Lactonase Specificity Is Key to Quorum Quenching in *Pseudomonas aeruginosa*

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The human opportunistic pathogen *Pseudomonas aeruginosa* orchestrates the expression of many genes in a cell density-dependent manner by using quorum sensing (QS). Two acyl-homoserine lactones (AHLs) are involved in QS circuits and contribute to the regulation of virulence factors production, biofilm formation, and antimicrobial sensitivity. Disrupting QS, a strategy referred to as quorum quenching (QQ) can be achieved using exogenous AHL-degrading lactonases. However, the importance of enzyme specificity on quenching efficacy has been poorly investigated. Here, we used two lactonases both targeting the signal molecules *N*-(3-oxododecanoyl)-*L*-homoserine lactone (3-oxo-Č₁₂HSL) and butyryl-homoserine lactone (Č₄HSL) albeit with different efficacies on Č₄HSL. Interestingly, both lactonases similarly decreased AHL concentrations and comparably impacted the expression of AHL-based QS genes. However, strong variations were observed in *Pseudomonas Quinolone Signal* (PQS) regulation depending on the lactonase used. Both lactonases were also found to decrease virulence factors production and biofilm formation in vitro, albeit with different efficiencies. Unexpectedly, only the lactonase with lower efficacy on Č₄HSL was able to inhibit *P. aeruginosa* pathogenicity in vivo in an amoeba infection model. Similarly, proteomic analysis revealed large variations in protein levels involved in antibiotic resistance, biofilm formation, virulence and diverse cellular mechanisms depending on the chosen lactonase. This global analysis provides evidences that QQ enzyme specificity has a significant impact on the modulation of QS-associated behavior in *P. aeruginosa* PA14.

**Keywords:** quorum sensing, *Pseudomonas aeruginosa*, quorum quenching, acyl-homoserine lactones, lactonases, virulence, biofilm

**INTRODUCTION**

The human pathogen *Pseudomonas aeruginosa* is commonly involved in healthcare associated infections and frequently displays drug or multidrug resistance (Sievert et al., 2013; Weiner et al., 2016). This latter constitutes a serious therapeutic threat. Yet, the quest for new alternative treatments to fight bacterial infections is highly challenging. Disruption of quorum sensing (QS),
a cell-density dependent communication system used by this bacterium to regulate the expression of several traits including virulence, has emerged as a non-bactericidal curative approach to address issues of antibiotics resistance (Fiqua and Greenberg, 2002; Bzdrenga et al., 2017; Rémy et al., 2018).

In *Pseudomonas aeruginosa*, three main QS systems have been described namely Las, Rhl, and PQS (i.e., *Pseudomonas Quinolone Signal*) involving the signal synthases LasI, RhII, PqsABCDEH, and the receptors LasR, RhII, and PqsR, respectively (Lee and Zhang, 2015; Papenfort and Bassler, 2016). These systems use three signaling molecules referred to as autoinducers. Two acyl-homoserines lactones (AHL) N-(3-oxododecanoyl)–L-homoserine lactone (3-oxo-C12 HSL) and butyryl-homoserine lactone (C4 HSL) are used by Las and Rhl systems while PQS is based on 2-alkyl-4-quinolones (Lee and Zhang, 2015; Papenfort and Bassler, 2016). These systems are interconnected and Las is considered as the global activator of all three systems (Latifi et al., 1996; Déziel et al., 2004).

Interferences with QS are termed quorum quenching (QQ) and mainly involve chemical inhibitors (QSIs) or autoinducer degrading enzymes (Grandclément et al., 2016; Rémy et al., 2018). QQ enzymes able to target AHLs belong to three classes: lactonases (EC 3.1.1), acylases (EC 3.5.1) and oxidoreductases (EC 1). Among lactone-degrading enzymes, paraoxonases, metallo-β-lactamase like lactonases (MLL) and phosphotriesterase-like lactonases (PLL) are the main studied families (Elias and Tawfik, 2012). They share a common catalytic mechanism and their differences in AHL substrate preference mainly lie on how the acyl chain can be accommodated into the catalytic site (Elias and Tawfik, 2012; Bergonzi et al., 2019).

In this study, we investigated the effects of two QQ lactonases with distinct AHL specificities, from PLL and MLL families, on *P. aeruginosa* PA14. We used W263I variant of SsoPox, an engineered variant of a PLL isolated from the thermophilic archaea *Saccharolobus solfataricus* (previously *Sulfolobus solfataricus*) that favors long aliphatic lactones as substrates, such as 3-oxo-C12 HSL (Hiblot et al., 2013). Its QQ efficiency for biofilm and virulence factors reduction was previously demonstrated in *P. aeruginosa* with two model strains (PA01 and PA14) and 51 clinical isolates (Hraiech et al., 2014; Guendouze et al., 2017). This lactonase was also shown to reduce mortality in a rat pneumonia model (Hraiech et al., 2014; Guendouze et al., 2017). This lactonase was also shown to reduce mortality in a rat pneumonia model (Hraiech et al., 2014; Guendouze et al., 2017). The MLL GcL isolated from *Parageobacillus caldoxylosilyticus* which was recently characterized and exhibits broad AHL specificity was chosen as the second lactonase (Bergonzi et al., 2016, 2019; Mahan et al., 2020). SsoPox W263I and GcL, which mainly differ in their ability to hydrolyze C4 HSL, were used independently and in combination, to disrupt QS of *P. aeruginosa* PA14 and to investigate the role of lactonase specificity on phenotypes, gene expression and proteome regulation. This work constitutes an extensive molecular and phenotypic comparative study of enzyme-based QQ in *P. aeruginosa* which highlight the importance of lactonase specificity in QQ treatment.

### RESULTS

#### Lactonase Specificity on *P. aeruginosa* AHLs

Using an *in vitro* colorimetric assay, the ability of SsoPox W263I and GcL to degrade synthetic *P. aeruginosa* AHLs was first investigated and the kinetic parameters of both enzymes were determined (Table 1). GcL efficiently hydrolyzed both C4 and 3-oxo-C12 HSL, whereas SsoPox W263I efficiently degraded 3-oxo-C12 HSL but exhibited poor activity toward C4 HSL as indicated by $k_{cat}/K_M$ values. SsoPox W263I is 930 times more specific for 3-oxo-C12 than for C4 HSL whereas GcL displays catalytic efficiencies ($k_{cat}/K_M$) in the same order of magnitude for both substrates. While both enzymes demonstrated similar catalytic efficiencies on 3-oxo-C12 HSL, SsoPox W263I showed stronger affinity for this substrate than GcL (lower $K_M$). Moreover, enzymes strongly diverged regarding their ability to degrade C4 HSL by more than two order of magnitude in favor of GcL. At saturating concentration of substrate, GcL and SsoPox showed only a 3-fold difference in specific activities (U·mg$^{-1}$) on 3-oxo-C12 HSL, while GcL displayed a 133-fold higher activity on C4 HSL as compared to SsoPox W263I in the conditions used for this experiment. Although, it can also degrade C4 HSL, SsoPox W263I is mainly active on 3-oxo-C12 HSL while GcL can almost equally degrade both substrates. To take into account protein addition in the culture medium, SsoPox variant 5A8, which demonstrated no detectable activity on any AHL (Table 1) was used as a negative control, in the same amounts as active enzymes (Bergonzi et al., 2019).

