

Disrupting quorum sensing as a strategy to inhibit bacterial virulence in human, animal, and plant pathogens

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Abstract

The development of sustainable alternatives to conventional antimicrobials is needed to address bacterial virulence while avoiding selecting resistant strains in a variety of fields, including human, animal, and plant health. Quorum sensing (QS), a bacterial communication system involved in noxious bacterial phenotypes such as virulence, motility, and biofilm formation, is of utmost interest. In this study, we harnessed the potential of the lactonase SsoPox to disrupt QS of human, fish, and plant pathogens. Lactonase treatment significantly alters phenotypes including biofilm formation, motility, and infection capacity. In plant pathogens, SsoPox decreased the production of plant cell wall degrading enzymes in *Pectobacterium carotovorum* and reduced the maceration of onions infected by *Burkholderia glumae*. In human pathogens, lactonase treatment significantly reduced biofilm formation in *Acinetobacter baumannii*, *Burkholderia cepacia*, and *Pseudomonas aeruginosa*, with the cytotoxicity of the latter being reduced by SsoPox treatment. In fish pathogens, lactonase treatment inhibited biofilm formation and bioluminescence in *Vibrio harveyi* and affected QS regulation in *Aeromonas salmonicida*. QS inhibition can thus be used to largely impact the virulence of bacterial pathogens and would constitute a global and sustainable approach for public, crop, and livestock health in line with the expectations of the One Health initiative.

Keywords: quorum sensing; bacteria; virulence; biofilm; lactonase; One Health

Introduction

The rise of antimicrobial resistance (AMR) has led the international community to reinforce hygiene measures to limit the spread of infections. Priorities include reducing the use of antimicrobials, which is a major issue, as their excessive use in livestock, human health, and agriculture induces serious AMR concerns worldwide. In this context, the World Health Organization (WHO) has initiated a “Global Action Plan” to tackle AMR (GAP-AMR) (Harbarth et al. 2015, World Health Organization 2015). Developing sustainable and global approaches to reducing bacterial infections with neither toxic side effects nor associated resistance is extremely challenging. Moving from conventional chemical approaches to novel bio-based alternatives is thus an attractive prospect. Bacteriophages, antimicrobial peptides, biological control, and enzymes are appealing candidates for a variety of applications (Nigam et al. 2014, Khardori et al. 2020). Enzymes in particular may be of interest, as their catalytic performances may allow them to be used in small quantities while having a broad effect. Nevertheless, to limit the emergence of AMR, rethinking how to inhibit bacterial virulence remains a major concern. Over the last two decades, enzymes able to disrupt quorum sensing (QS) have gained considerable interest with regards to limiting bacterial infections while not challenging bacterial sur-

vival (Rémy et al. 2016). QS is a bacterial communication process relying on the production and detection of autoinducible signaling molecules (AIs) that bacteria use to coordinate behaviors in a cell-density-dependent manner, including infection-related behaviors. AIs are chemically diverse, including acyl-homoserine lactones (AHLs), furanosyl diester AI-2, peptides, and hormones (e.g. epinephrine) that can be recognized by one or several bacteria depending on the signal (Rémy et al. 2018). Among AIs, special attention has been paid to AHLs, as they are widely used by Gram-negative bacteria, including human, animal, and plant pathogens. Enzymes such as lactonases, acylases, and oxidoreductases have been studied for their ability to interfere with AHL-based QS, a strategy referred to as quorum quenching (QQ). AHLs are signaling molecules presenting a conserved lactone ring and an acyl chain moiety that may vary in length (from C₄ to C₁₈), substitutions (e.g. hydroxyl and carbonyl), and degree of saturation. Basically, AHL-based QS relies on a synthase, usually noted “I,” that produces one or several AHLs that diffuse freely through cell membranes, and a sensor regulator, noted “R” which, upon AHL binding, becomes active and induces the expression of numerous genes that often include the synthase I, thus generating autoinduction (Papenfort and Bassler 2016). As a broad panel of AHLs is involved in QS regulation, QQ enzymes (QQE) require

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Table 1. SsoPox variants and concentration used for each strain.

Strains	Culture medium	SsoPox variants	Concentration (mg ml ⁻¹)
<i>A. baumannii</i>	LB	A	0.1
<i>A. salmonicida</i>	TSB	A	0.5
<i>B. cepacia</i>	SSM	B	0.09
<i>B. glumae</i>	LB	B	0.09
<i>D. chrysanthemi</i>	TSB	C	0.16
<i>D. dadantii</i>	TSB	C	0.16
<i>P. carotovorum</i> (DSM)	TSB	D	0.055
<i>P. carotovorum</i> (Vegepolys)	TSB	D	0.055
<i>P. aeruginosa</i>	MOPS	A	0.1
<i>V. harveyi</i>	AB	C and D	0.5 for both

large substrate acceptance in order to efficiently target numerous bacteria. Here, we aimed to evaluate SsoPox lactonase, a hyperstable enzyme of great interest for biotechnological applications, on a wide panel of AHL-using bacteria. Our objective was to probe the potential of lactonase technology to deal with bacterial threats in different fields of application. As the development of an alternative to antibiotics and conventional antimicrobials is challenging, this study aims to demonstrate that the same lactonase, and its related variants, can be used as a global alternative in a range of important applications. To this end, four variants of the lactonase SsoPox, obtained by semirational engineering strategies, were considered on the basis of their distinct specificities toward AHLs (Billot et al. 2022, Daude et al. 2023). As all bacteria do not necessarily use the same AHLs for their respective QS, a wide panel of pathogenic bacteria involved in plant infections (*Burkholderia glumae*, *Dickeya chrysanthemi*, *Dickeya dadantii*, and *Pectobacterium carotovorum*), human infections (*Acinetobacter baumannii*, *Burkholderia cepacia*, *Pseudomonas aeruginosa*), and animal infections (*Aeromonas salmonicida* and *Vibrio harveyi*) was selected. The ability of these strains to produce AHLs was checked by the identification of an AHL synthase in their respective genomes (Table 1). The chosen enzymatic variants showed different abilities to interfere with pathogenic bacteria. Nevertheless, each strain was sensitive to at least one variant, resulting in alteration of QS-related behaviors in these strains. These results suggest that enzyme-mediated QQ, combining one or more variants, may be considered as a multipurpose approach to strengthening the antimicrobial arsenal, as promoted by the GAP-AMR. Lactonases could thus constitute a sustainable approach to address health threats in the animal–human–environmental interface in line with the One Health initiative (McEwen and Collignon 2018, One Health 2024).

Material and methods
Strains and bacterial cultures

The following strains were used in this study: *A. baumannii* ATCC 17978, *A. salmonicida* ATCC 33658, *B. cepacia* DSM 7288 (ATCC 25416), *B. glumae* DSM 9512, *Chromobacterium violaceum* CV026, *D. chrysanthemi* DV1910-2771 (Vegepolys field isolate from carrot), *D. dadantii* DSM 18020, *P. carotovorum* DSM 30168 (from the German Collection of Microorganisms and Cell Cultures (DSMZ)), *P. carotovorum* DV1708-1686 (Vegepolys field isolate from tomato), *P. aeruginosa* PA14, and *V. harveyi* DSM 19623. All the strains were first precultivated from a single colony in Lysogeny Broth (LB: 10 g l⁻¹ NaCl, 10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, and pH 7), except for *D. dadantii*, *D. chrysanthemi*, and both *P. carotovorum*, which were precultivated in Tryptic Soy Broth (TSB). A culture step was then

performed as indicated below, before the different phenotypic assays. *Chromobacterium violaceum* CV026 was cultivated in LB at 37°C and used as a reporter strain for the detection of AHLs.

Regarding phytopathogenic bacteria, the inoculation of *B. glumae* from a 16-hour preculture was carried out at 1:100 dilution in fresh LB. Subsequently, 3 ml of this culture was poured into 12-well plates and was then incubated for 24 hours at 33°C, with stirring at 350 rpm. The cultures of *D. dadantii*, *D. chrysanthemi*, and both *P. carotovorum* from the DSMZ and Vegepolys collections, were performed from a 6-hour preculture diluted at 1:100 in TSB. A quantity of 1.5 ml of the culture was transferred to 24-well plates and then incubated for 16 hours at 30°C, with agitation at 400 rpm.

For the strains related to human health, *A. baumannii* was inoculated at a 1:100 dilution in fresh LB from a 16-hour preculture. Then, 3 ml of the inoculated culture was poured into 12-well plates and incubated for 48 hours at 37°C under static conditions. *Burkholderia cepacia* was inoculated at 1:1000 from a 16-hour preculture in standard succinate media (Meyer 1978) (6 g l⁻¹ K₂HPO₄, 3 g l⁻¹ KH₂PO₄, 0.2 g l⁻¹ MgSO₄·7H₂O, 1 g l⁻¹ [NH₄]₂SO₄, 0.4 g l⁻¹ acid succinic, and pH was adjusted to 7 with NaOH). A quantity of 2 ml of culture was poured into 24-well plates and incubated for 24 hours at 37°C, with stirring (350 rpm). For *P. aeruginosa*, a 6-hour preculture was diluted 1:1000 in MOPS minimal medium, as described previously (Rémy et al. 2020) (50 mM MOPS, 4 mM Tricine, 50 mM NaCl, 1 mM K₂SO₄, 50 μM MgCl₂, 10 μM CaCl₂, 0.3 μM (NH₄)₆Mo₇O₂₄, 40 μM H₃BO₃, 3 μM Co(OAc)₂, 1 μM CuSO₄, 8 μM MnSO₄, 1 μM ZnSO₄, and adjusted to pH 7 and filter sterilized), complemented with nitrogen (15 mM NH₄Cl), iron (5 μM Fe₂SO₄), phosphate (4 mM K₂HPO₄), and carbon (25 mM glutamate) sources. A quantity of 3 ml of culture was added to 12-well plates and incubated for 24 hours at 37°C with a 350 rpm agitation.

