Differential modulation of Quorum-Sensing signaling through QslA in *Pseudomonas aeruginosa* strains PAO1 and PA14

Running title: Differential QS modulation by QslA

Sana TG[1,2]*, Lomas R[3], Gimenez MR[1], Laubier A[1], Soscia C[1], Chauvet C[1], Conesa A[4], Voulhoux R[1], Ize B[1], and Bleves S[1]*

[1] LISM (Laboratoire d'Ingénierie des Systèmes Macromoléculaires-UMR7255), IMM (Institut de Microbiologie de la Méditerranée), Aix-Marseille Univ and CNRS, 31 chemin J. Aiguier, Marseille 13402 Cedex 20, France.

[2] Present address: Laboratory of Molecular Microbiology, Global Health Institute, School of Life Sciences, Station 19, EPFL-SV-UPBLO, Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015, Lausanne, Switzerland.

[3] Genomics of Gene Expression Laboratory Centro de Investigación Príncipe Felipe, Valencia, Spain.

[4] Microbiology and Cell Science, IFAS, Genetics Insitutte, University of Florida, Gainesville, FL, United States

*Corresponding authors: Thibault G Sana. Email address: thibault.sana@epfl.ch Sophie Bleves. Email address: bleves@imm.cnrs.fr

Abstract

Two clinical isolates of the opportunist pathogen *Pseudomonas aeruginosa* named PAO1 and PA14 are commonly studied in research laboratories. Despite being closely related, PA14 exhibits increased virulence compared to PAO1. To determine which players are responsible for the hypervirulence phenotype of the PA14 strain, we elected for a transcriptomic approach through RNA sequencing. We found 2029 genes that are differentially expressed between the two strains, including several genes that are involved with or regulated by Quorum-Sensing (QS), known to control most of the virulence factors in *P. aeruginosa*. Among them, we chose to focus our study on QslA, an anti-activator of QS whose expression was barely detectable in the PA14 strain according our data. We hypothesized that lack of expression of *qslA* in PA14 could be responsible for higher QS expression in the PA14 strain, possibly explaining its hyper-virulence phenotype. After confirming QslA protein was highly produced in PAO1 but not in the PA14 strain, we provided evidence showing that a PAO1 deletion strain of *qslA* has faster QS gene expression kinetics compared to PA14. Moreover, known virulence factors activated by QS such as (i) pyocyanin production, (ii) H2-T6SS (Type VI Secretion System) gene expression, and (iii) Xcp-T2SS (Type II Secretion System) machinery production and secretion were all lower in PAO1 compared to PA14 strain, due to higher *qslA* expression. However, biofilm formation and cytotoxicity towards macrophages, although increased in PA14 compared to PAO1, were independent of QslA control. Altogether, our findings implicated differential *qslA* expression as a major determinant of virulence factor expression in *P. aeruginosa* strains PAO1 and PA14.

Importance

Pseudomonas aeruginosa is an opportunistic pathogen responsible for acute nosocomial infections, and chronic pulmonary infections. *P. aeruginosa* strain PA14 is known to be hyper-virulent in different hosts. Despite several studies in the field, the underlining molecular mechanisms sustaining this hyper-virulent phenotype remain enigmatic. Here, we provide evidence that the PA14 strain has faster Quorum-Sensing (QS) kinetics compared to the PAO1 strain, due to the lack of QslA expression, an anti-activator of QS. QS is a major regulator of virulence factors in *P. aeruginosa*, therefore we propose that the hyper-virulent phenotype of PA14 strain is, at least partially, due to the lack of QslA expression. This mechanism could be of great importance, as it could be conserved amongst other *P. aeruginosa* isolates.

Introduction

Pseudomonas aeruginosa is an ubiquitous Gram-negative opportunistic pathogen responsible for various nosocomial infections in immunocompromised or intubated-ventilated patients, as well as chronic respiratory infections in cystic fibrosis sufferers (1). Its genome encodes a myriad of virulence factors and regulatory mechanisms that allow the pathogen to adapt efficiently to various hostile environments and to cause distinct infections (2). Virulence gene expression in *P. aeruginosa* is under a sophisticated and dynamic regulation network, and responds to largely unidentified environmental signals. This complex regulatory network involves alternative sigma factors, dozens of two-component systems, and Quorum-Sensing (QS) systems (3).

