at 30 °C for 8 h and fluorescence was measured using a micro-plate reader (Synergy HT, BioTek, USA) and the Gen5.1 software, with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Fluorescence values were later normalized by OD 600 nm for each well.

2.5. Phenotypic screenings

2.5.1. Pseudomonas aeruginosa (Pa14)

Pa14 was pre-cultivated in LB for 8 h at 37 °C under agitation at 350 rpm. The pre-culture was diluted 1/1000 in MOPS minimal medium complemented with nitrogen (15 mM NH_4Cl), iron (5 μM Fe_2SO_4), phosphate (4 mM K_2HPO_4) and glutamate (25 mM) as carbon source (MOPS glutamate)[17], and then split into a 96-well plate. An enzymatic lysate plate was thawed prior to the experiment and lysates were added to the wells in determined concentration. The final volume was 200 μL per well. A screening plate was then incubated at 37 °C for 20 h. The plates were centrifuged (1500 g, 20 min, 10 °C) and pyocyanin production was measured in supernatants at OD 690 nm.

2.5.2. Chromobacterium violaceum

C. violaceum 12472 was pre-cultivated in LB for 8 h at 30 °C under agitation at 350 rpm. The pre-culture was diluted 1/1000 in LB. A homogenized mix was then split into a 96-well plate. An enzymatic lysate plate was thawed prior to the experiment and lysates were added to the wells in determined concentration. The final volume was 200 μL per well. A screening plate was then incubated at 30 °C and violacein production was monitored by scanning the screening plate after 16 h growth. Pictures were processed with ImageJ software: gray levels of each well were measured to obtain a quantitative value.

2.5.3. Vibrio harveyi

V. harveyi DSM19623 was pre-cultivated in LB for 16 h at 30 °C under agitation at 350 rpm. The pre-culture was diluted 1/500 in AB medium (0.3 M NaCl, 0.05 M $MgSO_4$, and 0.2 % vitamin free casamino acids (Difco), supplemented with 200 μL of sterile 1 M potassium phosphate (pH 7.0), 200 μL of 0.1 M L-arginine, and 250 μL of Glycerol 80 % for a final volume of 20 mL) [18]. The homogenized mix was then split into a 96-well plate. An enzymatic lysate plate was thawed prior to the experiment and lysates were added to the wells in determined concentration. The final volume was 200 μL per well. A screening plate was then incubated at 30 °C for 9 h and bioluminescence was monitored with Fusion FX (Vilber-Lourmat) with 90-second exposure. Gray levels of each well were measured with ImageJ software to obtain a quantitative value.

2.6. Production and purification of SsoPox variants

Productions were performed using the *E. coli* BL21(DE3)-pGro7/GroEL (TaKaRa) chaperone expressing strain. Starter cultures were produced in an auto-inducible ZYP medium (supplemented with 100 μg.mL^{-1} ampicillin and 34 μg.mL^{-1} chloramphenicol). When the OD 600 nm reached a value of 0.8–1, $CoCl_2$ was added (final concentration 0.2 mM) as well as L-arabinose (final concentration 2 g.L^{-1}) to induce the production of chaperones GroEL/ES, and the temperature was decreased to 23 °C for 16–20 h. Cells were harvested by centrifugation (4400 g, 4 °C, 20 min) and resuspended in a lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 0.2 mM $CoCl_2$, 0.25 mg.mL^{-1} lysozyme, 0.1 mM PMSF and 10 μg.mL^{-1} DNase I) and were stored at − 80 °C. Cells were thawed and lysed in three steps of 30 s of sonication (Qsonica, Q700; Amplitude 45). Cell debris was removed by centrifugation (20,000 g, 10 °C, 15 min). As *Sso*Pox and its variants are hyperthermostable, a pre-purification step was performed by heating the lysate for 30 min at 80 °C. Precipitated host proteins were removed by centrifugation (20,000 g, 10 °C, 15 min). *Sso*Pox and its variants were collected by ammonium sulfate precipitation (75 %) and resuspended in 8 mL of activity buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 0.2 mM $CoCl_2$). The remaining ammonium sulfate was eliminated by injection on a desalting column (HiPrep 26/10 desalting, GE Healthcare; ÄKTA Avant) and concentrated to 2 mL for separation on exclusion size chromatography (HiLoad 16/600 Superdex TM 75 pg, GE Healthcare; ÄKTA Avant). Final purity was monitored by SDS-PAGE, and the protein concentration was measured with Bradford assay [19].

2.7. Lactonase activity

The time course for hydrolysis of lactones was performed in lactonase buffer (2.5 mM Bicine pH 8.3, 150 mM NaCl, 0.2 mM $CoCl_2$, 0.25 mM Cresol purple and 0.5 % DMSO) as previously described [20,21], over a concentration ranging from 0 to 2 mM. Cresol purple (pKa 8.3 at 25 °C) is a pH indicator used to follow lactone ring hydrolysis at 577 nm ($\varepsilon = 2923\ M^{-1}.cm^{-1}$) by acidification of the medium. For all experiments, each point was made in triplicate and Gen5.1 software was used to evaluate the initial velocity at each lactone concentration. Mean values were fitted to the Michaelis-Menten equation using Graph-Pad Prism 7 software to obtain the catalytic parameters.

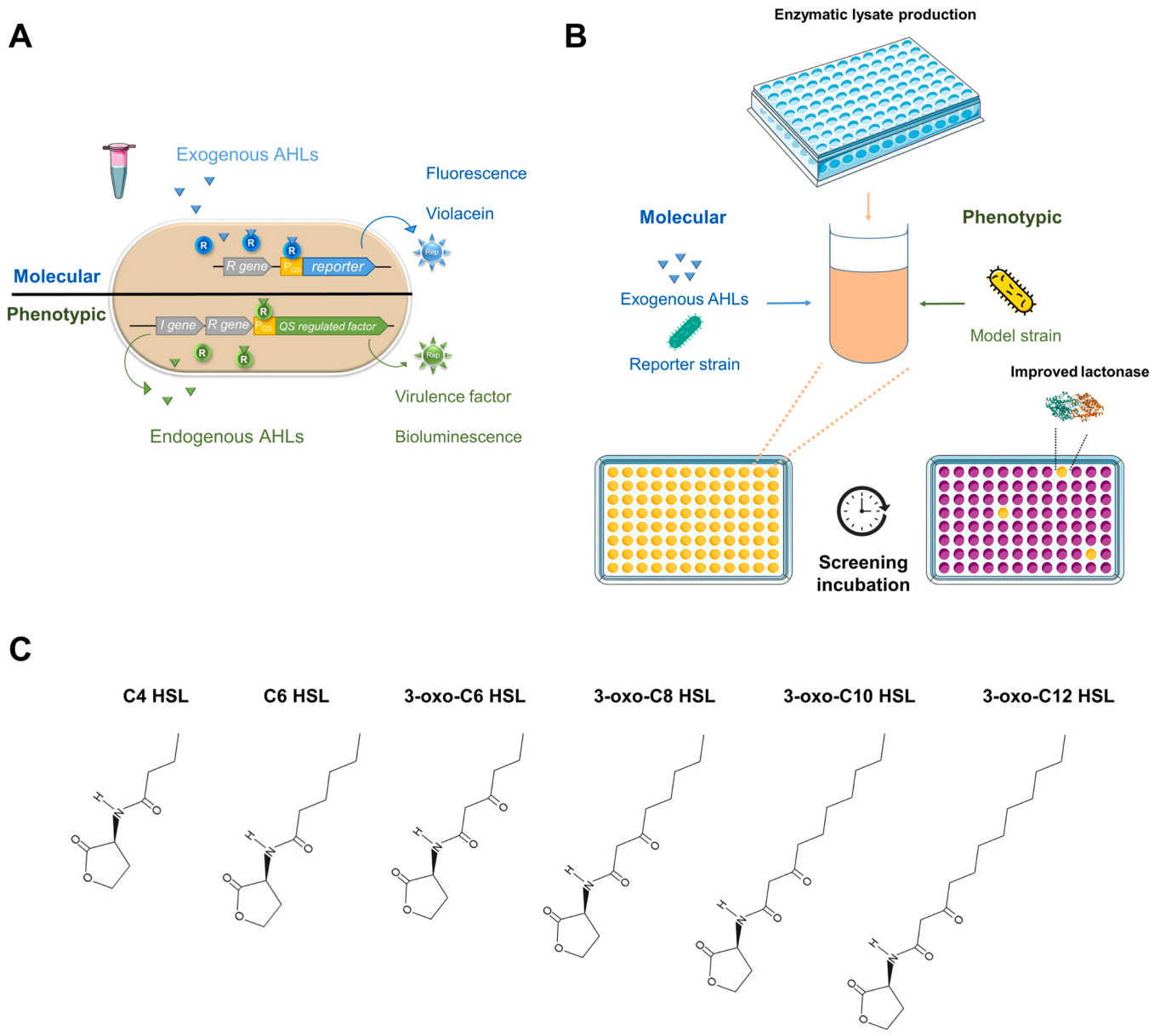

Fig. 2. (A) Quorum sensing system for reporter strains used in molecular screenings (blue) and model strains used in phenotypic screenings (green). (B) General pathway for quorum quenching lactonases screening assays. (C) Acyl-homoserine lactones used for molecular screenings.

2.8. Quorum quenching phenotypic assays

2.8.1. Violacein extraction

Violacein was extracted from 500 µL of culture by adding 500 µL of ethyl acetate [22]; the mixture was vortexed then centrifuged (5 min, 10,000 g, 23 °C), and 200 µL of the upper phase was collected. The absorbance was measured at 565 nm using a microplate reader (Synergy HT, BioTek).