Finally, for the veterinary strains, cultures of *A. salmonicida* were performed from a 16-hour preculture and diluted at 1:1000 in TSB. Cultures were grown for 24 hours at room temperature without stirring in a 24-well plate. *Vibrio harveyi* was precultivated in LB for 16 hours and inoculated 1:100 in autoinducer bioassay (AB) medium (Greenberg et al. 1979) [0.3 M NaCl, 0.05 M MgSO₄, and 0.2% vitamin-free casamino acids (Difco), adjusted to pH 7.5 with KOH and 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]. The cultures were transferred to 24-well plates and incubated for 24 hours at 30°C with stirring (350 rpm).

SsoPox variants were added during the cultures, and their respective concentrations were adjusted according to their specific kinetic parameters. The variant and the concentration used for each strain are indicated in Table 1.

Production of SsoPox enzymes and determination of their concentration

Enzyme production was performed as described previously (Hiblot et al. 2013). Briefly, *Escherichia coli* BL21 (DE3)-pGro7/GroEL strain containing the plasmid of the SsoPox variant (W263I, V82I-A266G-A275Y, V82I-A275G, and V82I-K271L) was used. Variants W263I, V82I-A266G-A275Y, V82I-A275G, and V82I-K271 L are hereafter referred to as A, B, C, and D, respectively. Precultures were incubated at 37°C for 16 hours in LB supplemented with antibiotics (ampicillin at 100 μg ml⁻¹ and chloramphenicol at 34 μg ml⁻¹). Bacteria were then inoculated at 1:100 in ZYP-5052 autoinducer medium supplemented with both antibiotics described above and incubated at 37°C with 180 rpm agitation. When cultures reached

an OD 600 nm between 0.8 and 1, bacteria were induced by adding 0.2% L-arabinose for chaperon production and CoCl_2 at 0.2 mM final concentration, to stabilize the active site of SsoPox lactonase. The temperature was reduced to 23°C and cultures were grown for 20 hours with stirring at 180 rpm. Cells were harvested by centrifugation at $4\,000 \times g$ for 20 minutes at 10°C. Pellets were solubilized in lysis buffer (HEPES 50 mM pH 8, NaCl 150 mM, DNaseI $10\,\mu\text{g ml}^{-1}$, lysozyme $0.25\,\text{mg ml}^{-1}$, and PMSF 0.1 mM). Cells in the lysis buffer were stored at -80°C for at least 16 hours. Thawed cells were sonicated three times for 30 seconds, with an amplitude of 45% (QSonica sonicator Q700 sonicator®, QSonica, USA). Finally, cells were heated to 80°C for 30 minutes and then centrifuged to pellet the debris. The supernatants were collected and saturated with 75% of ammonium sulfate at 4°C for 16 hours to precipitate SsoPox. The enzyme was pelleted down by centrifugation at $10\,000 \times g$ for 15 minutes at 10°C. The pellets were resuspended in 8 ml of HEPES 50 mM pH 8.0 and NaCl 150 mM buffer and then filtered at 0.8 μm . Ammonium sulfate was removed via desalting (HiPrep 26/10 desalting, ÄKTA pure, GE Healthcare, USA ÄKTA pure). The sample was concentrated using 30 kDa centricon, and then injected into a size-exclusion chromatography column (GF Hiload 16/600 Superdex 75 pg) to purify the enzyme. Enzyme purity was then checked by SDS-PAGE and protein concentration was measured using a Bradford assay (Bradford 1976).

Measurement of lactonase activity

Kinetic parameters were determined as previously described (Rémy et al. 2020), by following the degradation of lactones ($\text{C}_4\text{-HSL}$, 3-OH- $\text{C}_4\text{-HSL}$, $\text{C}_6\text{-HSL}$, 3-oxo- $\text{C}_6\text{-HSL}$, $\text{C}_8\text{-HSL}$, 3-oxo- $\text{C}_8\text{-HSL}$, $\text{C}_{10}\text{-HSL}$, 3-oxo- $\text{C}_{10}\text{-HSL}$, $\text{C}_{12}\text{-HSL}$, and 3-oxo- $\text{C}_{12}\text{-HSL}$) at different concentrations (in a range of concentrations between 0 and 2 mM), by each enzymatic variant in a cresol buffered solution (1.25 mM Bicine, 150 mM NaCl, 0.2 mM cresol purple, 10% of DMSO were supplemented for $\text{C}_{10}\text{-HSL}$, and pH was adjusted to 8.3). The degradation of AHLs was followed for 10 minutes at 577 nm in 200 μl using a plate reader (SynergyHT, BioTek). Mean values were fitted to the Michaelis-Menten equation using GraphPad Prism 7.04 software to obtain catalytic parameters.

Detection of AHL

Six-well plates containing 3 ml of LB agar supplemented or not supplemented with enzyme, were prepared to identify AHL production. A quantity of 10 μl of bacterial culture was streaked in line on the agar next to another line of 10 μl of the reporter strain *C. violaceum* CV026 from a 16-hour preculture in LB. This strain produces violacein when exogenous AHLs are detected (McClean et al. 1997). SsoPox action on QS was evaluated by the abolition of violacein production upon degradation of AHL produced by the tested strains. Plates were incubated at room temperature for 48 hours to observe violacein production by CV026.

Assessment of biofilm formation

Biofilm formation was measured for each strain using the crystal violet staining method (Sigma®) (Hoffman et al. 2005). Inoculation for each strain was performed as detailed above in the section "Strains and bacterial cultures." After incubation for 16 hours (*P. carotovorum* and *Dickeya* sp.), 24 hours (*A. salmonicida*, *Burkholderia* sp., *P. aeruginosa*, and *V. harveyi*) and 48 hours (*A. baumannii*) at the appropriate temperatures, planktonic cells were carefully removed from the 12- or 24-well plates. Wells were washed with phosphate buffered saline (PBS) and completely dried at 37°C. Then, 3 ml (for 12-well plates) and 2 ml (for 24-well plates) of

0.05% crystal violet was added to stain the attached biofilm, and each plate was stained for 10 minutes. Crystal violet was gently removed, and the wells were washed with PBS. The fixed crystal violet was finally dissolved in 3 or 2 ml of absolute ethanol, depending on the plate, and 200 μl was transferred to a 96-well plate. Biofilm formation was quantified by measuring the absorbance at OD 595 nm in a microplate reader. The following classification was applied for the determination of biofilm formation: Absorbances below 0.3 indicate the absence of an attached biofilm, absorbances between 0.3 and 1 reveal a weak biofilm formation, and absorbances above 3 are evidence of a strong biofilm (Xu et al. 2016).

Measurement of plant cell wall degrading enzymes

Plant cell wall degrading enzymes (PCWDE) assays were performed by measuring the activity of pectate lyase, polygalacturonase, and cellulase.

The pectate lyase plates were prepared according to a previously reported procedure (Starr et al. 1977). Briefly, the plates were composed of 1% (w/v) polygalacturonic acid, 1% (w/v) yeast extract, 10% (v/v) 0.1% bromothymol blue, and 1.5% agar in distilled water. The solution was heated, without boiling, until all the components were dissolved, and the pH was adjusted to 7. If necessary, the pH was readjusted after autoclave sterilization. Supernatants from overnight cultures treated or not treated with SsoPox were harvested, and 10 μl of the supernatants was added to the plates and incubated for 4 hours at 37°C. Polygalacturonic acid degradation halos were revealed by pouring 4 N HCl on the plates to precipitate nondegraded polygalacturonic acid. Pectate lyase activity in the supernatants was assessed by the presence of clear halos in the dish.

Polygalacturonase activity measurement was performed as follows (Cui et al. 2020). Briefly, plates were poured with a mixture of 0.5% polygalacturonic acid, 0.2% sucrose, 0.2% $(\text{NH}_4)_2\text{SO}_4$, and 1.5% agar. A quantity of 10 μl of cell-free supernatants was spotted on the plates. After incubation for 4 hours at 37°C, the plates were revealed with 4 N HCl. The formation of halos indicated the degradation of polygalacturonic acid by polygalacturonase in the supernatants.

Finally, the detection of cellulase activity was measured, as previously described (Cui et al. 2020). Plates were prepared with 0.1% carboxymethyl cellulose, 0.8% agarose and 0.38% sodium phosphate, and pH was adjusted to 7. A quantity of 10 μl of supernatants was spotted on the plate and after 4 hours of incubation at 37°C the plates were revealed with 5 minutes incubation at room temperature with 0.1% Congo red, followed by two washes with 1 M NaCl. Carboxymethyl cellulose degradation was detected by the formation of a white halo, indicating the presence of cellulase activity in the supernatants.