#### Both Quorum Quenching Lactonases Similarly Impact AHL Systems but Differently Influence PQS

To decipher the impact of lactonases, mainly differing in their activity toward C4 HSL, on the QQ of PA14, SsoPox W263I and GcL were used, alone or in combination, at adjusted concentrations in order to have the same activity regarding the substrate 3-oxo-C12 HSL (i.e., equal number of enzymatic Units per volume) in the MOPS minimal medium cultures. PA14 growth in MOPS medium was followed over 26 h. After 20 h, cells in the culture reached stationary phase in all tested conditions (Supplementary Figure 1). Moreover, lactonase activities in the culture medium were not impacted after 20 h, therefore this time point was chosen for further experiments (Supplementary Figure 2).

In order to evaluate the respective impact of both lactonases, alone or in combination, on AHL quantities, the concentration of both C4 HSL and 3-oxo-C12 HSL in the culture medium was estimated using reporter strains. Surprisingly, although the lactonases were proved to have very distinct specificity toward AHLs, neither C4 HSL nor 3-oxo-C12 HSL were detectable (Figures 1A,C). To further investigate the potential different role of the lactonases on PA14 regulation, the expression of major QS genes was monitored by RT-qPCR. Consistently
with AHL measurements, both enzymes similarly impacted Las and Rhl systems (Figures 1B,D). lasR expression was significantly decreased while last expression was not impacted and the expression of both rhlI and rhlR was significantly decreased. Nevertheless, strong variations between lactonases were observed regarding PQS system. Although, pqsR expression was decreased by both lactonases, a major difference arose for pqsA, a gene involved in the synthesis of the PQS autoinducer (Figure 1F). pqsA expression was strongly reduced with SsoPox W263I, whereas GcL did not impact pqsA transcript levels as compared to the controls. When treated with both enzymes combined, pqsA expression followed the same trend as with SsoPox W263I alone with a strong decrease in transcript levels (Figure 1F). To confirm these observations at the molecular level, the amount of 2-heptyl-3,4-dihydroxyquinoline (PQS) was measured in culture supernatants. PQS concentration drastically dropped after treatment with SsoPox W263I alone or combined with GcL as compared to controls and treatment with GcL alone (Figure 1E). Interestingly, the level of 4-hydroxy-2-heptylquinoline (HHQ), a precursor of PQS, was also affected and was mainly detected in culture supernatants of PA14 treated by GcL alone (1.5 μM) as compared to other conditions (<0.15 μM) (Supplementary Figure 3).

Altogether, these results demonstrate that lactonases with distinct AHL specificities similarly impacted the tested AHL-based systems but yielded significant changes in PQS production and regulation. To further probe the impact of these variations on PA14 behavior, phenotypic characterization was performed.

### Both Quorum Quenching Lactonases Inhibit the Production of Virulence Factors in vitro

Three representative virulence factors of *P. aeruginosa*, namely pyocyanin, protease and elastase were measured with different enzyme concentrations and combinations. Protease and elastase production decreased with increasing lactonase concentrations, while the addition of inactive variant had no effect (Figures 2A,B). Pyocyanin production slightly increased for the lowest concentrations of enzymes and decreased from 0.04 U.mL⁻¹ (Figure 2C). The highest concentration of enzymes reduced the production of elastase by more than 75% and the production of pyocyanin and protease by more than 95% in all conditions. GcL significantly reduced the production of all three virulence factors at lower concentrations than SsoPox W263I (0.04 U.mL⁻¹ versus 0.4 U.mL⁻¹). Furthermore, the combination of the two lactonases showed combinatory effects and decreased pyocyanin and protease from 0.004 U.mL⁻¹ as compared to each enzyme alone (Figures 2A,C).

Reduction of *in vitro* virulence factor production was thus achieved with both enzymes, GcL being efficient at lower concentrations than SsoPox W263I and the combination of both lactonases showed synergistic effect particularly regarding protease inhibition.

### The Two Quorum Quenching Lactonases Have Differential Abilities to Inhibit in vitro Biofilm Formation and Biofilm-Associated Tolerance to Antimicrobials

Biofilm formation was reduced in a dose-dependent manner in every condition (Figure 3). However, SsoPox W263I showed a greater anti-biofilm effect than GcL. SsoPox W263I nearly completely inhibited biofilm formation (reduction > 99% for SsoPox W263I and of 76% for GcL at 2 U.mL⁻¹) whereas small bacterial aggregates were still observable with GcL even when increasing the concentration up to 7 U.mL⁻¹ (Figure 3). The simultaneous utilization of both enzymes showed a combinatory effect at 0.004 U.mL⁻¹ as compared to each enzyme alone and inhibited biofilm like SsoPox W263I alone, and unlike GcL, at high concentration.

As biofilm may reduce antimicrobial molecules efficacy, we evaluated the role of QQ to increase bacterial susceptibility to antiseptics and antibiotics. Using the minimal biofilm eradication concentration (MBEC) assay, the sensitivity of PA14 biofilms to antimicrobials following lactonase exposure was determined for tobramycin and gentamicin as well as for H₂O₂ (Harrison et al., 2010). In this assay, only adherent cells grown on a peg support are transferred and exposed to antimicrobials. Without antimicrobial application, similar cell quantities were recovered from the peg lid support in every condition (Supplementary Figure 4). However, after pretreatment with SsoPox W263I, PA14 was more sensitive to all three of these antimicrobial agents (Table 2 and Supplementary Figure 4). For PA14 pretreated with SsoPox W263I, the MBEC values were reduced by 10-fold after

| Enzyme | SsoPox W263I | GcL | SsoPox 5A8 |
|--------|-------------|-----|-------------|
| **AHL** | **3-oxo-C₁₂ HSL** | **C₄ HSL** | **3-oxo-C₁₂ HSL** | **C₄ HSL** | **3-oxo-C₁₂ HSL** | **C₄ HSL** |
| Kᵢ (µM) | 40 ± 10 (4.3 ± 0.1) x 10¹ | (4.3 ± 0.1) x 10¹ | 97 ± 9 (9.8 ± 0.7) x 10² | (9.8 ± 0.7) x 10² | N.D. | N.D. |
| kcat (s⁻¹) | 2.9 ± 0.1 | 0.37 ± 0.01 | 10 ± 1 | 43 ± 1 | N.D. | N.D. |
| kcat/Km (M⁻¹.s⁻¹) | (8 ± 2) x 10⁴ | 86 ± 1 | (1.1 ± 0.1) x 10⁶ | (4.3 ± 0.3) x 10⁴ | N.D. | N.D. |