2.8.2. Pyocyanin extraction

Pyocyanin was extracted by mixing 250 µL of chloroform in 500 µL of cell-free supernatant. After centrifugation at 10,000 g for 1 min, 200 µL of the lower chloroform phase was transferred into a quartz 96-well plate. As blank, 200 µL of chloroform was used. The absorbance was measured at 690 nm [23]. Results for each condition were plotted after blank absorbance subtraction.

2.8.3. Proteolytic activity measurement assay

Protease activity was measured by using azocasein (Sigma Aldrich) degradation [24]. Briefly, 25 µL of cell-free supernatant were mixed with 675 µL of phosphate saline buffer pH 7.0 and 50 µL of azocasein solution (30 mg. mL^{-1} in water). After 3 h at 37 °C with agitation (300 rpm), 125 µL of 20 % (w/v) trichloroacetic acid were added. Then, non-degraded azocasein was pelleted down by centrifugation (10,000 g, 5 min). Afterward, 200 µL of supernatant was used to measure the optical density at 366 nm. As blank, an equivalent volume of sterile MOPS medium was used. Results for each condition were plotted after blank absorbance subtraction.

2.8.4. Elastase activity assay

Elastase B activity was measured by using elastin-Congo red conjugate (Sigma Aldrich) degradation assay [25]. In a 96-well plate, 50 µL of cell-free supernatant was mixed with 150 µL of elastin-Congo red solution (5 $mg.mL^{-1}$ in 10 mM Tris-HCl and 1 mM $CaCl_2$ buffer at pH 7.2). After 24 h incubation at 37 °C with agitation (300 rpm), the plate was

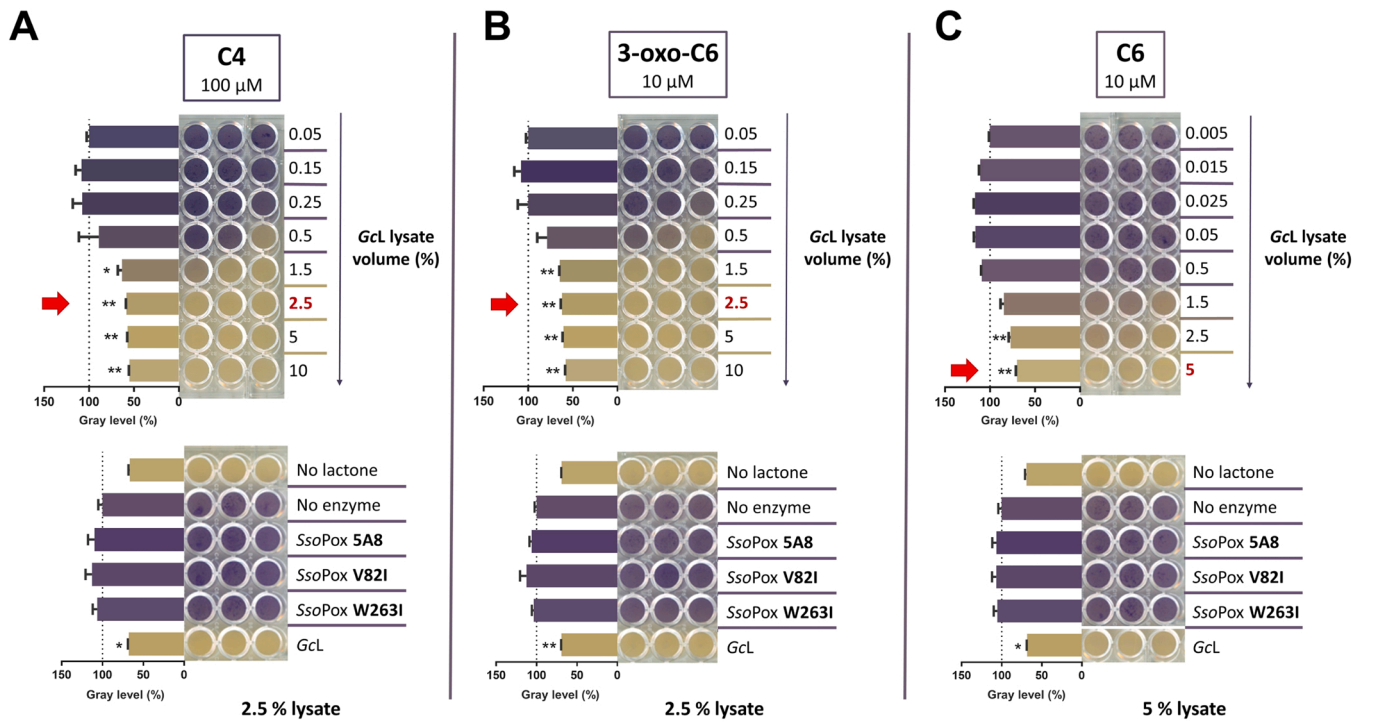

Fig. 3. Screening assay optimization using biosensor CV026. CV026 is inoculated 1/1000 with synthetic lactones (A: C4 HSL; B: 3-oxo-C6 HSL and C: C6 HSL) and enzyme lysate in LB media for 16 h. Absence of violacein implies a proficient lactonase able to degrade synthetic AHL. Violacein production was quantified by measuring the gray level of each well, which is displayed as percentage of the gray value measured for the control. Statistical analyses were calculated with Dunnett's multiple comparisons test, with the condition lacking lysate as reference. Error bars represent the standard deviations. * p-values < 0.05 ** p-values < 0.01; *** p-values < 0.001; **** p-values < 0.0001.

left to rest for 10 min at ambient temperature in order to pellet undigested elastin-Congo red. Afterwards, 100 µL of the reaction was carefully transferred into an empty well and then absorbance was measured at 490 nm. Sterile MOPS medium was used as blank. Results for each condition were plotted after blank absorbance subtraction.

2.8.5. Bioluminescence assay

V. harveyi bioluminescence was monitored after 8 h growth in AB medium with Fusion FX (Vilber-Lourmat) with 120 s exposure. Gray levels of each well were measured with ImageJ software to obtain a quantitative value.

2.9. Biofilm assay

Biofilm was measured using crystal violet (Sigma Aldrich) biomass staining [26]. After culture in 24-well plates, planktonic cells were carefully removed by pipetting. Wells were washed with 1.5 mL of phosphate buffered saline (PBS) solution (BioMérieux), dried at 37 °C and stained with 1.5 mL of 0.05 % (w/v) crystal violet solution. After removing crystal violet, wells were washed with 2 mL of PBS and fixed crystal violet was dissolved with 1.5 mL of 80 % ethanol. Using 200 µL, absorbance was measured at 595 nm. As blank, sterile culture medium was used in the same culture conditions. Results for each condition were plotted after blank absorbance subtraction.

3. Results

3.1. General principle

The objective was to develop miniaturized screening procedures to allow for the evaluation of recombinant QQ enzyme libraries in microplate formats. Two approaches, molecular and phenotypic screenings, were considered and used to evaluate the ability of crude enzymatic lysates to degrade endogenous and exogenous acyl-homoserine lactones (AHL), respectively.

Molecular screenings relate the ability of the enzymatic lysate to degrade exogenous AHL to a signal using a reporter strain as biosensor (e.g., pigment production or fluorescence; Fig. 2A-C). On the other hand, phenotypic screenings relate to the ability of the enzymatic lysate to inhibit phenotypes that are dependent on AHL. Phenotypic assays are useful because many bacteria are known to produce, sense or respond to multiple AHL molecules. Thus, phenotypic assays provide a more comprehensive evaluation of the quenching effect of the tested enzymes [27]. To this end, three bacteria known to use AHL-based QS systems and producing easy-to-monitor QS-dependent readouts were chosen. First, *V. harveyi* DSM19623 was chosen for its ability to activate bioluminescence production through a complex quorum sensing system consisting of three parallel signal transduction pathways using the autoinducers molecules 3-OH-C4 HSL, AI-2 and CAI-1 [28]. It was shown that its bioluminescence can be disrupted by recombinant lactonase lysate [29]. *P. aeruginosa* Pa14 was chosen for its ability to produce the blue/green siderophore pyocyanin in a QS-dependent manner involving two LuxIR circuits, termed RhlIR and LasIR, with their respective signal lactones: C4 and 3-oxo-C12 HSL. Biofilm formation and proteolytic activities are also regulated through QS in this strain [30,31]. Finally, *C. violaceum* 12472 was chosen for the production of the purple pigment violacein under QS regulation via the *vio* operon [32]. This strain has a LuxIR circuit named CviIR and uses numerous AHL such as C9, C10, C11, 3-OH-C10, 3-OH-C11, 3-oxo-C10 and 3-oxo-C12 HSL [33]. Besides their quantifiable QS-regulated factors, these bacteria are also of utmost interest in different fields and constitute serious targets for QQ strategies. *P. aeruginosa* is an opportunistic human pathogen causing healthcare-associated infections (HAIs) [34] and pulmonary infections in cystic fibrosis patients [35]. This bacteria is also prone to antibiotic resistance acquisition and considered a priority pathogen according by the WHO [36]. *C. violaceum* is an environmental bacterium, found in soil