Swarming motility assay

Swarming motility was monitored for *B. glumae*. Briefly, 3 μl from a 24-hour culture was spotted on 0.7% LB agar supplemented or not supplemented with SsoPox. 24-well plates were incubated at 30°C for 48 hours, maintaining a high humidity atmosphere to avoid agarose drying. Motility areas were measured using ImageJ. Experiments were performed in four replicates.

Virulence assay on onion slices

Red onions (*Allium cepa*), purchased from a local market, were used in this assay to assess the virulence of *B. glumae*. The onions were

disinfected for 15 minutes in a 70% ethanol bath, and thoroughly rinsed with sterile water. A sterile knife was used to cut the onions into six pieces. The slices were separated and perforated with tips prior to infection. Bacteria were centrifuged at $10\,000 \times g$ for 10 minutes and supernatants were removed.

The onions were then infected with $10\,\mu\text{l}$ of culture adjusted to $0.25 \times 10^8\text{ CFU ml}^{-1}$ with PBS. To evaluate the effect of SsoPox B on *B. glumae* virulence, when indicated, onion slices were pretreated with 0.09 mg ml^{-1} of lactonase and left to dry. The onion slices were then infected with bacterial culture. PBS was inoculated as negative control. The onion slices were placed on Petri dishes on a filter paper soaked with 3 ml of sterile water and placed in a box containing paper towel moistened with 30 ml of distilled water to maintain high humidity. The onion slices were incubated in the dark at 30°C for 5 days (Karki et al. 2012). Three onions were used in this assay, and 15 slices were included in each condition.

To quantitatively assess virulence, the macerated tissues were removed using a spatula and the percentage of macerated tissues was quantified by weighing the slices before and after removing maceration (Carstens et al. 2018).

Cytotoxicity of *P. aeruginosa* toward J774.1 macrophages

J774.1 macrophages were used to evaluate the cytotoxicity of *P. aeruginosa*. These macrophages were cultivated in Roswell Park Memorial Institute (RPMI) 1640 broth (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS) and 1% streptomycin and penicillin (Gibco™, Thermo Fisher Scientific) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 . When macrophages reached 75% confluence, cells were harvested in a 50-ml tube and centrifuged for 5 minutes at $750 \times g$ and 4°C . Cells were resuspended in RPMI supplemented with 10% FBS to remove the antibiotics, and then centrifuged again. Finally, cells were placed in a 96-well plate at 10^5 cells per well in $100\,\mu\text{l}$ RPMI with 10% FBS. Cells were incubated for 20 hours at 37°C in a humidified atmosphere containing 5% CO_2 .

Pseudomonas aeruginosa, cultivated as described above, was centrifuged and the pellet was diluted in RPMI broth supplemented with 10% FBS. A quantity of $10\,\mu\text{l}$ of bacteria was added to J774.1 macrophages at a multiplicity of infection (MOI) of 10. Supernatant toxicity was also evaluated by adding $10\,\mu\text{l}$ to the J774.1 macrophages. Centrifugation (10 minutes, $500 \times g$, 4°C) was performed to bring bacteria into contact with the macrophages, before incubating the 96-well plates for 45 minutes at 37°C with 5% CO_2 . Finally, supernatants were collected after a 5-minute centrifugation step at $200 \times g$ and 4°C .

Cytotoxicity was assessed by a CyQUANT™ lactate dehydrogenase (LDH) cytotoxicity assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions, by measuring LDH released by macrophages damaged by bacteria. The negative control was measured by adding PBS, corresponding to 0% cytotoxicity, and the positive control by adding the commercial lysis buffer, corresponding to 100% toxicity, to noninfected macrophage cells.

RNA extraction and QS gene expression measurement

Aeromonas salmonicida was grown as previously described, with or without the addition of SsoPox enzyme. A volume of $200\,\mu\text{l}$ of culture was used for RNA extraction with the RNA PureLink® mini kit (Thermo Fisher). After extraction, a DNase treatment was performed with the TURBO DNA-free™ kit (Thermo Fisher) to elim-

Table 2. Sequences of primers used for RT-qPCR experiments.

Primers	<i>A. salmonicida</i>
<i>recA</i>	Fwd: ATTGCGGAAGCCCAGAAGAA Rev: GTATCCGGCTGGGAGATCAG
<i>asaI</i>	Fwd: ATGGGAGGTAGAAAACGAGCTT Rev: GGCCTTCTTCGTCCTCGAT
<i>asaR</i>	Fwd: ACCAACTGCTTGAGTACCTCG Rev: TGATCAGTGCGAACCGGTAG

inate DNA contamination. Reverse transcription was then performed using the Taqman® Reverse Transcription reagents kit (Thermo Fisher) and random hexamers to obtain cDNA. Finally, qPCR was performed with LightCycler® SYBR® Green I Master (Roche) in CFX thermocycler (BioRad). The primers used are indicated in Table 2. As a control gene, *recA* was used and *asaIR* were targeted as *luxIR* homologs (Swift et al. 1997). qPCR data were analysed using the comparative Ct method (Tinh et al. 2007, Schmittgen and Livak 2008, Pande et al. 2013), to calculate $2^{-\Delta\text{Ct}}$. Statistical analyses were performed with GraphPad Prism 8 software, multiple t-test analyses were used with statistical significance of 0.05. The experiment was performed twice, with three replicates each time ($n = 6$).

Results and discussion

Selection of SsoPox candidates for a broad QQ potential study

The importance of QQE specificity to efficiently tackle QS-regulated bacterial virulence has been thoroughly demonstrated (Koch et al. 2014, Rémy et al. 2020), and reviewed (Murugayah and Gerth 2019, Billot et al. 2020, Sikdar and Elias 2020). These publications underlined that the right QQE may disrupt a precise QS signal with maximum efficacy. Protein engineering strategies were implemented, particularly on the highly robust SsoPox, leading to drastic changes in activities toward AHLs (Bzdrenga et al. 2017, Billot et al. 2020). Hence, to demonstrate the potential of the QQ strategy toward a wide range of pathogens relying on different QS systems, four SsoPox variants, generated by our group (Hiblot et al. 2013, Billot et al. 2022, Daude et al. 2023), with distinct specificities on AHL substrates, were carefully selected and tested from a collection of previously mutated enzymes. Briefly, the collection was rationally designed through site saturation mutagenesis targeting residues involved in substrate binding and active site flexibility, leading to catalytic activity modulation toward AHLs (Hiblot et al. 2013, Billot et al. 2022). Here, four variants were selected from laboratory screenings, referred to as SsoPox A–D (A: W263I, B: V82I-A266G-A275Y, C: V82I-A275G, and D: V82I-K271L), and were thoroughly characterized (Table 3), revealing (i) improved degradation activities toward several AHLs and (ii) distinct and complementary specificities towards different types of AHLs. SsoPox A was selected for its high catalytic activity towards long-chain AHLs such as 3-oxo- C_{12} -HSL, with a $k_{\text{cat}}/K_M > 10^5\text{ M}^{-1}\text{ s}^{-1}$ (Hiblot et al. 2013). This enzyme has already been shown to efficiently tackle the QS-related virulence of various pathogens, notably several strains of *P. aeruginosa* (Hraiech et al. 2014, Guendouze et al. 2017, Rémy et al. 2020), and *C. violaceum* (Mion et al. 2021). This enzyme thus constitutes a good candidate to target pathogens using long-chain AHLs. SsoPox B is a multiple variant with improved specificity towards AHLs carrying a 6-carbon acyl chain, such as C_6 -HSL and 3-oxo- C_6 -HSL ($10^4\text{ M}^{-1}\text{ s}^{-1}$ and $10^3\text{ M}^{-1}\text{ s}^{-1}$, respectively). This variant

Table 3. Kinetic parameters of different SsoPox variants.