QS is a cell density-based interbacterial communication system that involves the diffusion of small autoinducer molecules that are used to coordinate gene expression (4–6). Once a crucial threshold concentration of signal molecules has been reached, QS target genes can be either activated or repressed. In *P. aeruginosa* there are two QS systems based on acyl homoserine lactones (HSL) signaling: the LasR/3-oxo-C_{12}-HSL and RhlR/C_4-HSL systems. The *las* system is placed above the *rhl* system in the HSL-dependent QS hierarchy, since LasR/3-oxo-C_{12}-HSL activates both *rhlR* and *rhlI* (7, 8). These two QS systems control the expression of about 6% of genes encoded on *P. aeruginosa* genome (9–11). Among them, QS activates the expression of many genes encoding virulence factors, like the elastase LasB secreted by the Type II Secretion System (T2SS) (12, 13), two Type VI Secretion Systems (T6SS) named H2- and H3-T6SS (14–16), the pyocyanin (17) and biofilm maturation (18). In addition to the HSL-mediated QS, *P. aeruginosa* also produces the *Pseudomonas* quinolone signal (PQS: 2-heptyl-3-hydroxy-4-quinolone) (19). The quinolone signaling system is intertwined in a hierarchical manner to the HSL-based QS systems of *P. aeruginosa*, as LasR and RhlR respectively positively and negatively control the levels of PQS by binding the

promoter region of *pqsR* regulator gene (20, 21). In regards to regulation of the QS systems, there are several QS regulators described in the literature, such as MvaT (22), CdpR (23), the IQS system (24), QscR (25), QteE (26), and QslA (27). QscR, QteE, and QslA are thought to play crucial roles in determining the activation threshold of QS (25–27).

In laboratory settings, two clinically isolated strains of *P. aeruginosa*, named PAO1 and PA14, are principally studied and are noteworthy for their differences in virulence. While the PAO1 strain displays moderate virulence in most model systems, PA14 is highly virulent in mouse, nematode, and plant models of infection (28); for the historical discovery of PA14 strain, see (29)). Studies aimed at identifying the determinants mediating PA14 hypervirulence have found that the PA14 genome has two pathogenicity islands (108 kb and 11 kb in size), called PAPI-1 and PAPI-2 (*P. aeruginosa* Pathogenicity Island-1 and 2 respectively) (30). These pathogenicity islands encode virulence factors such as the type III secretion system (T3SS) effector ExoU, a potent and detrimental cytotoxin producing rapid cell death (31, 32). Nonetheless, enhanced PA14 virulence is not only a consequence of genomic acquired virulence determinants (33). In addition to the pathogenicity islands, the PA14 strain has a mutated version of the *ladS* gene (34), encoding a sensor of the RetS/LadS/GacS signaling cascade, which is associated with virulence and the switch between acute and chronic infections of *P. aeruginosa* (35, 36). This mutation leads to derepression of the T3SS regulon and thus higher cytotoxicity towards mammalian cells (34). More recently, differences in the expression of the three T6SSs have also been highlighted in PA14 compared to PAO1 and PAK backgrounds, presumably because of the *ladS* mutation (37). Finally, PA14 strain has been shown to secrete high levels of pyocyanin compared to PAO1, which is another important virulence factor of *P. aeruginosa* (38).

To better understand the divergent virulence profiles between PAO1 and PA14 and determine if there are other unknown players that are involved in this complex mechanism, we

chose to perform an unbiased transcriptomic approach using high-throughput RNA sequencing (RNAseq). From RNAseq data, we found significant differences in the expression of many QS targets; overall, QS-activated genes were overexpressed in PA14, while QS-repressed genes were overexpressed in PAO1 strain. Interestingly, the expression of *qslA*, encoding a QS inhibitor (27), is only detectable in PAO1 strain. QslA (QS LasR-anti-activator) is known to bind LasR, and disrupt LasR dimerization, preventing its binding to target promoters (27, 39). Since many virulence factors are activated by QS in *P. aeruginosa*, we hypothesized that decreased expression of *qslA* in PA14 strain would drive increased QS gene expression and subsequently overexpression of QS target genes, thus leading to the hyper-virulent phenotype of PA14. To test this hypothesis, we first demonstrated that the QslA protein is indeed readily produced in PAO1 strain while it is undetectable in PA14. We further confirmed that genes encoding QS systems are upregulated in PA14 strain compared to PAO1, and that it is due to lower level of QslA. Next, we provided evidence that many QS activated genes encoding important *P. aeruginosa* virulence factors are differentially regulated between these two strains, such as pyocyanin production, H2-T6SS gene expression, XcpP production, and Xcp-dependent secretion of the elastase LasB. Finally, we observed that whereas biofilm formation and cytotoxicity towards macrophages were higher in PA14, this was independent of *qslA* expression. Altogether, the level of the QslA protein and presumably the expression level of the *qslA* gene is a key player in QS target genes expression, which consequently contributes to higher pathogenic potential of the PA14 strain.