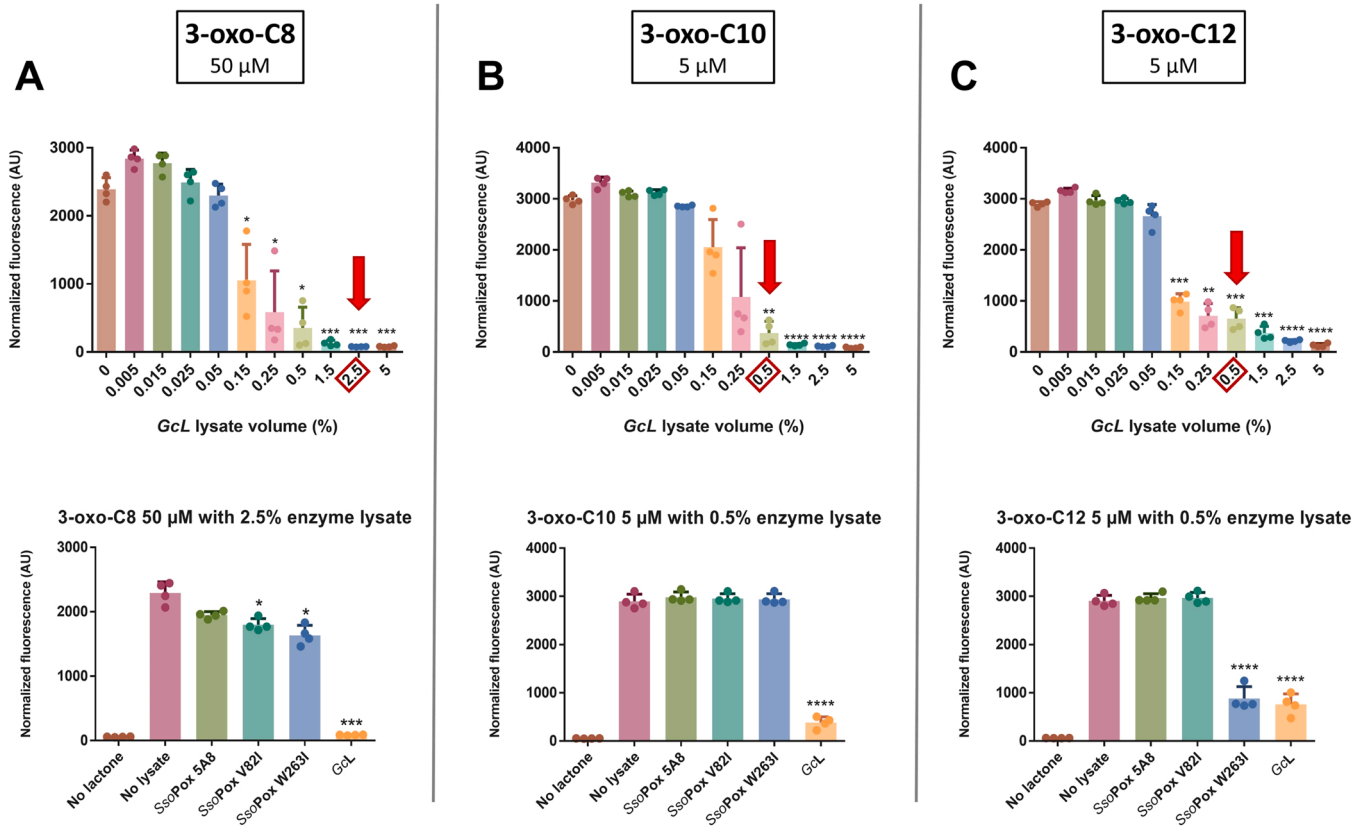

Fig. 4. Screening assay optimization using biosensor *P. putida* KS35. *P. putida* KS35 is inoculated 1/1000 with synthetic lactones (A: 3-oxo-C8 HSL; B: 3-oxo-C10 HSL and C: 3-oxo-C12 HSL) and enzyme lysate in LB media for 8 h. Decrease in fluorescence implies hydrolysis of the synthetic lactone. Statistical analyses were calculated with Dunnett's multiple comparisons test, with the condition lacking lysate as reference. Error bars represent the standard deviations. * p-values < 0.05 ** p-values < 0.01; *** p-values < 0.001; **** p-values < 0.0001.

and water [37], that can cause fatal septicemia [38], urinary tract infections [39] or pneumonia [40] in humans and uses several AHL signaling molecules [41]. Resistance to various antibiotics, such as penicillin, ampicillin, and cephalosporin was also reported [42]. Finally, *V. harveyi* DSM19623 is an aquatic pathogen responsible for huge economic losses in aquaculture, in particular in shrimp farming [43]. The strain developed multiple antibiotic resistance, possibly due to the massive use of prophylactic antibiotics [44–47]. Selecting efficient QQ enzymes that can interfere with the QS of these pathogens is of high interest to develop sustainable alternatives to antibiotics.

3.2. Molecular screenings

3.2.1. Chromobacterium violaceum (CV026)

CV026 is an AHL-biosensor strain derived from *C. violaceum* ATCC 31532 [48] and the wild-type strain produces a purple pigment known as violacein in response to the accumulation of AHL molecule. The CV026 biosensor strain is a *cviI* mutant and is thus unable to synthesize its own AHL [49]. However, this strain is still capable of producing violacein in response to exogenous short-chain AHL molecule (i.e., from C4 to C8-HSL). This strain is widely used as a QS sensor, and a simple micro-plate screening assay was developed to detect lactonases with high activity on short-chain AHL.

The minimal AHL concentration required to activate violacein production was determined by a dose-response experiment (Supplementary Figure 1). From this initial dose-response experiment, working AHL concentrations for the assays were determined: 100 µM for C4 HSL, 10 µM for C6 HSL and 10 µM for 3-oxo-C6 HSL. The volume of enzyme lysate for which a quenching response is only observed for the improved *Sso*Pox variants was determined. To this end, the proficient *Gc*L was used as a positive control for the various tested AHL molecules (Fig. 3). *Gc*L lysate volumes were gradually increased until complete inhibition of violacein production, while simultaneously checking the reporter strain growth behavior (Supplementary Figure 3). 2.5 % lysate was found to be the minimum volume allowing total quenching of violacein production after growth with C4 and 3-oxo-C6 HSL, while a minimum of 5 % lysate volume was required for C6 HSL screening (Fig. 3). To select for improved *Sso*Pox variants, the desired lysate volumes must discriminate enzymes with activities greater than *Sso*Pox V82I or W263I. Since the same lysate volume of *Sso*Pox V82I and *Sso*Pox W263I was insufficient to quench violacein production, these conditions were used to identify improved *Sso*Pox variants.

3.2.2. Pseudomonas putida KS35

P. putida KS35 is a reporter strain based on an AHL-negative *P. putida* isolate from tomato roots, supplemented with an AHL sensor cassette $P_{lasB\text{-}gfp}$ that expresses green fluorescent protein (GFP) in the presence of various AHL [16]. In contrast to the CV026 strain, *P. putida* KS35 does not recognize short-chain AHL, such as C4 and C6 HSL, but responds to longer AHL carrying a C3-carbonyl group, such as 3-oxo-C8, 3-oxo-C10 and 3-oxo-C12 HSL (Supplementary Figure 2A-C). This reporter strain is thus complementary to the CV026 screening assay. As performed previously, the first step was to determine the optimal AHL concentration to run the assay. GFP fluorescence produced by *P. putida* KS35 with increasing concentrations of AHL was measured in a dose-response experiment (Supplementary Figure 2D-F). Based on these results, the parameters chosen for the screening assay were as follows: 50 µM 3-oxo-C8, 5 µM 3-oxo-C10 and 5 µM 3-oxo-C12 HSL. Optimal volumes of enzyme lysates were then determined by measuring fluorescence quenching when increasing lactonase concentration. The most active