Enzyme	Kinetic parameter	C ₄	C ₆	3-oxo-C ₆	C ₈	3-oxo-C ₈	C ₁₀	3-oxo-C ₁₀	3-oxo-C ₁₂
A	k _{cat} (s ⁻¹)	0.37 ± 0.01 [°]	0.46 ± 0.02 [°]	0.18 ± 0.03 [°]	1.03 ± 0.03	0.85 ± 0.04 [°]	0.90 ± 0.13 ^{\$}	0.60 ± 0.09 [□]	1.80 ± 0.05 [□]
	K _M (μM)	4.3 (± 0.1) × 10 ^{3°}	9.99 (± 0.72) × 10 ^{2°}	2.81 (± 0.69) × 10 ^{4°}	1.79 (± 0.22) × 10 ²	8.74 (± 0.84) × 10 ^{2°}	6.64 (± 2.08) × 10 ^{2°}	1.61 (± 0.44) × 10 ^{3°}	1.78 (± 0.49) × 10 ^{3°}
	k _{cat} /K _M (s ⁻¹ .M ⁻¹)	8.6 (± 0.1) × 10 ^{1°}	4.58 (± 0.48) × 10 ^{2°}	4.34 (± 1.79) × 10 ^{1°}	5.75 (± 0.90) × 10 ³	9.69 (± 1.35) × 10 ^{2°}	1.35 (± 0.60) × 10 ^{2°}	3.74 (± 1.17) × 10 ^{2°}	1.01 (± 0.28) × 10^{5°}
B	k _{cat} (s ⁻¹)	0.37 ± 0.04 [‡]	6.70 ± 0.17 [‡]	2.23 ± 0.11 [‡]	2.05 ± 0.11	1.15 ± 0.04	N/D	0.20 ± 0.01	0.08 ± 0.01
	K _M (μM)	9.54 (± 0.20) × 10 ^{2‡}	2.77 (± 0.27) × 10 ^{2‡}	9.40 (± 1.06) × 10 ^{2‡}	4.21 (± 0.61) × 10 ²	6.72 (± 0.62) × 10 ²		2.01 (± 0.30) × 10 ²	6.51 (± 1.80) × 10 ¹
	k _{cat} /K _M (s ⁻¹ .M ⁻¹)	3.92 (± 1.17) × 10 ^{2‡}	2.42 (± 0.30) × 10^{4‡}	2.37 (± 0.39) × 10^{3‡}	4.86 (± 0.96) × 10 ³	1.70 (± 0.22) × 10 ³		9.84 (± 1.96) × 10 ²	1.25 (± 0.42) × 10 ³
C	k _{cat} (s ⁻¹)	2.13 ± 0.08 [‡]	8.42 ± 0.03 [‡]	2.24 ± 0.11 [‡]	1.74 ± 0.13	6.78 ± 1.12	N/D	1.79 ± 0.11	5.62 (± 0.23) × 10 ⁻²
	K _M (μM)	6.17 (± 0.68) × 10 ^{2‡}	1.06 (± 0.01) × 10 ^{4‡}	2.49 (± 0.18) × 10 ^{4‡}	1.37 (± 0.18) × 10 ³	3.57 (± 0.89) × 10 ³		6.84 (± 0.81) × 10 ²	1.15 (± 0.18) × 10 ²
	k _{cat} /K _M (s ⁻¹ .M ⁻¹)	3.45 (± 0.51) × 10^{3‡}	7.96 (± 0.63) × 10 ^{3‡}	9.02 (± 1.08) × 10 ^{2‡}	1.27 (± 0.25) × 10 ³	1.89 (± 0.30) × 10 ³		2.61 (± 0.47) × 10 ³	4.90 (± 0.96) × 10 ²
D	k _{cat} (s ⁻¹)	0.75 ± 0.02 [‡]	8.48 ± 0.14 [‡]	1.29 ± 0.06 [‡]	4.49 ± 0.26	1.85 (± 0.33) × 10 ¹	0.45 ± 0.02	3.00 ± 0.18	0.25 ± 0.011
	K _M (μM)	1.42 (± 0.07) × 10 ^{3‡}	3.55 (± 0.20) × 10 ^{2‡}	9.06 (± 0.84) × 10 ^{2‡}	3.07 (± 0.62) × 10 ²	2.10 (± 0.62) × 10 ³	8.52 (± 2.05) × 10 ¹	2.08 (± 0.54) × 10 ²	5.22 (± 0.65) × 10 ²
	k _{cat} /K _M (s ⁻¹ .M ⁻¹)	2.86 (± 0.22) × 10 ^{2‡}	2.39 (± 0.17) × 10 ^{4‡}	1.43 (± 0.20) × 10 ^{3‡}	1.46 (± 0.38) × 10⁴	8.80 (± 3.06) × 10³	5.25 (± 1.47) × 10 ³	1.44 (± 0.46) × 10⁴	4.69 (± 0.79) × 10 ²

[°]from Rémy et al. (2020).
[‡]from Billot et al. (2022).
[□]from Hiblot et al. (2013).
[‡]from Billot et al. (unpublished).
^{\$}from Mion et al. (2021), N/D: not detected.

is the best candidate so far to target these AHLs. Similarly, variant SsoPox D was selected for its broader specificity towards mid-range AHLs, such as C₆, C₈, and C₁₀ acyl chain AHLs. This enzyme could be qualified as broad-range, since it is the only one to reach a k_{cat}/K_M value $> 10^3 \text{ M}^{-1} \text{ s}^{-1}$ on six different AHLs, and up to $10^4 \text{ M}^{-1} \text{ s}^{-1}$ towards C₈-HSL and 3-oxo-C₁₀-HSL. Finally, SsoPox C was the variant selected to disrupt QS systems relying on short-chain AHLs, such as C₄-HSL. While its catalytic efficiency toward C₄-HSL remains relatively low ($10^3 \text{ M}^{-1} \text{ s}^{-1}$), it is the best activity reported so far for an SsoPox variant, the wild-type enzyme being almost inactive towards this substrate ($11.62 \text{ M}^{-1} \text{ s}^{-1}$) (Hiblot et al. 2013). Furthermore, its efficiency at disrupting the QS-regulated phenotypes of a C₄-HSL model bacterium has been previously established (Daude et al. 2023).

Taken as a whole, these four variants harbour complementary activity spectra, making it possible to tackle heterogeneous QS systems using different kind of AHLs more accurately and specifically. Hence, this narrow selection provides a sound basis for targeting the QS-regulated virulence phenotypes of a large spectrum of microorganisms causing deleterious issues in human health, fish farming, and food crops.

SsoPox inhibits virulence factors in phytopathogens

Many bacterial strains are of critical interest in agriculture and ornamental plant production, due to their pathogenicity. In particular, *Dickeya* and *Pectobacterium* are soft rot-inducing bacteria, present in the top 10 phytopathogenic bacteria (Mansfield et al. 2012), damaging a wide variety of crops (Charkowski 2018). Other bacterial species are also known to be noxious to specific cultures, for instance *B. glumae* to rice (Ham et al. 2011). Here, the ability of SsoPox lactonase to disrupt QS of strains from three relevant genera of phytopathogenic bacteria was evaluated.

Previous studies reported various AHL levels and types for *Dickeya* sp. and *P. carotovorum* spp., such as 3-oxo-C₆-HSL, 3-oxo-C₈-HSL, C₆-HSL, and C₁₀-HSL for the former, and 3-oxo-C₆-HSL, 3-oxo-C₈-HSL, and C₈-HSL for the latter (Crépin et al. 2012). In this respect, SsoPox C and SsoPox D were selected to perform QQ experiments (Table 3). The production of AHLs was checked for each selected phytopathogenic bacterium using the reporter strain *C. violaceum* CV026 (McClean et al. 1997). The production of AHLs was detected for each strain, leading to different levels of violacein production (Fig. 1). In the presence of SsoPox variants, no violacein was produced, indicating that the enzyme effectively degraded the AHLs produced by the phytopathogenic bacteria (Fig. 1).

The production of PCWDEs is a common bacterial virulence factor (Toth et al. 2003) that can be under AHL-mediated QS control (Barnard et al. 2007, Liu et al. 2008). Therefore, to evaluate QQ efficiency, semiquantitative assays were used to detect the activity of three PCWDEs, polygalacturonase, pectate lyase, and cellulase, in the supernatants from cultures of *D. dadantii*, *D. chrysanthemi*, *P. carotovorum* DMSZ30168, and *P. carotovorum* DV1708-1686 (Fig. 1).

The three PCWDE activities were detected in culture supernatants of both *Dickeya* strains, regardless of treatment with SsoPox lactonase, indicating that QQ is not sufficient to abolish the expression of PCWDEs in these strains (Fig. 1A and B). In addition to AHL-mediated QS, *Dickeya* sp. are known to use another signal known as virulence factor modulating (Vfm), which has been shown to be involved in PCWDE production in some strains (Nasser et al. 2013, Helman and Chernin 2015). However, in other *Dickeya* species, such as *D. chrysanthemi*, expression of the *pel* gene was shown to be under the control of C₁₀-HSL (Hosseinzadeh et

al. 2017). The interplay between the two QS systems, AHLs and Vfm, has not been fully described and may be strain-dependent. Here, we showed that AHL degradation did not lead to a decrease in PCWDE production, suggesting that another signal dominates the regulation of this phenotype.

In *Pectobacterium* sp., the three enzymatic activities pectate lyase, polygalacturonase, and cellulase were decreased in the culture supernatants after lactonase treatment (Fig. 1C and D). Specifically, pectate lyase and polygalacturonase activities dramatically decreased, while cellulase activity was only slightly reduced. This result is consistent with previous studies, which demonstrated that AHL-mediated QS regulates PCWDE in *Pectobacterium*, relying on gene inactivation and recombinant lactonase expression experiments (Dong et al. 2001, Smadja et al. 2004, Palmer et al. 2011, Kusada et al. 2017). However, this is the first demonstration that PCWDE production by *Pectobacterium* sp. can be quenched by the exogenous addition of a purified lactonase.

To extend these assays, biofilm formation, another commonly studied virulence factor, was assessed for all *Dickeya* and *Pectobacterium* strains. In previous studies, biofilm and pellicle production by *Dickeya* and *Pectobacterium* was shown to vary between strains and culture conditions (Mee-Ngan et al. 2005, Joshi et al. 2015). In the conditions tested, no biofilm production was obtained and therefore no difference between treatments was observed.