Values are represented as mean ± SD of n = 4 replicates. N.D., not detected. *kcat/Km for 3-oxo-C₁₂ HSL over kcat/Km for C₄ HSL. **Determined at saturating concentration of each AHL. ***Specific activity of GcL over specific activity of SsoPox W263I.
FIGURE 1 | QS molecules and QS gene expression are similarly impacted by the two lactonases except for PQS and its associated synthesis pathway. For each active enzyme or their mixture, 2 U.mL\(^{-1}\) activity on 3-oxo-C\(_{12}\) HSL was used during culture step. The inactive variant SsoPox 5A8 was used at the same protein quantity as SsoPox W263I. (A,C,E) QS molecules measurement after lactonase treatment at 2 U.mL\(^{-1}\) on 3-oxo-C\(_{12}\) HSL. For each condition, all \(n = 4\) independent samples are plotted with mean and standard deviation in colored histogram and black bars. (B,D,F) Relative expression of genes involved in Las, Rhl, and PQS systems after lactonase treatment at 2 U.mL\(^{-1}\) on 3-oxo-C\(_{12}\) HSL. The inactive variant SsoPox 5A8 was added in the same protein quantity as SsoPox W263I. All \(n = 8\) independent samples are plotted with mean and standard deviation as colored histograms and black bars. Statistical significance according to Sidak’s multiple comparison test was highlighted by black stars (multiplicity adjusted \(p\) value < 0.05*, < 0.01**, < 0.001***). When significantly different from the inactive SsoPox 5A8 control, fold change was added on top of the data.

relatively short exposure periods of 1.5 h to H\(_2\)O\(_2\) and 3 h for antibiotics. GcL, in contrast, only impacted H\(_2\)O\(_2\) sensitivity with a 2-fold reduction of the MBEC value (Table 2). The combined pretreatment with both lactonases increased sensitivity for all three antimicrobials with a similar-fold change as SsoPox W263I alone (Table 2 and Supplementary Figure 4). QQ enzymes
thus enhanced the efficacy of antimicrobial treatments in PA14. However, SsoPox W263I exhibited much greater ability than GcL (from 5- to 10-fold) to increase antimicrobial sensitivity of *P. aeruginosa* biofilms.

Biofilm formation and associated antimicrobial sensitivity were differently impacted by lactonases. Only SsoPox W263I decreased biofilm formation below detection limit and lowered MBEC values for both antibiotics and H$_2$O$_2$. Combining both...
Biofilm formation is reduced by GcL and undetectable with SsoPox W263I. (A) Biofilm formation was measured using crystal violet staining. For each active enzyme or their mixture, an equivalent activity on 3-oxo-C₁₂HSL was used. The inactive variant SsoPox 5A8 was used at the same protein quantity as SsoPox W263I. All n = 8 independent samples are plotted with their mean and standard deviation as colored histograms and black bars. Statistical significance according to Holm-Sidak’s multiple comparison test are highlighted by black stars (multiplicity adjusted p value < 0.05*, < 0.01**, < 0.001***). (B) Corresponding pictures of crystal violet stained biofilm after lactonase treatment for each concentration and conditions.

Both Quorum Quenching Lactonases Reduce Competing Capability of P. aeruginosa Toward Escherichia coli

The capacity of PA14 to compete with other prokaryotes was evaluated using Escherichia coli as bacterial competitor over 24 h (Allsopp et al., 2017). In the absence of QQ treatments, PA14 reduced the population of living E. coli by more than 10-fold as compared to E. coli alone (Figure 4). However, with any QQ treatment at 2 U.mL⁻¹, E. coli was not killed and a similar number of living cells were recovered as to E. coli control (Figure 4). Thus, both QQ lactonases, alone or combined, reduced with similar magnitude the competition of P. aeruginosa against another prokaryote, regardless of their substrate specificity.

Only the Lactonase With Substrate Preference for 3-Oxo-C₁₂ HSL Reduces Virulence in an Amoeba Infection Model

To evaluate the QQ impact of both lactonases on PA14 virulence toward a eukaryotic host, an in vivo amoeba assay was performed. This assay is based on the ability of Acanthamoeba polyphaga Linc AP1 to grow in the presence of quenched or non-quenched bacteria (Fenner et al., 2006; Mion et al., 2019). In both controls, amoebas were not able to grow in presence of PA14 demonstrating its virulence toward A. polyphaga (Figure 5). After quenching by SsoPox W263I, virulence toward amoebas was reduced in a dose–response manner and A. polyphaga was able to propagate even at the lowest enzyme concentration tested (Figure 5A). Conversely, GcL did not restore the ability of amoebas to grow, even at the highest concentrations (Figure 5B). Finally, when both enzymes were mixed, virulence was reduced to a degree comparable to that of SsoPox W263I alone (Figure 5C). Thus, in this eukaryotic virulence assay, these two lactonases with distinct substrate specificity had different impacts; SsoPox, even
when used at 17,500-fold lower concentration, showed greater effects than Gc.

**Treatment of PA14 With Different Lactonases Leads to Distinct Proteomic Profiles**

To further investigate the specific effects of signal disruption by the two lactonases and get a broader picture of the differences observed on phenotypes, we conducted proteomic analyses on *P. aeruginosa* PA14 cultures. A total of 515 (8.7%) out of the 5,886 proteins of PA14 were detected and identified (UniProt database). The relative abundances of 210 proteins were significantly changed (fold change \( > 2 \) and \( p_{\text{value}} < 0.05 \)) in at least one of the six possible comparisons (Supplementary Dataset S1). This number is in the same range as previous reports investigating QS effects by proteomic and transcriptomic analyses (Hentzer et al., 2003; Nalca et al., 2006; O’Loughlin et al., 2013; Sethupathy et al., 2016). It provides evidence of the great importance of signal disruption in the regulation of the PA14 proteome. Principal component analysis (PCA) of the 210 proteins resulted in 11 principal components with a cumulative explained variation (\( R^2 \)) of 98% and predicted variation (\( Q^2 \)) of 86% (Supplementary Figure 5). The two first components differentiate three distinct groups accounting for most of the sample variations (cumulated \( R^2 \) of 70%) (Supplementary Figures 5A,C). The first principal component highlights the significant difference between the control (SsoPox 5A8) and the three QQ treatments. The second principal component discriminates the treatments using either SsoPox W263I or both enzymes as opposed to GcL alone.

The 210 proteins with altered abundance were clustered by biological functions according to PseudoCAP classification (Winsor et al., 2016) (Figure 6A). 29% are associated with metabolic pathways including cofactor, nucleotide or amino acid biosynthesis, as well as the metabolism of energy, carbon compounds, or fatty acids. Proteins related to translation and post-translational modification as well as chaperones and heat shock proteins accounted for 14.3% of altered proteins. Membrane associated proteins represent 11.9% of the impacted proteins and specifically include porins and proteins involved in efflux or protein export. Other affected proteins relate to adaptation, protection and secreted factors (8.6%) and transcriptional regulator or transcription related proteins classes (5.7%). Changes of abundance in proteins involved in motility, attachments and chemotaxis (4.3%), cell wall/LPS/capsule (2.9%) and DNA processing and cell division (2.9%) were also observed. Finally, the remaining impacted proteins (20.5%) were categorized as hypothetical, unclassified, unknown proteins and putative enzymes.

Analyzing fold changes, strong variations were observed between GcL and SsoPox W263I treatments (Figure 6B). The impact of lactonases on QS-involved proteins was first evaluated. Although, LasI/R, RhlI/R, and PqsR proteins were not detected, probably due to concentrations below the detection limit of the method, proteins of the PQS synthesis pathway were detected and their level were strongly altered as a function of the used lactonase. The level of PqsD was only reduced with SsoPox W263I treatment (fold change of \(-5\) and \(-7\) vs. control and GcL respectively) whereas PqsE, an effector protein encoded by the *pqsABCDE* operon but not mandatory for PQS synthesis, was found more abundantly in the proteomic profile of PA14 treated with GcL (fold change of \(+17\) and \(+44\) vs. control and SsoPox W263I) (Drees and Fetzner, 2015; Rampioni et al., 2016).