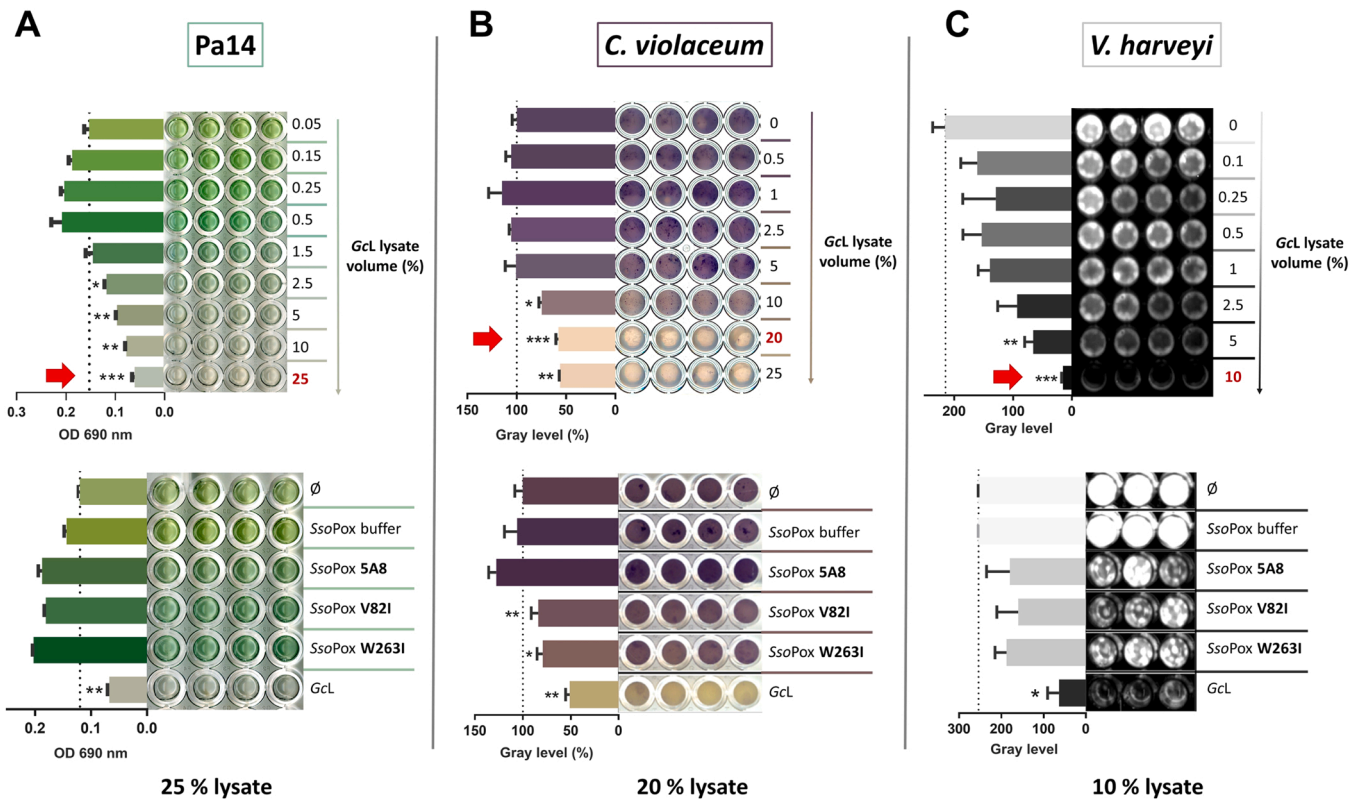

Fig. 5. Gradual disruption of (A) pyocyanin, (B) violacein and (C) bioluminescence production with lactonase GcL lysate (upper part) and differential disruption with reference enzymes (lower part). Violacein production was quantified by measuring the gray level of each well, which is displayed as percentage of the gray value measured for the control. Statistical analyses were calculated with Dunnett's multiple comparisons test, with the condition lacking lysate as reference. Error bars represent the standard deviations. * p-values < 0.05 ** p-values < 0.01; *** p-values < 0.001; **** p-values < 0.0001.

enzymes for each lactone were used as the standard: *Gc*L for 3-oxo-C8 and 3-oxo-C10 HSL and *Sso*Pox W263I for 3-oxo-C12 HSL. The ideal lysate volume would combine sufficient and reproducible fluorescence disruption and workability. Here, a minimum of 1 µL, corresponding to 0.5 % was required to reduce variability. Hence, for 3-oxo-C8 HSL, a volume proportion of 2.5 % enzyme lysate was chosen (Fig. 4A), while 0.5 % was retained for 3-oxo-C10 HSL and 3-oxo-C12 HSL (Fig. 4B and C). Lysates were checked for cytotoxicity towards the reporter strain (Supplementary Figure 3). These screening conditions were then used to detect potentially improved *Sso*Pox variants.

3.3. Phenotypic screenings

Phenotypic screenings were developed with the three previously described pathogens: *P. aeruginosa* Pa14, *C. violaceum* 12472 and *V. harveyi* DSM 19623. Similarly to the molecular screenings, the first step was to determine the quantity of lysate to add to the assay in order to fully quench the targeted QS readout. This was performed using the highly active lactonase GcL. Then, using the same lysate proportion, we compared the quenching ability of each enzyme with reference lactonases of average (*Sso*Pox V82I) and high catalytic efficiency (GcL). For each phenotypic assay, the desired lysate volumes were those for which the GcL lysate could fully disrupt the phenotypic marker while the *Sso*Pox V82I and *Sso*Pox W263I lysates would not. Optimal pyocyanin disruption in Pa14 required 25 % enzymatic lysate (Fig. 5A). For *C. violaceum*, a lysate proportion of 20 % was chosen (Fig. 5B) to reduce violacein production, while 10 % was sufficient to turn bioluminescence off in *V. harveyi* (Fig. 5C). Albeit large, these amounts of enzymatic lysates were proven to be non-cytotoxic to the cells, ensuring that monitored biomarkers were actually turned off through QS disruption (Supplementary Figure 3).

3.4. Applying molecular and phenotypic screenings to an SsoPox alanine-scanning library

Both molecular and phenotypic screening assays were combined to isolate potentially improved *Sso*Pox mutants in an alanine-scanning library. Briefly, 15 residues of the loop 8, a region previously reported as crucial in substrate recognition [14], were mutated into an alanine in order to probe the importance of each residue (Ala-scan). This resulted in a small enzyme library of 15 variants that were evaluated in quadruplicate to evaluate the robustness of screening procedures.

Molecular screenings were first tested using the previously determined AHL concentrations and enzymatic lysate volumes. Screening results showed that mutants R277A and S279A are improved against both C4 HSL and C6 HSL. Additionally, mutants K267A, P268A, E269A, Y270A, K271A, P272A, K273A and L274A all show increased ability to degrade C6 HSL and decreased violacein production as compared to the V82I enzyme (Fig. 6**A**). Similarly, several mutants are improved for 3-oxo-C8 HSL degradation (Fig. 6**B**), and mutants W263A, K267A, E269A, Y270A, K271A, K273A, L274A, R277A and S279A show enhanced degradation of 3-oxo-C10 HSL (Fig. 6**C**). For the degradation of 3-oxo-C12 HSL, screening results show that W263A, L274A and S279A are enhanced as compared to the starting enzyme (Fig. 6**D**). Overall, the fact that so many mutations result in improved lactonase activity suggests that the effect(s) on the active site may be non-specific, and may be related to the importance of the conformational space of loop 8, previously linked to substrate specificity [14,50].

To further evaluate the QQ potential of the variants from the alanine-scanning library, the previously described phenotypic screenings were performed. As for the molecular screenings, numerous mutants showed improved quorum quenching abilities through statistically-significant decreases in production of QS factors in *V. harveyi* (*Sso*Pox V82I/

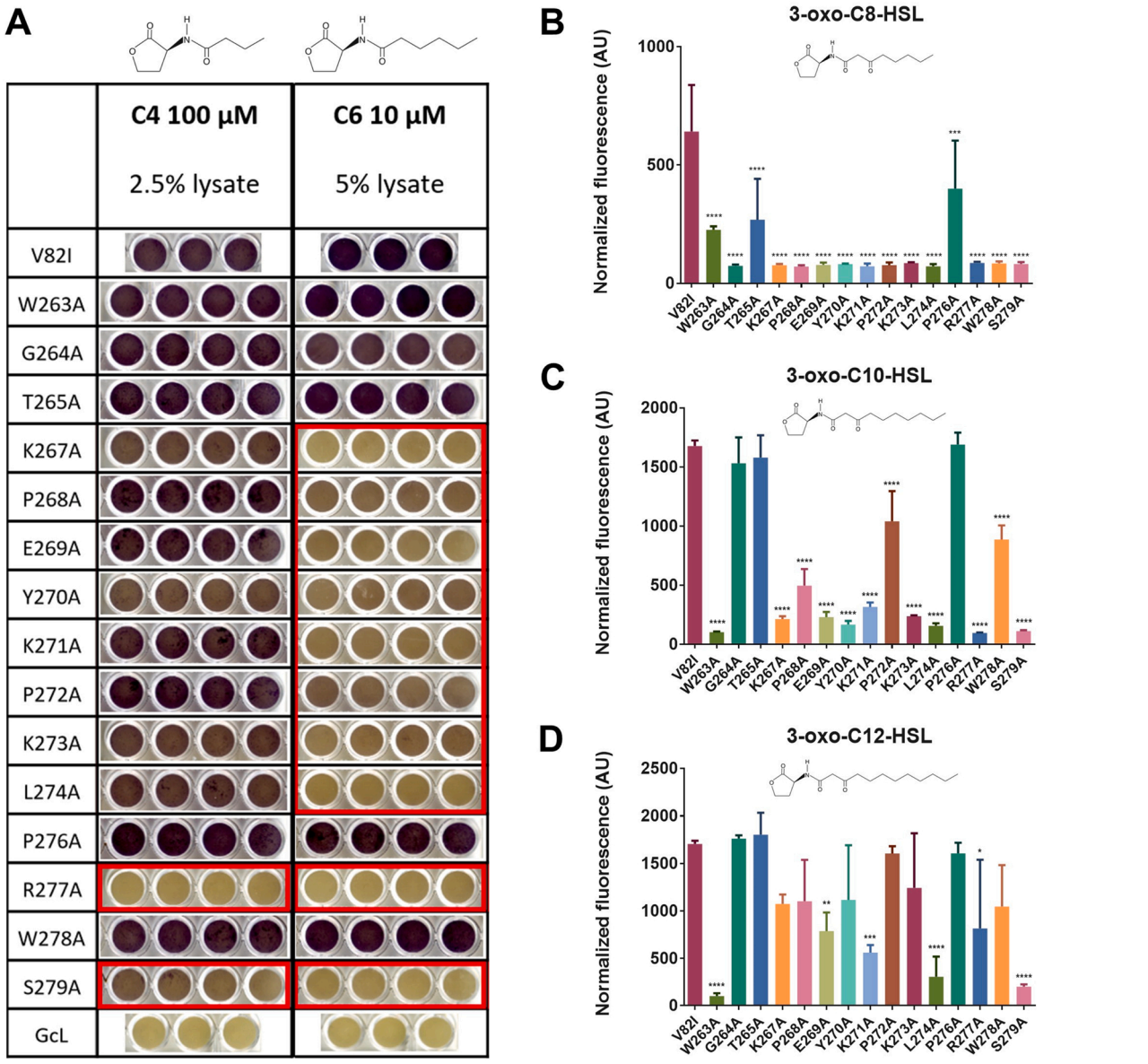

Fig. 6. (A) Violacein quenching of *Sso*Pox loop 8 alanine-scanning using reporter strain CV026, supplemented with C4 HSL 100 µM or C6 HSL 10 µM. (B, C, D) Fluorescence emitted by *P. putida* KS35, normalized by OD 600 nm, in the presence of (B) 3-oxo-C8 HSL, (C) 3-oxo-C10 HSL or (D) 3-oxo-C12 HSL and *Sso*Pox mutant lysates. Statistical analyses for (B,C,D) were calculated with Dunnett's multiple comparisons test. Error bars represent the standard deviations. ** p-values < 0.01; *** p-values < 0.001; **** p-values < 0.0001.