Another phytopathogenic bacterium tested was *B. glumae*, a Gram-negative rice pathogen causing bacterial panicle blight and grain rot (Shew et al. 2019, Choi et al. 2021). This bacterium is responsible for up to 75% of rice production losses in several producer countries, including Japan (Shew et al. 2019, Cui et al. 2021). Due to global warming, *B. glumae* infections could become more common and regular over the coming decades (Shew et al. 2019), especially due to the range of its optimal growth temperature (from 30°C to 35°C) (Mizobuchi et al. 2020). In addition to rice pathogenicity, *B. glumae* is involved in wilting symptoms in sunflowers, tomatoes, sesame, perilla, eggplants, and hot peppers (Jeong et al. 2003), and of rot in onions (Karki et al. 2012). *B. glumae* has been described to produce C₈-HSL through the TofIR QS system (Kim et al. 2004). The production of AHLs was evaluated using the reporter strain CV026. Violacein production was observed in untreated samples. Conversely, no violacein production was observed upon supplementation with SsoPox B, indicating AHL degradation by the lactonase (Fig. 2A). Biofilm formation was assessed, but no biofilm formation was obtained in the tested conditions and no impact of SsoPox treatment was observed (Fig. 2B). Swarming motility, a surface movement initiated by a group of bacteria and driven by rotating flagella (Kearns 2010), was evaluated. After 24 hours of incubation, untreated *B. glumae* were weakly motile and the zone surrounding the inoculation spot displayed a rounded shape. In contrast, bacteria grown on LB agar containing SsoPox B showed increased motility, together with a change in colony pattern, the shape of which became irregular (Fig. S1, Supporting Information). Previous studies showed that motility regulation and efficiency is multifactorial and depends on QS, temperature, and the production of rhamnolipids, among other factors (Nickzad et al. 2015). Here, we report that AHL degradation at 30°C modulates swarming motility. As motility was associated with virulence and pathogenesis of *B. glumae* in rice (Kim et al. 2018), the impact of SsoPox on the ability to infect plants was evaluated using a maceration assay on red onions. This infection model is an alternative to the rice infection model, and enables to assess the virulence of *B. glumae* with a high correlation between virulence in rice and onions (Karki et al. 2012). Onion slices were inoculated with *B. glumae* culture and measure-

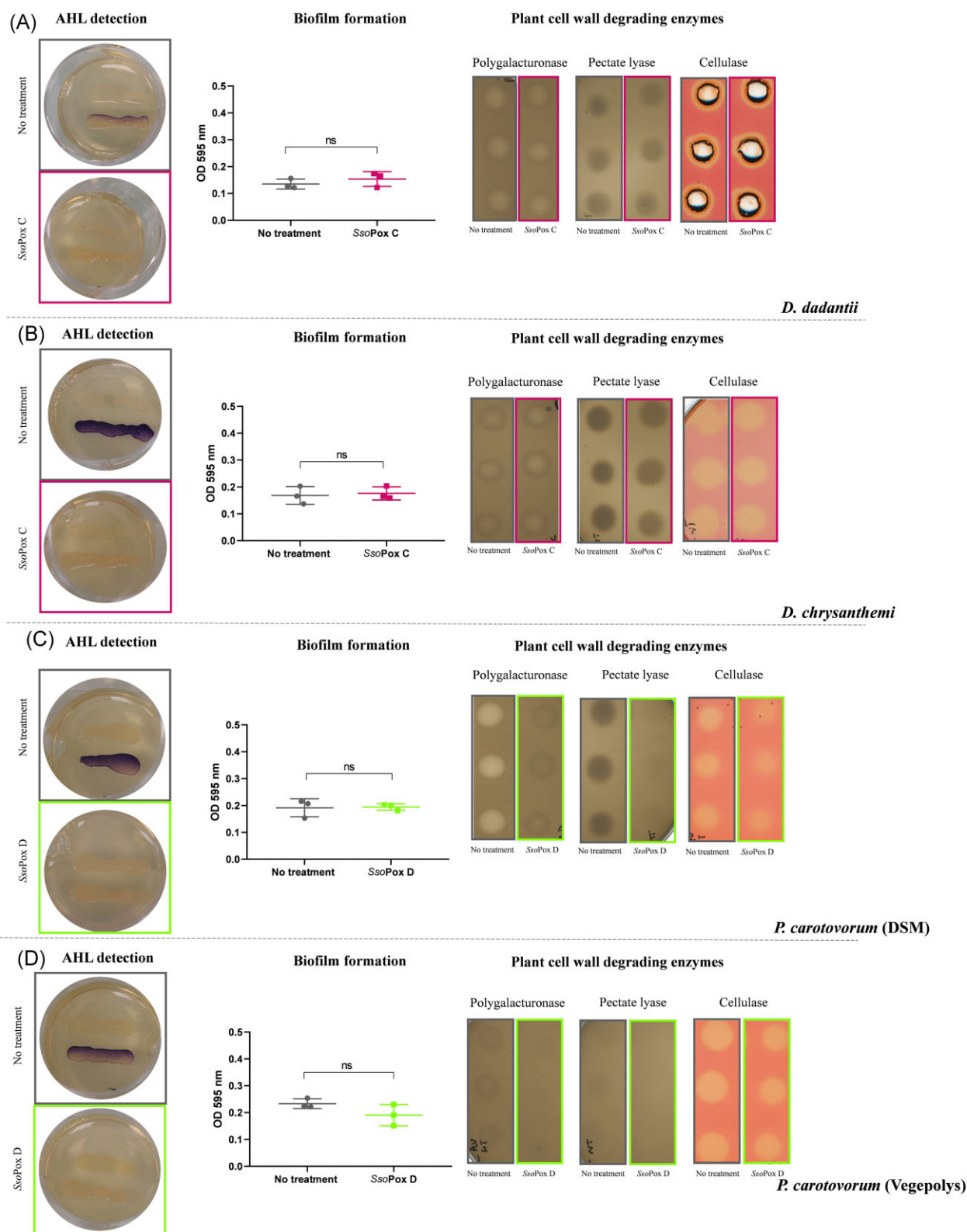


Figure 1. The effect of SsoPox variants on four phytopathogenic strains. Detection of AHL production using CV026, measurement of biofilm formation with crystal violet staining, and impact of SsoPox enzymes on PCWDE on (A) *D. dadantii*, (B) *D. chrysanthemi*, (C) *P. carotovorum* (DSM), and (D) *P. carotovorum* (Vegepolys). Statistical analysis was performed using nonparametric Mann-Whitney test, ns: nonsignificant, on GraphPad Prism 7.04.

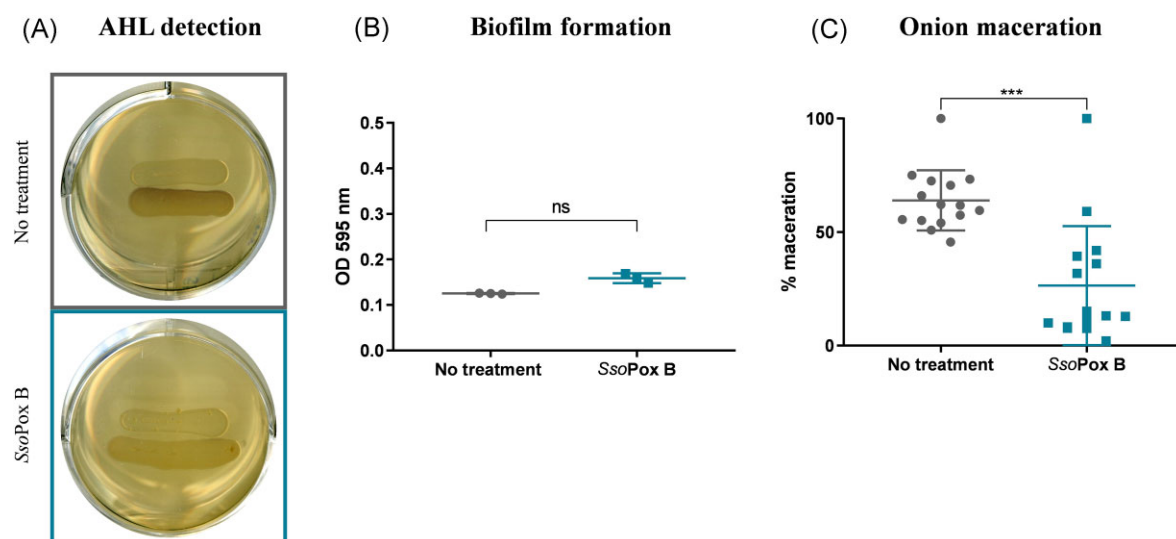


Figure 2. SsoPox B alters *B. glumae* phenotypes. (A) Detection of AHL production using CV026. (B) Measurement of biofilm formation with crystal violet staining. Statistical difference was measured according to Mann–Whitney test on GraphPad Prism 7.04, ns: nonsignificant (C) Onion maceration, $n = 15$ slices for each condition. *** P -value $< .001$ according to Mann–Whitney test on GraphPad Prism 7.04.

ments of the macerated area were performed. Onion slices infected with *B. glumae* were highly macerated (i.e. 64%), demonstrating the ability of *B. glumae* to infect onions (Fig. 2C). In contrast, the pretreatment of onion slices with SsoPox B significantly inhibited the infection by *B. glumae* (P -value $< .0001$), decreasing the average maceration to 26%, corresponding to a 2.5-fold decrease. In summary, these results demonstrate that SsoPox B degraded AHL produced by *B. glumae*, altered motility, and decreased maceration in an onion infection model. Previous studies using *B. glumae* strains expressing the *aiiA* lactonase gene also reported a decrease in AHL production, as well as reduced virulence in rice (Cho et al. 2007, Park et al. 2010). Taken as a whole, these results suggest that QS inhibition decreases the degree of infections caused by *B. glumae* and that motility increase *in vitro* is not necessarily correlated with an increase in maceration symptoms and onion infections. Further investigations *in planta* are needed to confirm the potential of QQ as a biocontrol solution against this phytopathogen.