Results

Global comparison of PAO1 and PA14 RNAseq transcriptomes

To determine if virulence factor genes are differentially expressed between the PAO1 and PA14 *P. aeruginosa* strains, we performed RNAseq on mRNAs extracted from cultures

grown in rich medium at the transition between exponential and stationary phase, in which most of the virulence factors are expressed (see Materiel and Methods). 2029 genes were identified as significantly and differentially regulated between the two strains (Figure 1A and Table 1), which represents about 39% of the *P. aeruginosa* genome, suggesting a massive gene expression variation between both isolates. Among the genes differentially expressed, PA3431/PA3432 expression levels were increased by respectively 108.7 and 105.3-fold in PAO1 (Table S1). However, we decided not to follow up on these genes encoding hypothetical proteins here. Further work will be required to address this interesting observation, more particularly as PA3431/PA3432 may constitute a holin-/antiholin-like system as PF04172 and PF03788 domains, are respectively present in PA3431 and PA3432, suggesting a potential role in bacterial lysis (40). Two other genes whose expression was very different between the two strains drew our attention: PA4685 and PA1244 (*qslA*). Expression of PA4685 was not detectable in PAO1, while expression of *qslA* was barely detectable in PA14 (Table S1 and Table 1).

Since we did not detect any PA4685 transcript in our PAO1 transcriptomic data (Table S1), we first decided to test whether the gene was present in the PAO1 genome. PAO1 is one of the most commonly *P. aeruginosa* strain used in research and is derived from the original Australian PAO isolate, isolated from a wound 50 years ago in Melbourne, and distributed worldwide to laboratories. Over decades, discordant phenotypes of PAO1 subcultures emerged (41). Notably, there is a 1 kb deletion within the PA4684 and PA4685 genes present in the Washington Genome Center PAO1 strain compared to the published PAO1 sequence (42) (which served as a reference for our *in silico* analysis). To test whether this locus deletion is present in the PAO1 strain used in this study (named "PAO1 Marseilles"), PCR was performed using pairs of oligonucleotides hybridizing to the coding sequence of PA4685 (TSO116-TSO117) or outside thereof (CCO1, CCO2) (Figure S1A). As shown, our results revealed that

PA4685 is absent in the PAO1 Marseilles strain, but present in the PA14 strain used in this study (Figure S1B). This explains the non-expression of PA4685 in PAO1 Marseilles strain observed in our RNAseq transcriptome.

The expression of *qslA* gene is increased by 55.8-fold in the PAO1 strain (Table 1). Interestingly, this gene encodes an anti-activator of QS, named QslA (27), and led us to hypothesize that the expression of QS-regulated genes may be different between the two isolates. As a result, we decided to look at the expression of all of the QS regulated genes in our RNAseq transcriptomic data. Interestingly, among the 353 genes known to be regulated by QS in *P. aeruginosa* (315 activated and 38 repressed (9)), 212 were significantly and differentially regulated between both strains (Figure 1A). Overall, QS activated genes were overexpressed in PA14 (shown as yellow dots) whereas QS repressed genes were overexpressed in PAO1 (shown as red dots), suggesting a major differential QS-dependent expression pattern (Figure 1A). All together these observations led us to hypothesize that the difference in QS target genes expression between these two strains could be mediated by the modulation of *qslA* expression levels.