G264A), *C. violaceum* (*Sso*Pox V82I/K273A, V82I/R277A, V82I/S279E) or Pa14 (*Sso*Pox V82I/W263A, V82I/G264A, V82I/K267A, V82I/E269A, V82I/K271A, V82I/L274A, V82I/P276A and V82I/W278A) (Fig. 7). Phenotypic screenings were then analyzed in the light of molecular screening results. Remarkably, the mutations identified for their increased QQ effects in *C. violaceum* (K273A, R277A and S279A) showed increased activity on 3-oxo-C10 HSL. This AHL is indeed involved in violacein production in this *C. violaceum* strain [33]. Regarding Pa14 phenotypic screening, 6 of the 8 mutants with improved QQ effects were found to exhibit higher activity against 3-oxo-C12 HSL in molecular screenings, a key signaling molecule for Pa14. Of note, mutants G264A and P276A showed increased QQ effects for Pa14, while they were not identified by the molecular screenings on C4 HSL and 3-oxo-C12 HSL, the two AHL involved in *P. aeruginosa* QS. This observation suggests a complex relationship between AHL-degrading activity levels and phenotypic effects in bacteria using more than one AHL. Although in most cases phenotypic changes can be related to AHL activity enhancement, the QQ effects of some variants were not predictable, highlighting the complementarity of both molecular and phenotypic levels.

To confirm the molecular and phenotypic screening results, four promising variants out of the 15 from loop 8 alanine scanning library were selected for further investigations. V82I/W263A was chosen because of the large improvement in long-chain AHL activity and increased ability to reduce pyocyanin in Pa14. The potential of this mutation has already been identified in a previous study [14], but remains to be characterized. V82I/R277A and V82I/S279A variants were selected because of their broad enhanced lactonase activity and increased inhibition of the production of violacein in *C. violaceum*. Finally, V82I/G264A was selected for its increased capacity to reduce the bioluminescence of *V. harveyi*.

These four variants were produced, purified and their kinetic parameters were determined (Table 2). *Sso*Pox V82I/W263A did not show enhancement to C4 and C6 HSL and thus did not alter violacein

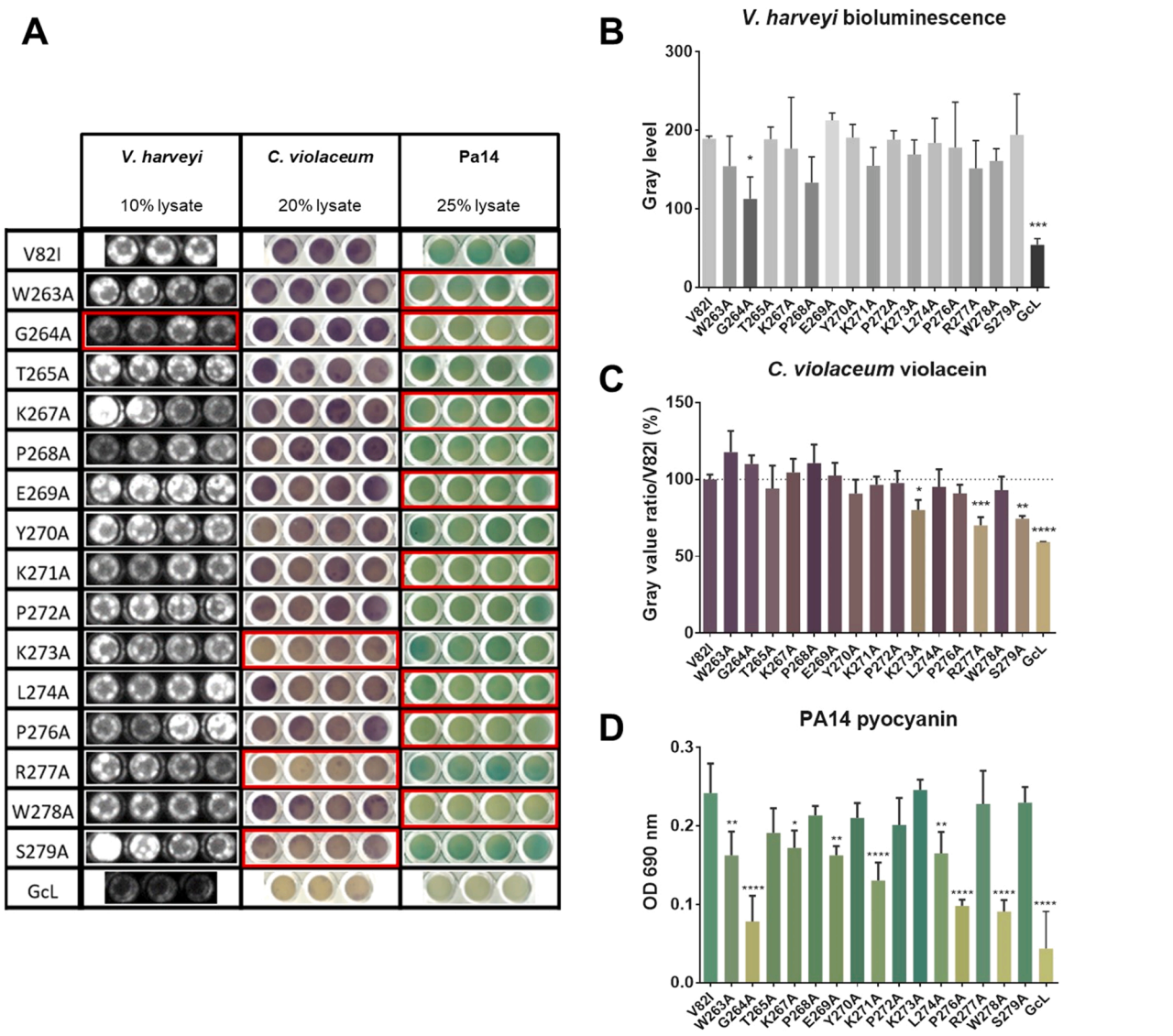

Fig. 7. Phenotypic screenings of *Sso*Pox loop 8 alanine scanning. For each screening, a pathogenic strain was supplemented with enzymatic lysate and specific quorum sensing factors were monitored after growth. (A) General view of phenotypic screening assay results. For *V. harveyi* (B) and *C. violaceum* (C), gray levels of each well were measured to estimate bioluminescence and violacein production, respectively. For Pa14, supernatant OD 690 nm was measured to assess pyocyanin production. Hits (red rectangle) were determined when a specific factor shift was significant, as compared to *Sso*Pox V82I, using Dunnett's multiple comparisons test. Error bars represent the standard deviations of three experiments. * p-values < 0.05; ** p-values < 0.01; *** p-values < 0.001; **** p-values < 0.0001.

production. As expected, however, this variant showed a very significant activity increase on 3-oxo-C12 HSL (45-fold) and a slightly increased activity on 3-oxo-C10 (2-fold) as compared to *Sso*Pox V82I. These results are consistent with molecular screenings and phenotypic screenings. The mutant V82I/G264A showed a slight, albeit non-significant, improvement for 3-oxo-C8 HSL as compared to V82I control. The mutant V82I/R277A, which showed improvement in both molecular and phenotypical screenings, was globally enhanced for AHL degradation (~2–8-fold increase, as compared to V82I). Similarly, the mutant V82I/S279A identified by molecular screening results also showed increases in AHL degrading activity (~2–12-fold increase, as compared to V82I; Table 2). Results also demonstrate that variants V82I/R277A and V82I/S279A exhibit broader substrate specificity compared to V82I, V82I/W263A and V82I/G264A. Overall, determination of the kinetic parameters largely corroborated the results from molecular screenings. This confirms the efficacy and utility of the screening methods presented here to identify improved variants for AHL degrading enzymes.

To further confirm the screening results, the ability of purified variants to quench phenotypic traits from Pa14, *C. violaceum* and *V. harveyi* was then evaluated in vitro. A Pa14 phenotypic screening assay showed that the lactonase *Sso*Pox V82I/W263A efficiently quenches this strain. To confirm this assumption, four phenotypic factors known to be controlled by the QS circuitry, namely pyocyanin, biofilm formation, elastolytic and proteolytic activities were monitored [51]. Consistent with the screening assay, this mutant was able to decrease pyocyanin production more efficiently than the parental enzyme *Sso*Pox V82I and was also more efficient at reducing biofilm formation and proteolytic activity (Fig. 8A). Interestingly, no changes were observed for the elastolytic activity, likely because the background control V82I is sufficiently active to quench this virulence factor.