SsoPox activity against human pathogens

The ESKAPEE group designates pathogens known to escape drug therapies because of their multi resistance to antibiotics (Cieřlik et al. 2021). The acronym ESKAPEE refers to *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* species, and *E. coli*. These pathogens have been classified by the WHO in a priority list of antibiotic-resistant bacteria for which the development of new therapeutic solution is urgent (World Health Organization 2017). Among these species, several bacteria use a QS system to regulate their virulence (Galloway et al. 2012), and the disruption of this system of communication is studied as an alternative to antibiotic molecules (Nigam et al. 2014).

First, we tested the impact of QQ on *A. baumannii*. This Gram-negative bacterium causes a broad array of infections, including pneumonia, urinary tract infections, bloodstream infections, and skin infections, and has been found in many healthcare environments (Bhargava et al. 2010, Antunes et al. 2011). Several lactones were reported in *A. baumannii*, mainly long-chain AHLs varying from C_{10} to C_{16} . 3-OH- C_{12} -HSL have been further described as the

main AHL in *A. baumannii* (Saipriya et al. 2020). AHLs have also been shown to influence biofilm formation which is an important virulence factor involved in antibiotic resistance and survival properties (Bhargava et al. 2010, Mayer et al. 2020). The SsoPox A variant was used to assess its QQ activity against *A. baumannii*. As the kinetic parameters could not be performed on 3-OH- C_{12} -HSL, which is not commercially available, the SsoPox variant was chosen with respect to its activity against long-chain AHLs, including 3-oxo- C_{12} -HSL (Table 3). Culture of *A. baumannii* failed to induce violacein production in the reporter strain CV026 (Fig. 3A). Nevertheless, an AHL synthase *abaI* was identified in the genome of the *A. baumannii* strain used in this study. In addition, *C. violaceum* CV026 is particularly adapted to the detection of AHLs from C_4 to C_8 , and was shown not to produce violacein in the presence of long-chain AHLs, even at high concentrations (McClean et al. 1997). Therefore, biofilm formation was directly evaluated as a QS phenotype. After 48 hours of culture, a high biofilm formation was found in the untreated condition, whereas supplementation with SsoPox A lactonase induced a 4-fold decrease in the quantity of biofilm (Fig. 3A). Similar results were obtained with *A. baumannii* ATCC 17978 and the lactonase Aii20J, significantly decreasing the formation of biofilm (Mayer et al. 2020), as well as in clinical isolates of *A. baumannii* (López et al. 2017), underlining the potential use of QQ as an alternative treatment to fight *A. baumannii* infections.

Burkholderia cepacia, another human pathogen, was then considered. *B. cepacia* is an important pathogen involved in cystic fibrosis (Geisenberger et al. 2000), known to produce AHLs. C_8 -HSL has been described as the main AHL, while the production of C_6 -HSL has also been reported (Riedel et al. 2001, Vial et al. 2007). Here, 24-hour cultures of *B. cepacia* treated or not treated with SsoPox B were used for AHL detection, but no violacein production was observed in any of the samples, although the AHL synthase *cepI* was identified in the genome of the strain used for this assay (Fig. 3B). Therefore, biofilm formation was studied as a direct readout for QS and QQ using crystal violet staining, as previously described. In our conditions, the amount of biofilm was reduced upon treatment with SsoPox B (Fig. 3B). Biofilm formation is a phenotype known to be under QS regulation in members of *B. cepacia* complex, such as

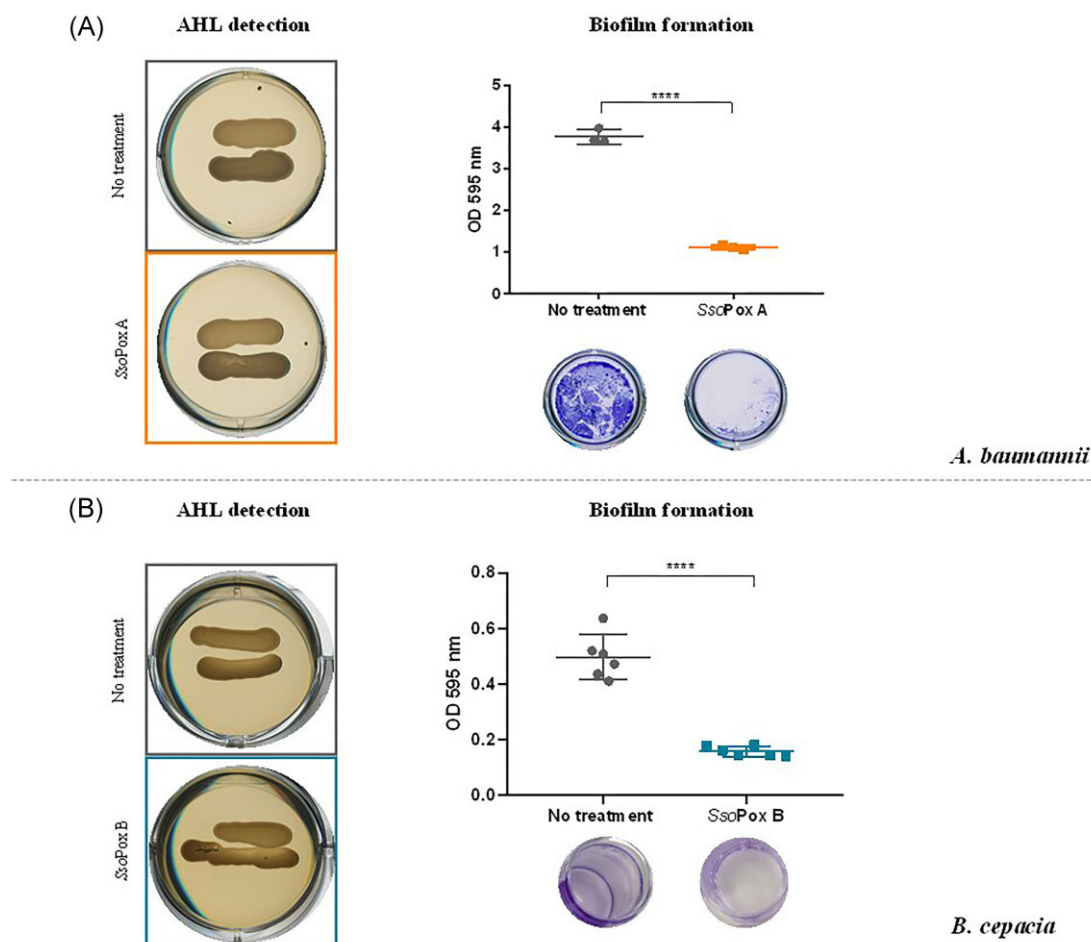


Figure 3. SsoPox A decreases biofilm formation in human pathogenic bacteria. Detection of AHL production using CV026 and measurement of biofilm formation with crystal violet staining with (A) *A. baumannii*. Experiment performed in $n = 3$ replicates. ****P-value $< .0001$ according to Student's t-test on GraphPad Prism 7.04. and with (B) *B. cepacia*. Experiment performed in $n = 6$ replicates. ****P-value $< .0001$ according to Student's t-test on GraphPad Prism 7.04.

B. cepacia H111 (Huber et al. 2001). Here, the results suggest that QQ may also influence biofilm formation in *B. cepacia* ATCC 25416 strain, although further investigations would be required to confirm QQ efficiency on other specific phenotypes.