Several proteins involved in biofilm formation were highlighted by the proteomic analysis. PA14_61190 (PA4624/CdrB), transporter of the adhesin CdrA, was decreased with both lactonases but to a stronger degree with SsoPox W263I as compared to GcL (fold change of \(-13\) and \(-2\) vs. control and GcL) (Borlee et al., 2010; Reichhardt and Wong, 2018).
Similarly, the abundance of PA14_01490 (PA0122/RahU), a Rhl activated lipid binding protein, was decreased with both lactonase treatments but to a greater extent with SsoPox W263I (fold change of $-959$ and $-107$ vs. control and GcL) (Miklavič et al., 2015). Conversely, GcL induced a stronger decrease of Tsp (or AlgO), a protein part of alginate synthesis regulation, than SsoPox W263I (fold change of $-115$ and $-35$ vs. control and SsoPox W263I) (Qiu et al., 2007; Hay et al., 2014; Delgado et al., 2018). However, AlgU, the alginate regulator, and OprF, a pleiotropic porin also involved in biofilm formation, were only significantly reduced with SsoPox W263I (Hay et al., 2014; Chevalier et al., 2017). On the other hand, Ndk, a protein related to alginate synthesis and DppA1, related to aggregation, were both only increased with GcL (Sundin et al., 1996; Kim et al., 1998; Lee et al., 2018).

Several proteins involved in oxidative stress response and antibiotic sensitivity exhibited a modified expression level. The abundance of three proteins (PA14_18690, OpdB, and OprG) were increased and seven others (PA14_47550, PA14_18330/50, ObgE, MexG, MexH, and OmpD) were reduced with both treatments as compared to the control (Aendekerk, 2005; Breidenstein et al., 2008; Dotsch et al., 2009; Balasubramanian et al., 2013; Verstraeten et al., 2015; Sakhtah et al., 2016; Chevalier et al., 2017; Gaviard et al., 2018; Wolloscheck et al., 2018). Several other protein levels showed differences between the two lactonase treatments. The abundance of five proteins (OprF, OprM, HlfK, OmpH, and Ppk) were reduced only with SsoPox W263I while two others (OprD and OprH) were increased only with GcL treatment (Fraley et al., 2007; Dotsch et al., 2009; Lister et al., 2009; Hinz et al., 2011; Somprasong et al., 2012; Chevalier et al., 2017; Sanz-García et al., 2019).

Regarding proteins associated with virulence, both lactonase treatments reduced the abundance of PA14_48590 and PA14_05510 (PA0423/PasP in PAO1) (Marquart et al., 2005; Feinbaum et al., 2012). SsoPox W263I and GcL also reduced the levels of phenazine synthesis pathway enzymes PhzB, D, E, F and the known phenazine transporter MexGHI-OpmD as compared to the control (Sakhtah et al., 2016). Interestingly, a stronger reduction was observed with SsoPox W263I than with GcL on PhzB and D, as well as a drastic decrease of PhzM with SsoPox W263I only (fold change of $-77$ and $-14$ vs. control and GcL). Changes in protein abundance levels were also observed in type 3 secretion system (T3SS) structural and effector proteins (CtpA, PopD, SpcU, and ExoU), associated with virulence toward amoeba, they were more abundant upon treatment with GcL as compared to SsoPox (Pukatzki et al., 2002; Matz et al., 2008; Seo and Darwin, 2013). Important fluctuations in the abundance of H3 type 6 secretion system (H3-T6SS) proteins, a system...
FIGURE 6 | Fluctuations in P. aeruginosa PA14 proteomes reflect enzyme-mediated phenotypic changes. For each active enzyme or their mixture, 2 U.mL$^{-1}$ activity on 3-oxo-C$_{12}$ HSL was used. The inactive variant SsoPox 5A8 was used at the same protein quantity as SsoPox W263I. (A) Global modifications of PA14 proteome in response to lactonase treatment. The mean of normalized intensities for each condition are plotted. Only the 210 significantly changed proteins were used. The proteins are grouped according to PseudoCAP classification (http://www.pseudomonas.com). *Metabolism: Amino acid biosynthesis and metabolism/Biosynthesis of cofactors, prosthetic groups and carriers/Carbon compound catabolism/Central intermediary metabolism/Energy metabolism/Fatty acid and phospholipid metabolism/Nucleotide biosynthesis and metabolism. (B) Highlighted modifications of PA14 proteome in response to lactonase treatment. Heat map of the log$_{10}$ relative fold change mean for the six comparisons.
which could participate in competition toward eukaryotic and prokaryotic organisms (Lesic et al., 2009; Sana et al., 2013; Jiang et al., 2014), were also detected. The abundance of HsiB3 was higher in lactonase treated samples than in control conditions especially in SsoPox W263I (fold change of +170 and +43 vs. SsoPox W263I and GcL). However, Hcp3 was only reduced with SsoPox W263I treatment (fold change of −10 vs. control).

Altogether these results evidence the large effects of AHL signal disruption using QQ lactonases on the proteome of PA14. Furthermore, these results emphasize that QQ effects between lactonases SsoPox W263I and GcL are distinct while their mixture behaves similarly to SsoPox W263I alone.

**DISCUSSION**

In this study, we used two different QQ lactonases, SsoPox W263I that proficiently degrades 3-oxo-C12 HSL, but has lower activity toward C4 HSL, and GcL, a broad-spectrum lactonase that degrades both lactones with high proficiency. Using these two molecular tools to disrupt the QS circuits of *P. aeruginosa*, we collected phenotypic and molecular evidences that lactonase specificity toward AHLS modulates QQ outputs in *P. aeruginosa*.

First, the effects of both lactonases on PA14 autoinducer concentrations and QS gene expression were investigated. Despite a significant difference in C4 HSL hydrolase activity, both enzymes similarly reduced C4 HSL and 3-oxo-C12 HSL concentrations, consistently the expression levels of las and rhl genes were comparable with both lactonases. A similar reduction of 3-oxo-C12 HSL and Las system is consistent with the fact that enzymes were used at similar activity levels toward this substrate. However, similar reductions of C4 HSL and Rhl systems is more surprising as SsoPox W263I is less active than GcL against this AHL. The efficient reduction of C4 HSL observed with SsoPox W263I could originate from a slow but sufficient degradation of this AHL throughout the growth of the bacteria leading at the end of the growth to similar results than GcL. Expression of *pqsR* was also reduced with both enzymes suggesting that interfering with AHLS affects other QS systems underlining the interconnection of the three QS systems. We also investigated several pathogenicity-related phenotypes such as pyocyanin, protease, elastase production, biofilm formation and ability to compete. We showed that the lactonase GcL, which is highly proficient against both AHLS, exhibited inhibitory activity at concentrations lower than SsoPox W263I for the production of pyocyanin, protease, and elastase. At concentrations of 0.4 and 2 U.mL\(^{-1}\), these three factors were quenched by either enzymes or the combination of both enzymes, evidencing that QQ of these factors is dose-dependent. At low enzyme concentrations, pyocyanin production was increased for all lactonase treatments. This result is consistent with a previous report in which pyocyanin could be induced using a QSI antagonizing RhIR, probably by alleviating RhIR inhibition of PQS system (Welsh et al., 2015). Similarly, low lactonase concentrations could lead to reduced levels of C4 HSL that partially alleviate RhIR inhibition on PQS and lead to the induction of pyocyanin production. Pyocyanin was the only tested factor to be induced at low enzyme concentrations. Finally, the competition between PA14 and *E. coli* was also equally affected by any enzymatic treatment with a restoration of *E. coli* survival upon lactonase treatment. These results are supported by the proteomic data which showed that levels of proteins involved in phenazine synthesis and the type VI secretion system were similarly impacted by any enzyme or combination. Thereby, lactonase specificity does not impact QQ of these phenotypes which regulation may mainly rely on AHL based QS systems.