The mutants *Sso*Pox V82I/R277A and V82I/S279A were isolated during phenotypic screenings for their capacity to decrease violacein production in *C. violaceum*. Consistently, these purified enzymes showed greater efficacy than *Sso*Pox V82I in decreasing violacein production at equivalent enzyme concentrations (Fig. 8**B**), confirming the screening results. These enzymes were also more efficient in reducing biofilm formation. The mutant V82I/G264A was identified from phenotypic screenings for its ability to reduce *V. harveyi* bioluminescence. As

Table 2
Kinetic characterization of *Sso*Pox variants. ND: non detectable. Statistical analyses were calculated with Dunnett's multiple comparisons test, with V82I as reference. Error bars represent the standard deviations. * p-values < 0.05 ** p-values < 0.01; *** p-values < 0.001.

Substrate	*Sso*Pox	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}.M^{-1}$)	Enhancement/ V82I
C4 HSL	V82I	0.13 ± 0.01	1240 ± 136	1.02 (± 0.17) x 10^2	1
	V82I/ W263A	ND	ND	ND	–
	V82I/ G264A	0.25 ± 0.08	3966 ± 1776	6.29 (± 4.9) x 10^1	0.6
	V82I/ R277A	2.39 ± 1.29	10,050 ± 6203	2.37 (± 2.74) x 10^2	2.3
	V82I/ S279A	1.99 ± 0.85	8719 ± 4341	2.28 (± 2.11) x 10^2	2.2
C6 HSL	V82I	0.80 ± 0.02	373 ± 32	2.15 (± 0.24) x 10^3	1
	V82I/ W263A	0.54 ± 0.13	3241 ± 1112	1.66 (± 0.97) x 10^2	0.08
	V82I/ G264A	2.23 ± 0.46	2837 ± 860	7.85 (± 4) x 10^2	0.36
	V82I/ R277A	1.46 ± 0.08	191.3 ± 49	7.64 (± 2.39) x 10^3	3.6 *
	V82I/ S279A	3.49 ± 0.21	409.6 ± 79	8.52 (± 2.16) x 10^3	4 *
3-oxo-C8 HSL	V82I	0.70 ± 0.024	504.9 ± 51	1.39 (± 0.19) x 10^3	1
	V82I/ W263A	0.91 ± 0.051	915.1 ± 113	9.98 (± 1.78) x 10^2	0.7
	V82I/ G264A	3.30 ± 0.512	2189 ± 542	1.51 (± 0.61) x 10^3	1.1
	V82I/ R277A	2.07 ± 0.087	168.7 ± 34	1.22 (± 0.3) x 10^4	8.8 *
	V82I/ S279A	2.85 ± 0.11	160.4 ± 31	1.78 (± 0.41) x 10^4	12.8 **
3-oxo-C10 HSL	V82I	2.44 ± 0.1	197.8 ± 24	1.24 (± 0.2) x 10^4	1
	V82I/ W263A	7.29 ± 0.24	297.6 ± 29	2.45 (± 0.32) x 10^4	2
	V82I/ G264A	2.07 ± 0.19	617.5 ± 133	3.35 (± 1.03) x 10^3	0.3
	V82I/ R277A	5.18 ± 0.11	91.5 ± 8	5.66 (± 0.65) x 10^4	4.6 **
	V82I/ S279A	9.55 ± 0.34	112 ± 16	8.46 (± 1.47) x 10^4	6.9 ***
3-oxo-C12 HSL	V82I	0.64 ± 0.04	127 ± 26	5.01 (± 1.31) x 10^3	1
	V82I/ W263A	23.95 ± 0.95	106.7 ± 19	2.24 (± 0.48) x 10^5	44.5 ***
	V82I/ G264A	0.40 ± 0.013	95.4 ± 13	4.18 (± 0.68) x 10^3	0.8
	V82I/ R277A	0.82 ± 0.017	69.03 ± 7	1.18 (± 0.14) x 10^4	2.3
	V82I/ S279A	2.03 ± 0.105	73.67 ± 18	2.76 (± 0.8) x 10^4	5.5

expected, it is indeed significantly better than *Sso*Pox V82I to disrupt this typical quorum sensing regulated factor (Fig. 8C). Kinetic characterizations of the purified enzymes and in vitro assays of virulence factor quenching thus confirmed the reliability of the described molecular and phenotypic lactonase screening assays.

4. Discussion

In this study, five molecular screening methods, using reporter strains CV026 for C4, C6 and 3-oxo-C6 HSL activites and *P. putida* KS35 for 3-oxo-C8, 3-oxo-C10 and 3-oxo-C12 HSL activities, as well as three phenotypic screenings aiming QS disruption of pathogenic strains *P. aeruginosa, C. violaceum* and *V. harveyi* were adapted to the identification of improved lactonases from mutant libraries. For each screening assay, optimal parameters were investigated and determined to discriminate improved enzymes from parental, control lactonase. Benchmark enzymes (*Sso*Pox 5A8, *Sso*Pox V82I, *Sso*Pox W263I and *Gc*L) were used to identify key experimental parameters, leading to discriminative screening conditions. Molecular screenings are efficient tools to identify improved variants for specific lactones, while phenotypic screening can be used to select variants with enhanced quenching potential for a bacterial candidate.

Using these screening systems on a small, alanine-scanned library, we were able to find several beneficial mutations for the degradation of all tested AHL molecules (R277A and S279A). We also identified mutants with specific, improved degradation against one lactone (W263A), and mutants with increased ability to reduce bacterial QS-related phenotypes (W263A, G264A, R277A and S279A). These four mutants were further purified and their capacity to quench multiple virulence factors more efficiently than parental *Sso*Pox V82I was confirmed with the pathogenic strains. This confirms the capacity of the different screenings to identify mutants with increased catalytic parameters against AHL.

Here, we show that molecular screenings relying on the reporter cells CV026 and *P. putida* KS35, based on the measurement of the purple pigment violacein and GFP fluorescence, respectively, were suitable to evaluate QQ enzyme activity against exogenous AHL molecules. Interestingly, a high AHL degradation ability does not necessarily result in more quenched phenotypes. This observation may stem from the fact that pathogenic strains often use complex regulation networks to finely tune QS responses [8]. In addition, QS regulation is interconnected with many other regulatory systems, including metabolism and environmental stimuli; therefore, screening procedures in a whole bacterial context may be required to identify proficient QQ enzymes. To this end, three phenotypic screenings were developped. Phenotypic screenings allowed to screening directly for lactonases capable of interfering with three bacteria of interest, *P. aeruginosa*, *C. violaceum* and *V. harveyi*. Screening read-outs rely on measuring QS-dependant products: pyocyanin, violacein, and bioluminsecence, respectively.

These screening assays are thus adapted to probe the performance of AHL-interfering enzymes in a miniaturized 96-well format. Although such screening would not be compatible with high-throughput screening of large libraries generated through directed evolution strategies, they are compatible with small to medium, "smart" libraries generated though rational or semi-rational approches and containing up to thousands of variants. In addition, these procedures could be adapted to any pathogen with a measurable QS-dependant product or behavior, such as pigments, fluorescence or luminescence. Typically, the identification of engineered lactonases rely on molecular-type screenings, and identified

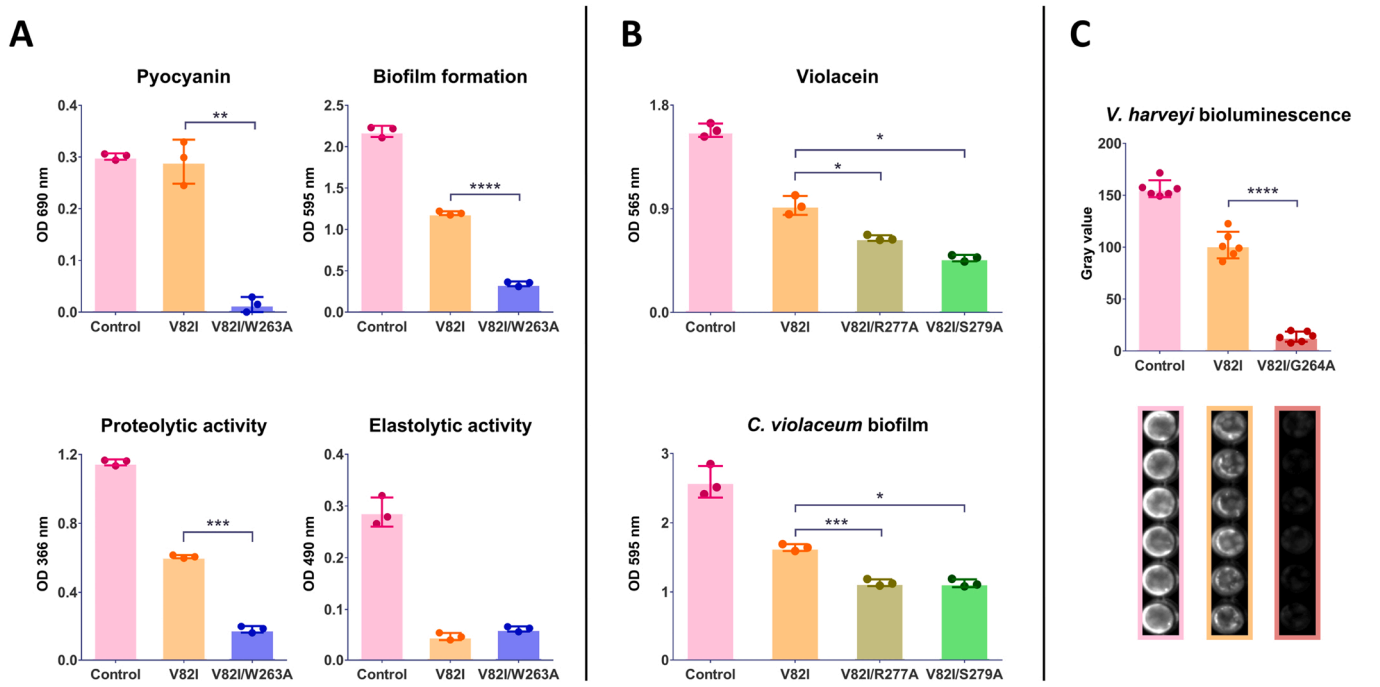

Fig. 8. Virulence factor reduction by quorum quenching lactonases. (A) Pa14 pyocyanin and biofilm production, as well as proteolytic and elastolytic activities, were measured after 40 h growth in MOPS medium supplemented with 1 mg.mL^{-1} lactonase. (B) *C. violaceum* violacein and biofilm production was measured after 40 h growth in LB supplemented with 20 μg.mL^{-1} lactonase. (C) *V. harveyi* bioluminescence was measured after 8 h growth in AB medium with 0.1 mg.mL^{-1} lactonase. Statistical analyses were performed with Tukey's multiple comparisons test and described with asterisks as: * : p-value < 0.05; * *: p-value < 0.01; *** : p-value < 0.001; and ****: p-value < 0.0001.