To first demonstrate a correlation between levels of *qslA* expression and QslA production, we engineered chromosomally encoded $QslA_{V5}$ translational fusions in both the PAO1 and PA14 strains. The production of $QslA_{V5}$ could be monitored and analyzed by Western blotting and immunodetection with an anti-V5 antibody (Figure 1B). The results for the PAO1 strain reflected a constant level of QslA during growth, whereas QslA was not detected under the same conditions in PA14, which coincides with our transcriptomic data.

qslA expression level drives gene expression of HSL-based QS regulators

Then, we asked whether this QS-dependent global differential expression could be due to differential expression of *qslA*. We reasoned that abolishing *qslA* expression in the PAO1

184 background may mimic the PA14 profile whereas QslA overproduction in the PA14 strain may
185 resemble the PAO1 pattern. Our transcriptomic data indicate an increased expression of genes
186 encoding the two HSL-based QS systems in PA14. Indeed, *rhlR*, *rhlI*, *lasR*, and *lasI* were
187 respectively 2.5, 1.8, 1.9, and 1.5-fold more expressed in the PA14 strain (Table 1).

188 Therefore, we decided to measure the relative levels of *lacZ* transcriptional fusions to
189 *lasR*, *rhlR* and *rhlI* promoters (7) in strains PAO1, PA14, PAO1Δ*qslA*, PAO1Δ*qslA* carrying
190 plasmid pTS51 encoding *qslA* to complement the *qslA* deletion, and PA14 overproducing QslA
191 from the pTS51 (Table 2). In PAO1, deletion of *qslA* gene increased the expression level of
192 QS regulator genes by 2 to 3-fold (Figure 2A, C, & E). WT level expression was restored by
193 complementation of the mutation *in trans* (compare columns 3 and 4, repression and activation
194 respectively of *qslA*, in graphs 2A, 2C & 2E). Overproduction of QslA caused a significant
195 decrease (1.5 to 2.5-fold) in β-galactosidase activities measured for all three of the
196 transcriptional fusions made in the PA14 strain (Figure 2B, D, &F). Thus, the expression levels
197 of *lasR*, *rhlR*, and *rhlI* were correlated in a dose-dependent manner with QslA production in
198 both *P. aeruginosa* strains. Interestingly, the increased fold expression in PA14 measured for
199 *lasR*, *rhlR*, and *rhlI* (Figure 2), recapitulates the increased fold expression of these genes in our
200 transcriptomic data between two strains (Table S1), suggesting that modulation of *qslA*
201 expression is solely responsible for this differential expression of QS genes.
202
203 **Biosynthesis of pyocyanin depends on *qslA* expression levels**
204 We next hypothesized that differential expression of *qslA* could explain the differential
205 expression of QS-regulated genes between PAO1 and PA14 strains. To test this hypothesis, we
206 chose to compare the expression level or production profile of several QS activated virulence
207 factors gene in both strains, producing or not QslA.

Pyocyanin is a blue, redox-active phenazine that contributes to *P. aeruginosa* virulence by inhibiting the oxidative burst of host phagocytic cells, by inducing apoptosis in host cells and through antibiotic activities. This respiratory pigment also participates in the reduction of iron and functions as an intracellular redox buffer (43). As the regulation of pyocyanin expression is both mediated by HSL- and PQS-based QS (17, 20), the study of its production is then a perfect example of the intrinsic and complex relationship between regulations by HSL- and PQS-based QS. Furthermore, in our transcriptomic data, expression of the operon *phz* encoding the genes involved in the production of pyocyanin, were 10 to 20-fold increased in PA14 (Table S1).

The production of pyocyanin was monitored after 3.5 and 24 hours of growth (Figure 3). Pyocyanin concentrations were higher in PA14 than in PAO1 reflecting the lack of expression of *qslA*. Moreover, the overproduction of QslA in PA14 led to a major decrease in pyocyanin production (5.2- and 22.4- fold at 3.5 and 24 hours) whereas the *qslA* deletion in PAO1 produced a significant increase (2- and 1.6- fold at 3.5 and 24 hours) that could be restored to WT PAO1 level by complementation. Therefore, the increased pyocyanin production in PA14 strain (38) is due to low *qslA* expression in this strain.