Nevertheless, several major differences arose from the different enzymatic treatments. First SsoPox W263I completely inhibited biofilm formation while cell aggregates were still observable with GcL treatment, including at high enzyme concentrations. Thereby the difference in QQ efficiencies concerning biofilm formation does not solely depend on the AHL hydrolytic activity levels, but lactonase specificity plays an important role for its inhibition. Additionally, SsoPox W263I was more efficient than GcL to increase susceptibility of *P. aeruginosa* PA14 biofilm to H\(_2\)O\(_2\) and antibiotics. Likewise, in the eukaryotic competition assay, only the treatment with SsoPox W263I decreased PA14 virulence and restored the growth of amoebas. Consistently with phenotypic results, a fraction of the observed variations in PA14 proteomes were largely different as a function of the lactonase substrate specificity. Specifically, distinct regulations occurred in antibiotic resistance, biofilm formation or virulence related proteins echoing alterations observed in phenotypes between lactonase treatments. Regarding QS gene expression levels, a drastic difference arose with the significant and strong reduction of *pqsA* expression in SsoPox W263I treated samples only. This result is consistent with proteomic observations since the levels of PqsD and PqeE, both encoded in the same operon as *pqsA*, were found less abundant in samples treated with SsoPox W263I as compared to GcL. Subsequently, we measured PQS and HHQ concentration levels in culture supernatants and showed that PQS levels were only decreased upon treatment with SsoPox W263I and that its precursor HHQ was increased upon treatment with GcL, suggesting that lactonases differentially impact QS hierarchy. Because PQS is known to contribute to virulence, biofilm and antibiotic susceptibility, variations in PQS concentrations may be involved in the observed differences between lactonase-induced phenotypes (Häussler and Becker, 2008; Lee and Zhang, 2015; Hazan et al., 2016; Maura et al., 2016; Rampioni et al., 2016; Maura and Rahme, 2017). Thereby lactonase specificity differently impacts QS at the molecular level resulting in different phenotypic outputs of QQ.

Unexpectedly, despite exhibiting a narrower substrate specificity spectrum, the lactonase SsoPox W263I inhibited a wider range of virulence-related phenotypes in PA14 than the broad spectrum GcL. In a final set of experiments, combining both enzymes resulted in the same phenotypes, gene expression profiles and proteomes as observed with SsoPox W263I alone. The differences observed in PA14 behaviors following the applied lactonase treatment may be completely explained by AHL substrate preferences, specifically in regards to 3-oxo-C12 HSL and C4 HSL, of both enzymes. It can also be related to other differences in their catalytic parameters. For example,
SsoPox W263I shows a higher affinity for 3-oxo-C12 HSL than GcL (i.e., lower $K_M$ value, meaning that SsoPox W263I is able to reach its maximum rate of reaction at lower concentration of substrate than GcL), and this might modulate the balance between 3-oxo-C12 HSL and C4 HSL concentrations. It is also possible that some of the observed changes are due to a different, unknown activity of these enzymes since both enzymes are known to be promiscuous. Indeed, both enzymes are known to proficiently hydrolyze various $\delta$- and $\gamma$-lactones and to exhibit other low hydrolytic activities against phosphotriesters (both enzymes) and arylesters (SsoPox W263I) (Merone et al., 2005; Hibi et al., 2013; Bergonzzi et al., 2019).

We demonstrated that the specificity of the used QQ lactonase is important to interference strategies: indeed, while both enzymes are capable of decreasing virulence factors and biofilm formation in vitro, the magnitude of inhibition and their performances in the tested systems, including in vivo, largely vary. Counterintuitively, the lactonase showing the broader activity toward AHLs did not induce the larger QQ impact in PA14. This informs us about the complexity of the interplay between the two AHL QS systems in PA14 and reveals that signal integration by the cell is likely neither linear nor additive. Nevertheless, QQ lactonases constitute promising tools to modulate bacterial communications and associated behaviors including host–bacteria and bacteria–bacteria interactions in complex ecosystems. Lactonases with various specificities should be further assayed on a wider selection of P. aeruginosa strains as its QS network is known to vary from one strain to another and to evolve in contact with hosts and other bacteria (Chugani et al., 2012; Feltner et al., 2016; Kostylev et al., 2019; Mahan et al., 2020). Even though QS regulation varies depending on growth conditions and strains, we provide evidence that more than the dose, the choice of enzyme is crucial to maximize QQ strategies.

**MATERIALS AND METHODS**

**Bacterial Strains**

For enzyme production, *Escherichia coli* BL21 (DE3)-carrying plasmids pGro7/GroEL for chaperones and pET22b with either SsoPox W263I, SsoPox 5A8 (V27G/P67Q/L72C/Y97S/Y263H) or N-terminal strep tagged GcL or 5A8 transformed with the plasmid pGEM®-3Zf(+) (constitutive expression of lacZ alpha peptide) was used in the virulence assay. For AHL measurement, the biosensor strains *Pseudomonas putida* KS35 and *Chromobacterium violaceum* CV026 were used (McCleam et al., 1997; Steidle et al., 2001). For all the phenotypic and proteomic studies, *Pseudomonas aeruginosa* PA14 (UCBBP-14) was used.

**Enzyme Production and Purification**

SsoPox W263I and 5A8 were produced as previously described (Guendouze et al., 2017). Overnight precultures were incubated at 37°C in Luria Bertani (LB) medium (10 g.L⁻¹ NaCl, 10 g.L⁻¹ tryptone, and 5 g.L⁻¹ yeast extract) complemented with chloramphenicol (34 µg.mL⁻¹) and ampicillin (100 µg.mL⁻¹). Then, cultures in ZYP-5052 medium complemented with the same antibiotics were inoculated and incubated at 37°C until optical density at 600 nm reached 0.8–1 (Studier, 2005). At this state, CoCl₂ and L-arabinose were added at a final concentration of 0.2 mM and 0.2% (w/v). Cultures were further incubated at 23°C for another 20 h. Afterward, cells were pelleted down by centrifugation (4,400 × g, 4°C, 20 min) and resuspended in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 0.25 mg.mL⁻¹ lysozyme, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg.mL⁻¹ DNaseI and pH 8.0). The cells were stored overnight at −80°C. The next day, cells were thawed and sonicated three times for 30 s with an amplitude of 45% (Qsonica sonicator Q700). Cell debris was pelleted down by centrifugation (12,000 × g, 4°C, 30 min) and discarded. The supernatant was heated at 80°C over 30 min to precipitate *E. coli* proteins which were removed afterward by centrifugation (12,000 × g, 4°C, 15 min). The remaining proteins were incubated overnight at 4°C in 75% ammonium sulfate in order to precipitate and concentrate SsoPox. After resuspension in HEPES buffer (50 mM HEPES, 150 mM NaCl and pH 8.0), ammonium sulfate was eliminated via desalting (HiPrep 26/10 desalting, GE Healthcare; ÄKTA Avant). The resulting fractions were pooled and concentrated with 10 kDa centricon (Millipore). The proteins were then loaded onto a size exclusion chromatography column (HiLoad 16/600 Superdex™ 75pg, GE Healthcare; ÄKTA Avant) and were eluted in HEPES buffer.