mutants are subsequently tested and characterized on a specific pathogen [33,52,53]. Therefore, the use and the development of such phenotypic screenings could significantly shorten the search for efficient enzymes with desired QQ effects. This is examplified by the hereby reported, rapid identification of the V82I/G264A variant, disrupting *V. harveyi* bioluminescence, while being unnoticeable with molecular screenings. These new assays would constitute efficient tools for isolating improved lactonases in the search for reliable alternatives to antibiotic therapy.

CRediT authorship contribution statement

Raphaël Billot: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Laure Plener:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Visualization, Supervision. **Damien Grizard:** Resources, Funding acquisition. **Mikael H. Elias:** Writing – review & editing, Visualization. **Éric Chabrière:** Resources, Supervision, Project administration, Funding acquisition. **David Daudé:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Acknowledgments

R. B. is a Ph.D. student supported by the French Agence Nationale de la Recherche et de la Technologie (ANRT) CIFRE Grant 2018/1543. This work was supported by the "Investissements d'avenir" program (Méditerranée Infection Grant 10-IAHU-03) of the French Agence Nationale de la Recherche (ANR). M.H.E. was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R35GM133487. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The authors would like to thank Dr. Raphael Lami for providing the *P. putida* KS35 reporter strain and MAPIEM lab for providing the CV026 strain.

Conflict of interest statement

EC has patent WO2014167140 A1 licensed to Gene&GreenTK. L.P., E.C. and D.D. have filed the patent EP3941206. R.B., L.P., D.D. and E.C. report receiving personal fees from Gene&GreenTK during the study. E. C. and D.D. are shareholders in Gene&GreenTK. D.D. is CEO of Gene&GreenTK. D.G. is Director of Gene&GreenTK.

M.H.E. is the co-founder, former Scientific Advisory Board member, and equity holder of Gene&GreenTK, a company that holds the license to WO2014167140 A1, FR 3068989 A1, FR19/02834. M.H.E. is an inventor of patents No. 62/816,403, WO2014167140 A1, WO2008145865A2, WO2015014971A1, FR3068989 A1, FR19/02834 and EP3941206. These interests have been reviewed and managed by the University of Minnesota in accordance with its Conflict of Interest policies.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.enzmictec.2022.110092.

References

[1] M.B. Miller, B.L. Bassler, Quorum sensing in bacteria, Annu. Rev. Microbiol. 55 (2001) 165–199, https://doi.org/10.1146/annurev.micro.55.1.165.
[2] W.R.J.D. Galloway, J.T. Hodgkinson, S.D. Bowden, M. Welch, D.R. Spring, Quorum sensing in gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways, Chem. Rev. 111 (2011) 28–67, https://doi.org/10.1021/cr100109t.
[3] M.R. Parsek, E.P. Greenberg, Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: a signaling mechanism involved in associations with higher organisms, PNAS 97 (2000) 8789–8793, https://doi.org/10.1073/pnas.97.16.8789.

[4] M. Schuster, D. Joseph Sexton, S.P. Diggle, E. Peter Greenberg, Acyl-homoserine lactone quorum sensing: from evolution to application, Annu. Rev. Microbiol. 67 (2013) 43–63, https://doi.org/10.1146/annurev-micro-092412-155635.

[5] F. Chen, Y. Gao, X. Chen, Z. Yu, X. Li, Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection, IJMS 14 (2013) 17477–17500, https://doi.org/10.3390/ijms140917477.

[6] J. Bzdrenga, D. Daudé, B. Rémy, P. Jacquet, L. Plener, M. Elias, E. Chabrière, Biotechnological applications of quorum quenching enzymes, Chem. -Biol. Interact. 267 (2017) 104–115, https://doi.org/10.1016/j.cbi.2016.05.028.

[7] R. Billot, L. Plener, P. Jacquet, M. Elias, E. Chabrière, D. Daudé, Engineering acyl-homoserine lactone-interfering enzymes towards bacterial control, jbc. REV120.013531, J. Biol. Chem. (2020), https://doi.org/10.1074/jbc.REV120.013531.

[8] C. Grandclément, M. Tannières, S. Moréra, Y. Dessaux, D.D. Faure, Quorum quenching: role in nature and applied developments, FEMS Microbiol. Rev. (2015) fuv038.

[9] B. Rémy, L. Plener, P. Decloquement, N. Armstrong, M. Elias, D. Daudé, É. Chabrière, Lactonase specificity is key to quorum quenching in pseudomonas aeruginosa, Front. Microbiol. 11 (2020) 762, https://doi.org/10.3389/fmicb.2020.00762.

[10] K. Mahan, R. Martinmaki, I. Larus, R. Sikdar, J. Dunitz, M. Elias, Effects of signal disruption depends on the substrate preference of the lactonase, Front. Microbiol. 10 (2020) 3003, https://doi.org/10.3389/fmicb.2019.03003.

[11] M. Wójcik, A. Telzerow, W. Quax, Y. Boersma, High-throughput screening in protein engineering: recent advances and future perspectives, IJMS 16 (2015) 24918–24945, https://doi.org/10.3390/ijms161024918.

[12] C. Bergonzi, M. Schwab, M. Elias, The quorum-quenching lactonase from *Geobacillus caldoxylosilyticus*: purification, characterization, crystallization and crystallographic analysis, Acta Crystallogr F. Struct. Biol. Commun. 72 (2016) 681–686, https://doi.org/10.1107/S2053230X16011821.

[13] B. Rémy, L. Plener, L. Poirier, M. Elias, D. Daudé, E. Chabrière, Harnessing hyperthermostable lactonase from Sulfolobus solfataricus for biotechnological applications, Sci. Rep. 6 (2016) 1–11, https://doi.org/10.1038/srep37780.

[14] J. Hiblot, G. Gotthard, M. Elias, E. Chabriere, Differential active site loop conformations mediate promiscuous activities in the lactonase SsoPox, PLoS ONE 8 (2013) 1–14, https://doi.org/10.1371/journal.pone.0075272.

[15] M. Elias, J. Dupuy, L. Merone, L. Mandrich, E. Porzio, S. Moniot, D. Rochu, C. Lecomte, M. Rossi, P. Masson, G. Manco, E. Chabriere, Structural basis for natural lactonase and promiscuous phosphotriesterase activities, J. Mol. Biol. 379 (2008) 1017–1028, https://doi.org/10.1016/j.jmb.2008.04.022.

[16] A. Steidle, K. Sigl, R. Schuhegger, A. Ihring, M. Schmid, S. Gantner, M. Stoffels, K. Riedel, M. Givskov, A. Hartmann, C. Langebartels, L. Eberl, Visualization of N-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere, Appl. Environ. Microbiol. 67 (2001) 5761–5770, https://doi.org/10.1128/AEM.67.12.5761-5770.2001.

[17] B. Mellbye, M. Schuster, Physiological framework for the regulation of quorum sensing-dependent public goods in pseudomonas aeruginosa, J. Bacteriol. 196 (2014) 1155–1164, https://doi.org/10.1128/JB.01223-13.

[18] E.P. Greenberg, J.W. Hastings, S. Ulitzur, Induction of luciferase synthesis in Beneckea harveyi by other marine bacteria, Arch. Microbiol. 120 (1979) 87–91, https://doi.org/10.1007/BF00409093.

[19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

[20] O. Khersonsky, D.S. Tawfik, Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase, Biochemistry 44 (2005) 6371–6382, https://doi.org/10.1021/bi047440d.

[21] L. Afriat, C. Roodveldt, G. Manco, D.S. Tawfik, The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase †, Biochem. (Mosc.) 45 (2006) 13677–13686.

[22] I. Castillo-Juárez, R. García-Contreras, N. Velázquez-Guadarrama, M. Soto-Hernández, M. Martínez-Vázquez, Amphypterygium adstringens anacardic acid mixture inhibits quorum sensing-controlled virulence factors of chromobacterium violaceum and pseudomonas aeruginosa, Arch. Med. Res. 44 (2013) 488–494, https://doi.org/10.1016/j.arcmed.2013.10.004.

[23] A. Price-Whelan, L.E.P. Dietrich, D.K. Newman, Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14, J. Bacteriol. 189 (2007) 6372–6381, https://doi.org/10.1128/JB.00505-07.