Finally, another human pathogen, *P. aeruginosa*, was tested. *Pseudomonas aeruginosa* is known to be responsible for hospital-acquired infections, particularly in immunocompromised patients and patients with cystic fibrosis (Eberl and Tümmler 2004, Palleroni 2010). The QS of *P. aeruginosa* PA14 involves two major AHL-based QS systems, the LasIR and RhlIR, which produce C_4 -HSL and 3-oxo- C_{12} -HSL (Li et al. 2022). In this experimental set-up, no AHL was detected by the reporter strain *C. violaceum* CV026 (Fig. 4A), but the same reporter strain detected AHLs in ethyl acetate extractions of *P. aeruginosa* supernatants (Rémy et al. 2020). The addition of SsoPox A, the variant selected for its degrading activity against 3-oxo- C_{12} -HSL (Table 3), induced a 3-fold decrease in biofilm formation (Fig. 4B), confirming previous results obtained with *P. aeruginosa* PA14 and PAO1 (Hraiech et al. 2014, Guendouze et al. 2017, Rémy et al. 2020). The QQ activity of SsoPox A was further evaluated in an *in vitro* cytotoxicity model using macrophages. The bacterial cytotoxicity at a MOI of 10 was assessed toward J774.1 macrophage cell lines. Untreated cultures of *P. aeruginosa* were strongly cytotoxic towards macrophages and 80% cytotoxic-

ity was measured, while upon enzymatic treatment the cytotoxicity against macrophages decreased down to 20%, demonstrating the efficiency of QQ at reducing *P. aeruginosa* cytotoxicity and virulence factor production in this model. The cytotoxicity of cell-free supernatants was also evaluated. Cytotoxicity was detected in untreated supernatants, where 40% of cytotoxicity was measured, while cytotoxicity decreased below 10% in supernatants treated with SsoPox A (Fig. 4C). These results demonstrate the impact of QS disruption by SsoPox A treatment to tackle *P. aeruginosa* virulence in an *in vitro* model. *Pseudomonas aeruginosa* strains were largely used for QQE probing. Using this bacterial model, several studies have demonstrated that QQE may reduce various virulence factors such as motility, biofilm formation, and virulence against model organisms (*Caenorhabditis elegans*, *Drosophila melanogaster*, and mice) (Hraiech et al. 2014, Sikdar and Elias 2020). In addition, recent studies have demonstrated that the use of a QQ lactonase can significantly modulate *P. aeruginosa* PA14 susceptibility or resistance to antimicrobials (Rémy et al. 2020, Sikdar and Elias 2022). The synergistic effect of combining the QQE and antimicrobials (e.g. bacteriophages and antibiotics) to counteract *P. aeruginosa* PA14 virulence was also evaluated demonstrating the promising potential of this enzyme against human pathogenic strains (Mion et al. 2019).

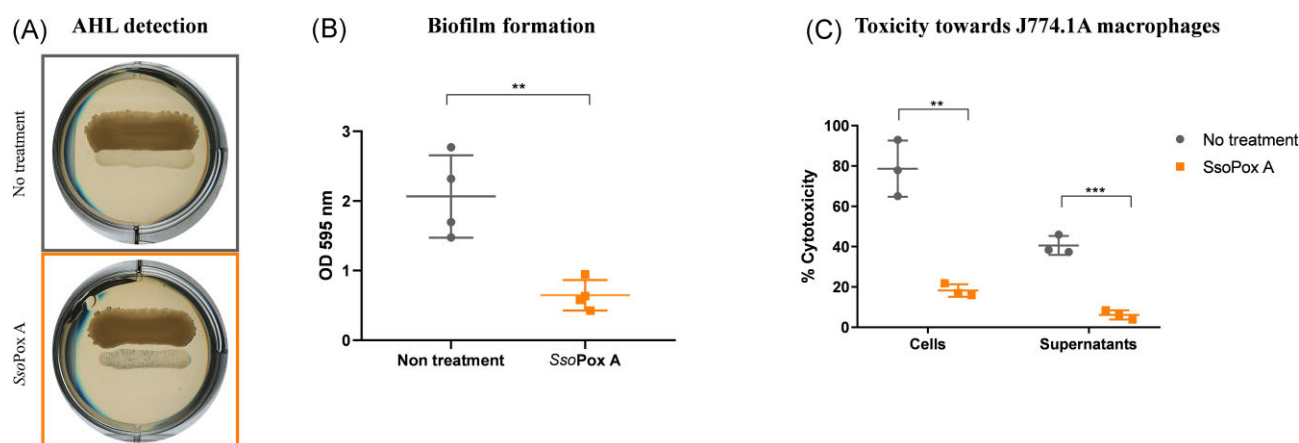


Figure 4. SsoPox A decreases biofilm formation and cytotoxicity of *P. aeruginosa*. (A) Detection of AHL production using CV026. (B) Measurement of biofilm formation with crystal violet staining. Experiment performed in $n = 3$ replicates. ** P-value $< .01$ according to Student's t-test on GraphPad Prism 7.04. (C) Cytotoxicity of *P. aeruginosa* cells and supernatants toward J774.1A macrophages with SsoPox A treatment or not. Experiment was performed in $n = 3$ replicates. ** P-value $< .01$, *** P-value $< .001$ according to Student's t-test on GraphPad Prism 7.04.

SsoPox inhibits virulence factors in aquaculture fish pathogens

In aquaculture, a significant proportion of periodical disease outbreaks is due to bacterial pathogens (Dadar et al. 2017). Among these diseases, vibriosis and aeromoniasis are of crucial interest in aquaculture (Jayaprakashvel and Subramani 2019). To treat these pathogens, vaccination, antibiotics, and bacteriophages are the most studied methods (Defoirdt et al. 2011, Richards 2014, Dadar et al. 2017), and QQ is a developing method, which is being considered to fight bacterial pathogens (Jayaprakashvel and Subramani 2019).

The fish pathogen *A. salmonicida*, was first tested. *Aeromonas* sp. are Gram-negative bacteria living ubiquitously in aquatic environments and are opportunistic pathogens of animals, mainly fish, and humans (Talagrand-Reboul et al. 2017, Vincent et al. 2019). *Aeromonas salmonicida* is responsible for furunculosis and septicemia in fish (Tewari et al. 2014, Kim et al. 2015, Menanteau-Ledouble et al. 2016), and is the cause of severe economic losses in the aquaculture industry (Vincent et al. 2017). *Aeromonas salmonicida* uses two QS systems, the AI-2 and the AHL system, based on AsaIR (Swift et al. 1997, Jangid et al. 2007).

Previous studies have reported that the *A. salmonicida* strain ATCC 33658 mainly produces C₄-HSL and C₆-HSL, as confirmed by thin layer chromatography (Swift et al. 1997, Talagrand-Reboul et al. 2017), as well as C₈-HSL, C₁₀-HSL, C₁₂-HSL, and C₁₄-HSL, as detected by gas chromatography (Cataldi et al. 2007, Talagrand-Reboul et al. 2017).

In this study, the impact of SsoPox A was assessed focusing on AHL production, biofilm formation, and *asaRI* gene expression (Fig. 5). First, the production of AHLs by *A. salmonicida* was confirmed by the reporter strain *C. violaceum* CV026, while the addition of SsoPox A resulted in the absence of violacein production, confirming its ability to degrade the corresponding AHLs (Fig. 5A). To study the impact of QS and QQ on *A. salmonicida* phenotypes, biofilm formation was then measured. Although *Aeromonas* strains are known to produce biofilm (Talagrand-Reboul et al. 2017), no biofilm was detected in our conditions (Fig. 5B). Therefore, the expression of the genes involved in *A. salmonicida* QS system was checked to evaluate the impact of the lactonase on the QS of the strain. The expression of *asaI* was significantly decreased in the presence of SsoPox A, as compared to the control

without the enzyme. However, the expression of the *asaR* gene was not significantly impacted by the presence of the enzyme in comparison to the control without the enzyme (Fig. 5C). These results show that SsoPox is able to affect QS in *A. salmonicida* ATCC 33658. Indeed, AHL degradation by SsoPox prevents AsaR activation by AHL binding, therefore limiting the induction of *asaI* expression. Further impact on *A. salmonicida* virulence remains to be studied but in two other *A. salmonicida* strains, the AsaIR system was correlated to virulence factor modulation. In *A. salmonicida* subsp. *achromogenes*, a Δ *asaI* mutant was not able to produce AHLs, and its virulence was decreased in Arctic charr (*Salvelinus alpinus* L.) (Schwentel et al. 2011). In *A. salmonicida* AE03, also a Δ *asaI* mutant, the production of C₄- and C₆-HSL was abolished, while C₈-, C₁₀-, and C₁₂-HSL were still detected and the maximum biofilm biomass and thickness were decreased (Liu et al. 2018).

A second marine gammaproteobacterium, *V. harveyi*, was then tested. *Vibrio harveyi* naturally lives in marine habitats, especially in warm waters and represents, together with other *Vibrio* species, a serious pathogen of wild and farmed fish and invertebrates (Zhang et al. 2020). Among its virulence arsenal, the productions of extracellular toxins, exoproteases, and siderophores (Defoirdt et al. 2008) are known to be regulated by QS. In addition to its ecological and economic significance, *V. harveyi* uses a QS system that differs from any other Gram-negative bacteria. This QS system relies on three AIs, including the AHL 3-OH-C₄-HSL (HAI-1), that are sensed extracellularly by their cognate sensor kinase, and the QS signal is transmitted to the LuxR regulator via a phosphorelay cascade (Cao and Meighen 1989, Chen et al. 2002, Ng et al. 2011). Finally, *V. harveyi* is also known for its easy QS readout, bioluminescence, which is involved in the milky sea effect (Lapota et al. 1988, Miller et al. 2005). As no degradation signal was obtained with any SsoPox variant using 3-OH-C₄-HSL, no kinetic parameters could be determined. However, two variants identified for their ability to degrade AHLs with short acyl chains were selected, namely SsoPox C and D (Table 3). Similarly, the reporter strain *C. violaceum* CV026 did not detect 3-OH-C₄-HSL, so that no direct evidence for degradation of this AHL by the selected variants could be observed (Fig. 6A; Fig. S2, Supporting Information). Nevertheless, both variants successfully inhibited bioluminescence (Fig. 6C), indicating that QS could be disrupted by SsoPox. In addition, QS disruption was checked using biofilm formation as a second QS biomarker. Interestingly, while biofilm formation was not at all impacted by the