For GcL, cultures were realized in the same conditions, but cells were resuspended in Tris-HCl lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 0.25 mg.mL⁻¹ lysozyme, 0.1 mM PMSF and 10 mg.mL⁻¹ DNaseI and pH 8.0). After being stored overnight at −80°C, resuspended cells were thawed and sonicated twice for 30 s with an amplitude of 45%. Cell debris was pelleted down by centrifugation (12,000 × g, 4°C, 30 min). The crude extract was then loaded onto a Strept-tag column (5 mL StreptTrap HP, GE Healthcare; ÄKTA Avant). The elution was performed in Tris-HCl buffer (50 mM Tris-HCl, 300 mM NaCl and pH 8.0) complemented with 2.5 mM of desthiobiotin (Sigma Aldrich).

After purification on chromatography column, the GcL or SsoPox containing fractions were pooled and concentrated with 30 kDa centricon (Millipore). The purity of each protein was checked by 12.5% SDS-PAGE separation and the concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

**Lactonase Activity Measurement**

The activity was measured on (L)-3-oxo-C12 and (L)-C4 HSL at ambient temperature using a colorimetric pH based assay (Bergonzzi et al., 2016). Briefly, lactone ring hydrolysis leads to acidification of the solution which is followed by the absorbance modification of a pH indicator (cresol). For kinetic parameters, the degradation of lactones at different concentrations by each enzyme in a cresol buffered solution (1.25 mM Bicine, 150 mM NaCl, 0.2 mM CoCl₂, 0.25 mM cresol purple, 3.5% or a minimum of 1.5% DMSO for respectively 3-oxo-C12 and C4 HSL, and pH 8.3) was followed in 200 µL at 577 nm using a plate reader (SynergyHT, BioTek). 5 µg of SsoPox W263I and 1.6 µg GcL
were used on 3-oxo-C12 HSL and 50 μg of SsoPox W263I and 1.6 μg of GcL on C4 HSL. For specific activity determination, one saturating concentration of AHL was tested (8 mM for C4 HSL and 1 mM for 3-oxo-C12 HSL). One enzymatic unit (U) corresponds to 1 μmol of substrate hydrolyzed per minute. For SsoPox 5A8, up to 500 μg of protein were used and it proved active toward neither C4 nor 3-oxo-C12 HSL.

**Culture Media and Conditions**

Selected medium and culture conditions were adapted from Welsh and Blackwell (2016). Briefly, *P. aeruginosa* PA14 was precultivated in LB in a 25 cm² culture flask (Corning) and incubated over 5–6 h at 37°C with a 300 rpm agitation (Titramax 3000, Heidolph) to then inoculate cultures at 1/1,000. Cultures incubated over 5–6 h at 37°C correspond to 1 μM HSL and 1 mM for 3-oxo-C12 HSL. One enzymatic unit (U) corresponds to 1 μmol of substrate hydrolyzed per minute. For SsoPox 5A8, up to 500 μg of protein were used and it proved active toward neither C4 nor 3-oxo-C12 HSL.

**AHL Extraction and Measurement**

Acyl-homoserine lactone extraction was performed using a modified method of ethyl acetate liquid-liquid extraction (Elasri et al., 2001). From 1 mL of MOPS bacterial culture, cells were removed by centrifugation (10,000 × g, 5 min) and the supernatant was extracted once with ethyl acetate (1:1 v/v). The organic upper phase was evaporated to dryness, and the residues were resuspended into 15 μL of DMSO. As blank, sterile MOPS medium was extracted the same way.

Acyl-homoserine lactones were quantified in the extract using two reporter strains: *P. putida* KS35 and *C. violaceum* CV026. *P. putida* KS35 harbored an integrated transposon carrying a *lasR* gene controlled by a *lac* promoter and a *gfp* gene fused to *lasB* promoter which can be activated by LasR binding 3-oxo-C12 HSL (Steidle et al., 2001). *C. violaceum* CV026 do not produce AHLs (*cvI* inactivated) but able to detect short-chain ABLs (McClean et al., 1997).

For 3-oxo-C12 HSL detection, *P. putida* KS35 was precultivated overnight in LB (supplemented with 50 μg/ml kanamycin) at 30°C and then diluted to 1/10 in 1 mL of fresh LB with 5 μL of extract. After 8 h of culture at 30°C, the fluorescence was measured using a plate reader (SynergyHT, BioTek) with an excitation wavelength of 485 nm and emission detection at 528 nm. For C4 HSL, *C. violaceum* CV026 was precultivated overnight in LB at 30°C and then diluted to 1/1,000 in 1 mL of fresh LB with 10 μL of extract. After 24 h of culture at 30°C, the violacein was quantified using a method based on ethyl acetate extraction (Collins et al., 1980). Briefly, 0.5 mL of cell culture was vigorously mixed with ethyl acetate (1:1 v/v) and then 200 μL of the organic upper phase were transferred into a quartz 96 well plate and the OD at 565 nm was measured. Results for each AHL measurement were plotted after background noise removal.

**PQS and HHQ Extraction and Measurement**

*Pseudomonas* quinolone signal and HHQ were extracted, as previously described for ABLs, from 1 mL of culture medium with equal volume of ethyl acetate. As blank, sterile MOPS medium was used. After evaporation of the organic phase, the residues were resuspended into 100 μL of HPLC-grade methanol.

For screening and measurement, liquid chromatography coupled to mass spectrometry (LC-MS) was used. Water, methanol and formic acid were ULC-MS grade (Biosolve, Dieuze). Analysis was performed with an Acquity I-Class UPLC chromatography system connected to a Vion IMS QToF ion mobility-quadrupole-time of flight mass spectrometer (Waters). Samples were maintained at 4°C and randomly injected (5 μL) into a reverse phase column maintained at 35°C (Acquity BEH C18 1.7 μm 2.1 × 50 mm, Waters). Mobile phase flow rate was 0.5 mL.min⁻¹ and a composition gradient was set as follows: using water (A) and methanol (B) each containing 0.1% formic acid: 30 to 95% of B (2 min), 95% of B (1 min), initial composition (1 min). Compounds were ionized in the positive mode with a Zspray electrospray ion source: capillary/cone 1.5 kV/20 V, source/desolvation 120/250°C. Ions were monitored using a High Definition MS(E) data independent acquisition method combining a traveling wave ion mobility survey and a tandem MS monitoring (50–1000 m/z, 0.1 s scan time, 6 and 20–30 eV for low and high energy alternate scans, automatic lockmass correction using Leucine Enkephalin at m/z 556.2766). The spectrometer was calibrated beforehand (Major Mix, Waters) to enable Collision Cross Section (CCS) and m/z values measurements. Ion components (retention time, ion mobility drift time and parents/fragments m/z values) were collected from raw data using the UNIFIT software (version 1.9.3, Waters). Structures were targeted as followed: 0.2 min retention time window, 2% CCS tolerance (experimental CCS values were 165/167 Å² for HHQ/PQS), 3 ppm m/z tolerance on parent [M + H] + adducts (m/z 244.1696/260.1645 for HHQ/PQS) and 10 mDa m/z tolerance on predicted fragments (including m/z 159.0679/175.06205 for HHQ/PQS). For calibration, HHQ/PQS stock solutions were prepared in methanol from pure standards (> 96%; Sigma Aldrich). Culture medium was spiked with both compounds at concentrations ranging from 2 nM to 20 μM for HHQ and from 4 nM to 40 μM for PQS, every 10-fold. The molecules were extracted in the same conditions as previously described and the calibration curve was fitted point to point in order to estimate the concentrations of HHQ/PQS compounds (<15% deviation on controls).