[24] J.-P. Chessa, I. Petrescu, M. Bentahir, J. Van Beeumen, C. Gerday, Purification, physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic Pseudomonas species, Biochim. Et. Biophys. Acta (BBA) - Protein Struct. Mol. Enzymol. 1479 (2000) 265–274, https://doi.org/10.1016/S0167-4838(00)00018-2.

[25] K.M. Smith, Y. Bu, H. Suga, Induction and inhibition of pseudomonas aeruginosa quorum sensing by synthetic autoinducer analogs, Chem. Biol. 10 (2003) 81–89, https://doi.org/10.1016/S1074-5521(03)00002-4.

[26] L.R. Hoffman, D.A. D'Argenio, M.J. MacCoss, Z. Zhang, R.A. Jones, S.I. Miller, Aminoglycoside antibiotics induce bacterial biofilm formation, Nature 436 (2005) 1171–1175, https://doi.org/10.1038/nature03912.

[27] B.L. Bassler, How bacteria talk to each other: regulation of gene expression by quorum sensing, Curr. Opin. Microbiol 2 (1999) 582–587, https://doi.org/10.1016/s1369-5274(99)00025-9.

[28] C.M. Waters, B.L. Bassler, Quorum sensing: communication in bacteria, Annu Rev. Cell Dev. Biol. 21 (2005) 319–346, https://doi.org/10.1146/annurev.cellbio.21.012704.131001.

[29] F. Bai, Y. Han, J. Chen, X.-H. Zhang, Disruption of quorum sensing in Vibrio harveyi by the AiiA protein of Bacillus thuringiensis, Aquaculture 274 (2008) 36–40, https://doi.org/10.1016/j.aquaculture.2007.11.024.

[30] T.G. Sana, R. Lomas, M.R. Gimenez, A. Laubier, C. Soscia, C. Chauvet, A. Conesa, R. Voulhoux, B. Ize, S. Bleves, Differential modulation of quorum sensing signaling through QslA in pseudomonas aeruginosa strains PAO1 and PA14, J. Bacteriol. 201 (2019), https://doi.org/10.1128/JB.00362-19.

[31] M. Fazli, H. Almblad, M.L. Rybtke, M. Givskov, L. Eberl, T. Tolker-Nielsen, Regulation of biofilm formation in *P seudomonas* and *B urkholderia* species: Regulation of biofilm formation, Environ. Microbiol 16 (2014) 1961–1981, https://doi.org/10.1111/1462-2920.12448.

[32] S.Y. Choi, S. Lim, G. Cho, J. Kwon, W. Mun, H. Im, R.J. Mitchell, *Chromobacterium violaceum* delivers violacein, a hydrophobic antibiotic, to other microbes in membrane vesicles, Environ. Microbiol 22 (2020) 705–713, https://doi.org/10.1111/1462-2920.14888.

[33] S. Mion, N. Carriot, J. Lopez, L. Plener, A. Ortalo-Magné, E. Chabrière, G. Culioli, D. Daudé, Disrupting quorum sensing alters social interactions in Chromobacterium violaceum, Npj Biofilms Micro 7 (2021) 40, https://doi.org/10.1038/s41522-021-00211-w.

[34] L.M. Weiner, A.K. Webb, B. Limbago, M.A. Dudeck, J. Patel, A.J. Kallen, J. R. Edwards, D.M. Sievert, Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2011–2014, Infect. Control Hosp. Epidemiol. 37 (2016) 1288–1301, https://doi.org/10.1017/ice.2016.174.

[35] J.W. Costerton, Cystic fibrosis pathogenesis and the role of biofilms in persistent infection, Trends Microbiol. 9 (2001) 50–52, https://doi.org/10.1016/S0966-842X(00)01918-1.

[36] L. Fernández, E.B.M. Breidenstein, D. Song, R.E.W. Hancock, Role of intracellular proteases in the antibiotic resistance, motility, and biofilm formation of Pseudomonas aeruginosa, Antimicrob. Agents Chemother. 56 (2012) 1128–1132, https://doi.org/10.1128/AAC.05336-11.

[37] T. Morohoshi, K. Fukamachi, M. Kato, N. Kato, T. Ikeda, Regulation of the violacein biosynthetic gene cluster by acylhomoserine lactone-mediated quorum sensing in *Chromobacterium violaceum* ATCC 12472, Biosci., Biotechnol., Biochem. 74 (2010) 2116–2119, https://doi.org/10.1271/bbb.100385.

[38] S. Sharmin, A.A. Jahan, S.M.M. Kamal, P. Sarker, Fatal Infection Caused by *Chromobacterium violaceum*: A case report from a tertiary care hospital in Bangladesh, Case Rep. Infect. Dis. 2019 (2019) 1–4, https://doi.org/10.1155/2019/6219295.

[39] N.D. Pant, S.P. Acharya, R. Bhandari, U.N. Yadav, D.B. Saru, M. Sharma, Bacteremia and Urinary Tract Infection Caused by *Chromobacterium violaceum*: Case Reports from a Tertiary Care Hospital in Kathmandu, Nepal, Case Rep. Med. 2017 (2017) 1–4, https://doi.org/10.1155/2017/7929671.

[40] H. Hagiya, T. Murase, M. Suzuki, K. Shibayama, Y. Kokumai, N. Watanabe, M. Maki, F. Otsuka, Chromobacterium violaceum nosocomial pneumonia in two Japanese patients at an intensive care unit, J. Infect. Chemother. 20 (2014) 139–142, https://doi.org/10.1016/j.jiac.2013.10.001.

[41] T. Morohoshi, M. Kato, K. Fukamachi, N. Kato, T. Ikeda, *N* -Acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472, FEMS Microbiol. Lett. 279 (2008) 124–130, https://doi.org/10.1111/j.1574-6968.2007.01016.x.

[42] C.-H. Yang, Y.-H. Li, Chromobacterium violaceum infection: a clinical review of an important but neglected infection, J. Chin. Med. Assoc. 74 (2011) 435–441, https://doi.org/10.1016/j.jcma.2011.08.013.

[43] G. Aguirre-Guzman, H. Mejia Ruiz, F. Ascencio, A review of extracellular virulence product of Vibrio species important in diseases of cultivated shrimp, Aquac. Res. 35 (2004) 1395–1404, https://doi.org/10.1111/j.1365-2109.2004.01165.x.

[44] F.C. Cabello, Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment, Environ. Microbiol 8 (2006) 1137–1144, https://doi.org/10.1111/j.1462-2920.2006.01054.x.

[45] T. Nakayama, E. Ito, N. Nomura, N. Nomura, M. Matsumura, Comparison of *Vibrio harveyi* strains isolated from shrimp farms and from culture collection in terms of toxicity and antibiotic resistance, FEMS Microbiol. Lett. 258 (2006) 194–199, https://doi.org/10.1111/j.1574-6968.2006.00225.x.

[46] C.-H. Kang, Y. Kim, S.J. Oh, J.-S. Mok, M.-H. Cho, J.-S. So, Antibiotic resistance of Vibrio harveyi isolated from seawater in Korea, Mar. Pollut. Bull. 86 (2014) 261–265, https://doi.org/10.1016/j.marpolbul.2014.07.008.

[47] Z.M. Zhu, C.F. Dong, S.P. Weng, J.G. He, The high prevalence of pathogenic *Vibrio harveyi* with multiple antibiotic resistance in scale drop and muscle necrosis disease of the hybrid grouper, *Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂), in China, J. Fish. Dis. 41 (2018) 589–601, https://doi.org/10.1111/jfd.12758.

[48] K.H. McClean, M.K. Winson, L. Fish, A. Taylor, S.R. Chhabra, M. Camara, M. Daykin, J.H. Lamb, S. Swift, B.W. Bycroft, G.S.A.B. Stewart, P. Williams, Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones, Microbiology 143 (1997) 3703–3711, https://doi.org/10.1099/00221287-143-12-3703.

[49] L. Steindler, V. Venturi, Detection of quorum-sensing *N* -acyl homoserine lactone signal molecules by bacterial biosensors, FEMS Microbiol. Lett. 266 (2007) 1–9, https://doi.org/10.1111/j.1574-6968.2006.00501.x.

[50] P. Jacquet, J. Hiblot, D. Daudé, C. Bergonzi, G. Gotthard, N. Armstrong, E. Chabrière, M. Elias, Rational engineering of a native hyperthermostable

lactonase into a broad spectrum phosphotriesterase, Sci. Rep. 7 (2017) 1–15, https://doi.org/10.1038/s41598-017-16841-0.
[51] M. Juhas, L. Eberl, B. Tummler, Quorum sensing: the power of cooperation in the world of Pseudomonas, Environ. Microbiol 7 (2005) 459–471, https://doi.org/10.1111/j.1462-2920.2005.00769.x.
[52] A. Guendouze, L. Plener, J. Bzdrenga, P. Jacquet, B. Rémy, M. Elias, J.P. Lavigne, D. Daudé, E. Chabrière, Effect of quorum quenching lactonase in clinical isolates of pseudomonas aeruginosa and comparison with quorum sensing inhibitors, Front. Microbiol. 8 (2017) 1–10, https://doi.org/10.3389/fmicb.2017.00227.
[53] G. Koch, P. Nadal-Jimenez, C.R. Reis, R. Muntendam, M. Bokhove, E. Melillo, B. W. Dijkstra, R.H. Cool, W.J. Quax, Reducing virulence of the human pathogen Burkholderia by altering the substrate specificity of the quorum-quenching acylase PvdQ, Proc. Natl. Acad. Sci. 111 (2014) 1568–1573, https://doi.org/10.1073/pnas.1311263111.