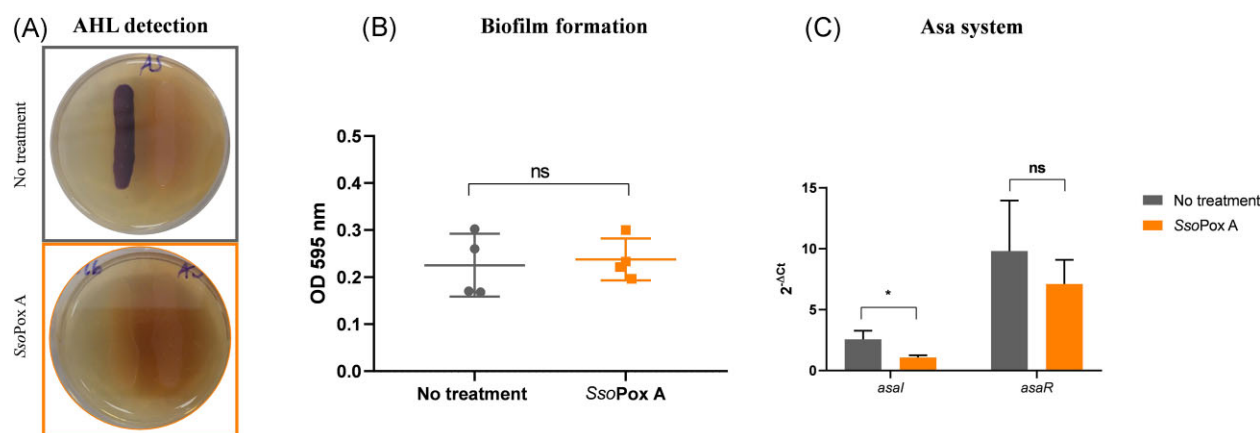


Figure 5. SsoPox A alters QS of *A. salmonicida*. (A) Detection of AHL production using *C. violaceum* CV026. (B) Measurement of biofilm formation with crystal violet staining. ns: nonsignificant, according to Mann–Whitney test on GraphPad Prism 7.04. (C) Relative expression of genes involved in the Asa system after lactonase treatment. The experiment was performed in $n = 6$ replicates. ns: nonsignificant, * P -value $< .05$, according to Multiple t-tests on GraphPad Prism 7.04.

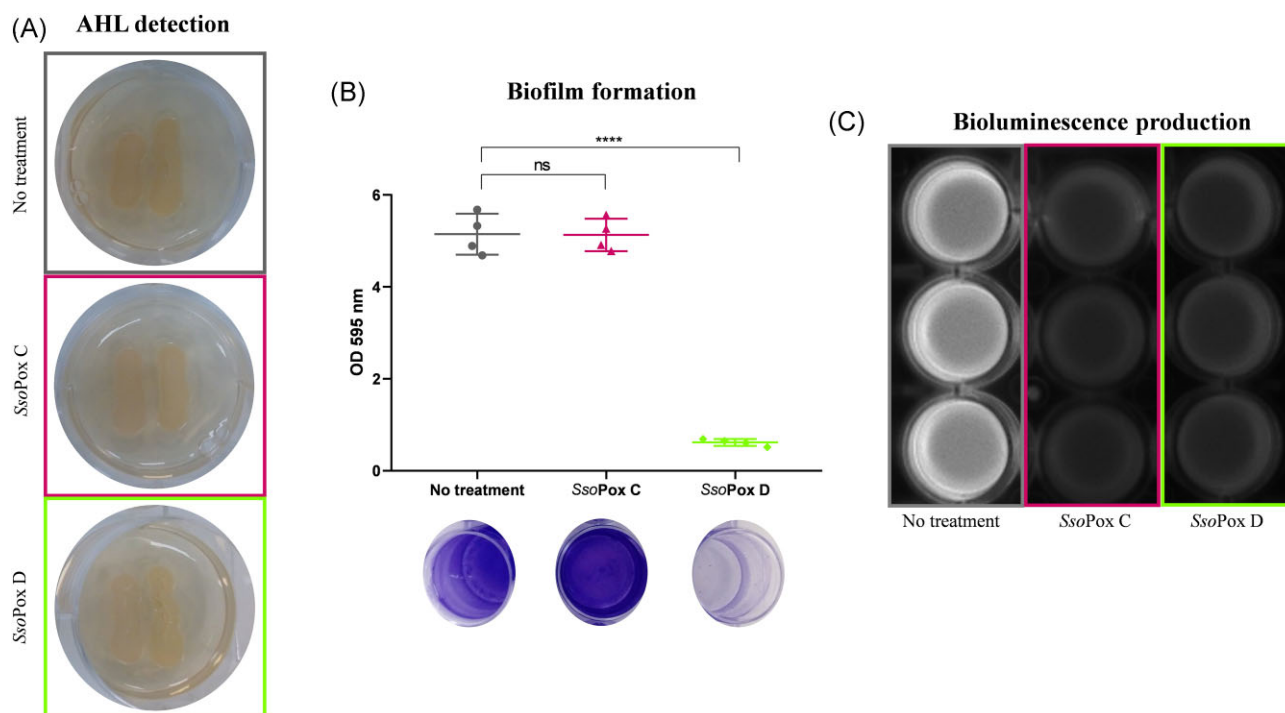


Figure 6. SsoPox alters biofilm formation and bioluminescence in *V. harveyi*. (A) Detection of AHL production using *C. violaceum* CV026. (B) Measurement of biofilm formation with crystal violet staining. The experiment was performed in $n = 4$ replicates. ns: nonsignificant, **** P -value $< .0001$ according to Student's t-test on GraphPad Prism 7.04. (C) Pictures of bioluminescence production.

variant SsoPox C, it was fully abolished using SsoPox D (Fig. 6B), suggesting a major difference in the kinetics of both variants to degrade the AHL used by *V. harveyi*. Taken as a whole, these results indicate that QS can be interrupted even when only one of the signals is degraded, probably by destabilizing the timing of autoinducer production (Anetzberger et al. 2012), or by perturbing the phosphorylation flow and the feedback loops necessary to maintain the robustness of the phosphorelay cascade (Plener et al. 2015, Papenfort and Bassler 2016). In the context of global warming, the increased presence of *Vibrio* species in northern seas could be correlated with the increase in sea surface temperature (Vezzulli et al. 2016, Vezzulli 2022), increasing the risk of disease outbreaks in marine organisms that could spread to humans through

infected water or seafood (Newton et al. 2012, Montánchez and Kaberdin 2020). Many potential vaccines have been developed in recent years against different *Vibrio* sp. but their use has thus far been limited to laboratories (Xu et al. 2022). To study the potential of QQ and particularly that of lactonases as a reliable strategy to limit antibiotic use against vibriosis, *in vivo* studies need to be performed (Tinh et al. 2007, Pande et al. 2013, Noor et al. 2019). Most studies are based on the use of bacterial mutants unable to produce and/or perceive QS signals. Therefore, the outcome might differ from QQ approaches that induce a metabolic sink by constantly degrading AHL produced by bacteria. Indeed, the addition of AHL-degrading bacteria (*Bacillus* sp.) protected giant river prawn larvae (*Macrobrachium rosenbergii*) against *V. campbellii* viru-

lence (Pande et al. 2013), and the direct injection of a lactonase in *Carassius auratus* significantly protected goldfish against infection by *A. hydrophila* (Peng et al. 2021).

Conclusion

Taken as a whole, these results show that lactonase-mediated QS interference is of utmost interest when it comes to targeting the virulence of bacterial pathogens. Although the different bacteria did not respond equally to lactonase treatments, all were nevertheless affected in at least one QS-related phenotype. Depending on the communication molecules used and the bacterial regulatory QS circuit, the very same lactonase can be engineered by specific point mutations to efficiently target a broad range of bacteria and tackle their virulence. *In vivo* studies are needed to reinforce the previous work and assess the effectiveness of SsoPox treatment. To harness this broad-spectrum potential for a variety of applications, enzymes need to be competitive in comparison with conventional approaches, and enzyme engineering seems an attractive way to make this technology cost effective. Moreover, cautious attention must be paid to regulatory requirements, as optimized enzymes issued from genetically modified microorganisms must fit within current regulatory frameworks, such as international regulations on health products, veterinary medicinal products, and plant protection products. Although several aspects need to be considered before reaching industrial applications, this study suggests that lactonases could constitute a sustainable approach to address health threats in the animal-human-environmental interface. These results, combined with the high stability of SsoPox, will pave the way for concrete applications and broaden the arsenal available to fight bacterial infections.

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Author contributions

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Supplementary data

Supplementary data is available at [FEMSPD](https://academic.oup.com/femspd/advance-article/doi/10.1093/femspd/ftae009/7667931) Journal online.

Conflict of interest: MG, BK, PJ, RB, EC, DG, LP and DD report receiving personal fees from Gene&GreenTK during the study. EC, LP, and DD have filed the patents FR3093894, EP3941206, and WO2020187861. RB, EC, LP, and DD have filed the patent WO2023156432. EC, LP and DD are shareholders in Gene&GreenTK. DD is CEO of Gene&GreenTK.

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