**RNA Extraction and QS Gene Expression Measurement**

From 200 μL of culture cells pellet, RNA was extracted and purified with PureLink™ RNA Mini Kit (Invitrogen) and
residual DNA were digested with TURBO DNA-Free™ kit (Invitrogen). Then cDNA was synthesized using TaqMan™ Reverse Transcription Reagents (Applied Biosystems) and provided random hexamers. Eventually, qPCR was realized using LightCycler® 480 SYBR Green I Master (Roche), and a CFX96 Touch™ Real Time PCR Detection System (BioRad). Primers used are indicated in Supplementary Table S1. The resulting data for each gene were normalized using housekeeping gene recA expression and analyzed using the 2−ΔΔCt method (Schmittgen and Livak, 2008). The results were plotted as relative expression level by dividing each 2−ΔΔCt values by the mean of the control without added protein.

**Virulence Factors Measurement**

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 were transferred into a quartz 96 well plate. As blank, 200 µL of chloroform were used. The absorbance was measured at 690 nm (Price-Whelan et al., 2007). Results for each condition were plotted after blank absorbance removal.

Protease activity was measured by using azocasein (Sigma Aldrich) degradation assay (Chessa et al., 2000). 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 2 h at 37°C with agitation (300 rpm), 125 µL of 20% (w/v) trichloroacetic acid were added. Then, degraded azocasein was pelleted down by centrifugation (10,000 × g, 5 min). Afterward, 200 µL of supernatant were 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 removal.

Elastase B activity was measured by using elastin-Congo red conjugate (Sigma Aldrich) degradation assay (Smith et al., 2003). In a 96 well plastic plate (Greiner), 50 µL of cell-free supernatant were mixed with 150 µL of elastin-Congo red solution (5 mg.mL−1 in 10 mM Tris-HCl and 1 mM CaCl2 buffer at pH 7.2). After 24 h incubation at 37°C with agitation (300 rpm), the plate was left to rest for 10 min at ambient temperature in order to pellet undigested elastin-Congo red. Afterward, 100 µL of the reaction were carefully transferred into an empty well and then absorbance was measured at 490 nm. As blank, an equivalent volume of sterile MOPS medium was used. Results for each condition were plotted after blank absorbance removal.

**Biofilm Formation Measurement**

Biofilm was measured using crystal violet (Sigma Aldrich) biomass staining (Hoffman et al., 2005). After culture in 12 well plates (Nunc™, Thermo Scientific), planktonic cells were carefully removed by pipetting. Wells were washed with 3 mL of phosphate buffered saline (PBS) solution (Biomériex), dried at 37°C and stained with 3 mL of 0.05% (w/v) crystal violet solution. After removing crystal violet, wells were washed with 4 mL of PBS and fixed crystal violet was dissolved with 3 mL of pure ethanol. Using 200 µL, absorbance was measured at 595 nm. As blank, sterile MOPS medium was used in the same culture conditions. Results for each condition were plotted after blank absorbance removal.

**Antimicrobial Sensitivity Assay**

The effect of tobramycin, gentamicin and hydrogen peroxide on PA14 was evaluated using the MBEC™ assay (Innovotech) (Harrison et al., 2010). As previously described, PA14 was grown in 180 µL MOPS medium in the 96 well plate of the MBEC™ assay device for 24 h at 37°C under orbital agitation (110 rpm). Afterward, the lid was transferred, first to a 96 well plate containing 190 µL of fresh non-complemented MOPS medium to wash the planktonic cells and then transferred to another plate containing antimicrobial agents. Tobramycin and gentamicin were used at concentrations ranging from 0 to 20 µg.mL−1 and H2O2 from 0 to 500 mM. PA14 was exposed under agitation (110 rpm) for 1.5 h and 3 h to H2O2 and antibiotics, respectively. The cells were washed in 200 µL of non-complemented MOPS medium and then transferred into 200 µL of recovery LB (LB supplemented with 20.0 g.L−1 saponin and 10.0 g.L−1 Tween-80). After 1 h, cells were diluted in non-complemented MOPS medium and plated on LB agar. CFU were counted after 3 days growth at 20°C.

**Competition Assay With E. coli**

Competition between P. aeruginosa PA14 and E. coli was evaluated using a modified protocol from Allsopp et al. (Allsopp et al., 2017). The E. coli strain was cultivated in LB with 100 µg.mL−1 of ampicillin in a 25 cm2 culture flask and incubated over 5–6 h at 37°C with a 300 rpm agitation to inoculate LB/ampicillin cultures at 1/1,000. After 20 h in 12 well plate, E. coli and MOPS cultivated PA14 were harvested from 1.2 mL of culture by centrifugation at 10,000 × g for 5 min. The pelletted cells were resuspended into 1.2 mL of PBS and 200 µL were used to evaluate cell density at 600 nm. The remaining 1 mL was centrifuged again, and the cells were resuspended in adequate volume of PBS in order to reach a final OD 600nm of 1 in all samples. E. coli was mixed with PA14 or PBS (control) at a volume ratio of 1:5 (corresponding to a cell ratio of approximately 1:10). Then, 20 µL was spotted onto 0.22 µm pore size hydrophilic PDVF membrane (Durapore® GVWP01300, Millipore) resting on a 1.5% agarose plate. After incubation for 24 h at 37°C, cells were recovered by washing the membrane with 200 µL of PBS. Before and after the incubation, cell counts were realized with serial dilutions in PBS plated on LB agar plate complemented with 80 µg.mL−1 of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

**Virulence Assay Toward Amoeba**

The virulence assay using amoeba was adapted from Fenner et al. (2006) and Mion et al. (2019). Acanthamoeba polyphaga AP1 was routinely cultivated in peptone yeast extract glucose medium (Fenner et al., 2006). After 2–3 days of cultivation at 28°C, the cells were pelleted down at 750 g and resuspended.
in Page’s amoeba saline (PAS) buffer (2 mM NaCl, 16 µM MgSO$_4$, 27 µM CaCl$_2$, 0.53 mM Na$_2$HPO$_4$, 1 mM KH$_2$PO$_4$, pH 6.9). The volume was adjusted to obtain a 10$^5$ cells.m$^{-1}$ final concentration. As for the bacterial growth, after culture in 6 well plates (Nunc$^\text{TM}$, Thermo Scientific), 3 mL of bacterial culture were pelleted down and resuspended in a minimum of 1 mL of PAS buffer. Depending on their concentration in suspension, the buffer volume was adjusted to have the same concentration of bacteria in each condition. Then a PAS agar plate was flooded with 1 mL of bacterial suspension. After drying at ambient temperature, 5 µL of A. polyphaga were spotted at the center and left to dry. Afterward, the plate was incubated at 30°C over 7 days. Each day, amoeba propagation was followed by directly measuring the central spot with a ruler. The results were plotted for each condition from day 0 to 7.

**Protein Extraction**

Cells were harvested by centrifugation (10,000 × g, 5 min) and washed with 2 mL of PBS and centrifuged again (10,000 × g, 5 min). Pellets were resuspended in 100 µL of UTSTS buffer [8 M Urea, 2 M Thiourea, 100 mM NaCl, 25 mM Tris-HCl, pH 8.2 and protease inhibitor (complete, Roche)] and sonicated on ice for 30 s with an amplitude of 15% (Vibra cells) until it became clear. Cell debris was removed by centrifugation (16,000 × g, 20 min) and the supernatant was the supernatant was carefully transferred into a dialysis cassette (Slides Alzyer dialysis cassette 2K MWCO, Thermo Scientific). The cassette was incubated for 4 h in 2 L with Urea/Ambic buffer (1 M Urea, 50 mM ammonium bicarbonate, pH 7.4) and overnight in 2 L of fresh Urea/Ambic buffer. Protein quantity was estimated with Bradford assay (BioRad) and 50 µg of proteins were mixed to Urea/Ambic buffer to a final volume of 50 µL. 1 µL of 0.5 M dithiothreitol in Urea/Ambic buffer was added for the reduction of disulfide bonds and the reaction was conducted over 1 h at 37°C. For alkylation, 2 µL of 0.5 M iodoacetamide in Urea/Ambic buffer were added and let to react over 1 h protected from light. Afterward, pH was checked to be above 7. Protein digestion was performed by adding 2 µL of 1 µg.mL$^{-1}$ trypsin (Agilent) and samples were incubated overnight at 37°C. Digestion efficiency was checked on 10% SDS-PAGE gel. Finally, a detergent removal spin column (Pierce$^\text{TM}$, Thermo Fisher) and a C18 spin column (Pierce$^\text{TM}$, Thermo Fisher) were used to clean the samples.

**Label-Free Quantitative Nano-LC-MS/MS Proteomics Analysis**

In a first step, protein digests were separated by Ultra Performance liquid chromatography (UPLC) using the NanoAcquity UPLC System (Waters) connected to a Synapt G2Si Q-TOF ion mobility hybrid mass spectrometer (Waters). The chromatographic system was used in 1D configuration with an analytical column (ACQUITY UPLC M-Class Trap Column Reversed-Phase 1.7 µm spherical Hybrid, CSH, 75 µm × 150 mm, Waters) after a trapping column (ACQUITY UPLC M-Class Trap Column Reversed-Phase 5 µm spherical silica, 180 µm × 20 mm, Waters). Eluted peptides were separated using a 100 min gradient (300 nL.min$^{-1}$; 0.5 to 40% acetonitrile–0.1% formic acid). Data-independent MS/MS analysis was performed with the ion mobility feature (HDMSe method). The ion source parameters were: capillary voltage 3 kV, sampling cone voltage 40 V, ion source temperature 90°C, cone gas flow 50 L.h$^{-1}$. Transfer collision low energy was set to 5 V, trap collision low energy was set to 4 V. The high energy ramp was applied from 4 V to 5 V for the trap collision and from 19 V to 45 V for the transfer collision enabling fragmentation of the ions after the ion mobility cell and before the time-of-flight (TOF) MS. On-column sample load was 800 ng (2 µL injected). Each sample was injected in duplicate.

**Proteomic Data Processing and Analysis**

The acquired files were imported into Progenesis QI software Version 2.0 (Non-linear Dynamics, Newcastle, United Kingdom) for label-free quantification analysis. The data were aligned automatically and normalized. Processing parameters were 150 counts for the low energy threshold, 30 counts for the elevated energy threshold. The database used to identify peptides contains the protein sequences of Pseudomonas aeruginosa PA14 (Trembl, 25/04/2017, 5,886 sequences). Search tolerance parameters were: peptide and fragment tolerance, 15 ppm, FDR < 1%; Minimum ion matching requirements were three fragments per peptide, seven fragments per protein and two peptides per protein. The enzyme specificity was trypsin allowing 1 missed cleavage, the accepted modifications were carbamidomethyl of cysteine (fixed), oxidation of methionine (variable), carbamyl of lysine and N-terminal (variable), deamidation (variable) of asparagine and glutamine. The protein normalization was performed according to the relative quantitation using non-conflicting peptides. To determine the significance of changes between samples, a significant ANOVA ($P_{\text{value}} < 0.05$) and a fold change superior to 2 were used as the thresholds to define differently expressed protein. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD017421.

The principal component analysis (PCA) was performed on normalized data using SIMCA 14. The data were Pareto scaled, autoscaled for principal components and the Hotelling’s $T^2$ was used to assess the possible presence of outlier. For the heat map, the logarithm with base 10 ($\log_{10}$) of the fold change was calculated. According to the reference condition, either $\log_{10}$ (higher expressed) or $-\log_{10}$ (lower expressed) was used in the representation. Non-significant fold changes ($P_{\text{value}} \geq 0.05$ and/or fold change < 2) were considered to have a value equal to 1 and were represented by a zero on the heat map.

**Statistical Analyses**

For virulence factors, biofilm, competition assay with E. coli and QS gene expression measurement data, statistical analyses were performed using GraphPad Prism 7. The significance level ($\alpha$), or the probability of committing a type I error, was set at 0.05. For all these data, normality distribution was checked with the D’Agostino and Pearson omnibus normality test.
For virulence factors and biofilm, statistical analyses were performed on raw optical density data (without blank removal). A two-way ANOVA was performed according to enzyme treatment and concentration. Then when ANOVA p-value was inferior to 0.05, the Holm-Sidak's multiple comparisons test was used to assess the difference between SoxPox 5A8 (inactive) and SoxPox 72631, GcL or SoxPox 72631 + GcL; SoxPox 72631 and GcL or SoxPox 72631 + GcL; GcL and SoxPox 72631 + GcL for each concentration.

For the QS gene expression, statistical analyses were performed on 2−ΔΔCT (Schmittgen and Livak, 2008). For the competition assay and QS gene expression, a one-way ANOVA was performed on raw optical density data (without blank removal). Then when ANOVA was used and if the ANOVA p-value was inferior to 0.05, the Sidak's multiple comparison test was used to assess the difference between: without protein and SoxPox 5A8 (inactive); SoxPox 5A8 (inactive) and SoxPox 72631, GcL or SoxPox 72631 + GcL; SoxPox 72631 and GcL or SoxPox 72631 + GcL; GcL and SoxPox 72631 + GcL.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the ProteomeXchange via the PRIDE database, accession PXD017421.

AUTHOR CONTRIBUTIONS

BR, LP, ME, DD, and EC designed the research. BR, LP, NA, and PD performed the research. BR, LP, and DD analyzed the data.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: ME and EC have a patent WO2014167140 A1 licensed to Genet&GreenTK. LP, DD, BR, ME, and EC report personal fees from Genet&GreenTK during the conduct of the study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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