H2-T6SS expression is driven by QslA levels

The H2-T6SS machinery is a virulence factor of *P. aeruginosa* that is known to be activated by QS in both PA14 and PAO1 strains (14, 15). Our transcriptomic data indicates a 1.5-fold increased expression of the first gene of the H2-T6SS operon (PA1656) in PA14 (Table 1). *P. aeruginosa* utilizes H2-T6SS to invade epithelial cells by manipulating the microtubular network and host kinase pathways (44–46) and to promote autophagy (44, 47–49). H2-T6SS also mediates antibacterial activity (44, 47–49), making it a trans-kingdom cell targeting machinery (48, 50). We used a *lacZ* transcriptional fusion of H2-T6SS promoter

region to evaluate its expression in these strains (Figure 4). Strain PAO1Δ*qslA* presented a 1.8-fold increase in the expression of H2-T6SS compared to the PAO1 WT strain. Moreover, production of QslA in the mutant strain was able to decrease H2-T6SS expression to comparable level than the WT strain. Similarly in PA14, overproduction of QslA significantly reduced the expression of H2-T6SS. The expression of H2-T6SS machinery genes was thus controlled at a transcriptional level by QslA in both strains.

Production of XcpP depends on *qslA* expression levels

XcpP is an inner membrane protein of the *P. aeruginosa* Xcp T2SS machinery, known to secrete various virulence factors (51). XcpP interacts with the outer membrane secretin XcpQ, which forms the pore through which the secreted protein reaches the extracellular medium (52–54) and is therefore an essential component of the T2SS process. The two operons encoding the Xcp machinery are activated by HSL-based QS (13), and our transcriptomic data indicates that *xcp* genes are upregulated in PA14 in comparison to PAO1 in transition phase (Table S1). We thus asked whether QslA levels could modulate XcpP production, using immunodetection at different time of the growth (Figure 5). Whereas XcpP could be readily detected after 2 hours of growth of the PA14 strain, the same level of proteins was obtained two hours later in PAO1. Moreover, the deletion of the *qslA* gene in PAO1 led to an earlier synthesis of XcpP and to higher levels of the protein that can be restored to a PAO1 WT profile by complementation. Accordingly, overproduction of QslA in the PA14 strain showed a PAO1-like pattern of XcpP. These data corroborated our hypothesis that the T2SS machinery is dependent on QslA for an optimal production.

Elastase secretion is dependent on *qslA* expression level

The elastase is an Xcp T2SS-secreted protease, encoded by the *lasB* gene. It degrades elastin, a major component of lung tissue, and cleaves a surfactant protein (SP-D), involved in several immune functions (55). The expression of elastase coding gene is activated by QS (12), and we measured an 7.7 fold increased expression of *lasB* in PA14 in comparison to PAO1during early stationary phase in our RNAseq analysis (Table 1). To measure the impact of QslA on this T2SS substrate, the secretion into the extracellular medium and the extracellular activity of elastase were monitored (Figure 6). The secretion of LasB was analyzed by Coomassie blue staining of the extracellular fraction at different growth times (Figure 6A). As expected, the deletion of *qslA* in PAO1 altered the kinetics, allowing earlier LasB secretion; after 4 and 6 hours of growth, LasB secretion was significantly higher in the mutant PAO1Δ*qslA* than in the WT PAO1 strain. This phenotype is restored to a WT phenotype by *trans* complementation since the protein profile observed for the PAO1Δ*qslA* strain producing QslA was identical to that observed for the WT PAO1 strain (Figure 6A, compare first and fourth lines). In contrast in PA14, overproduction of QslA led to a delayed and decreased secretion of LasB compared to the PA14 WT strain (Figure 6A, compare fifth and sixth lines).

These data were also confirmed by monitoring the LasB protease activity by observing the formation of a protein degradation halos on skim-milk plates (Figure 6B). We noticed an earlier halo in PA14 strain compared to PAO1 (Figure 6B, compare first and fourth lines). In addition, the delay of halo formation in PAO1 can be visualized in a *qslA* mutant strain (Figure 6B, compare first and second lines). As negative controls, we used mutants in T2SS Xcp machinery for both strains (Figure 6B, third and fifth lines). As shown, the halo was weak after 35 hours.

Altogether these data demonstrated that the functionality of the Xcp T2SS machinery measured by the secretion and extracellular activity of the protease LasB is higher in the PA14 strain, due to the decreased expression of *qslA* in this strain.

QslA does not control biofilm formation by *P. aeruginosa*

P. aeruginosa ability to form biofilm is a crucial virulence determinant, mainly regulated by QS (18). To assess if QslA has a role during biofilm formation, bacterial adherence to abiotic surface of various strains was visualized (Fig 7A) and quantified using crystal violet staining (Fig 7B). The production of exopolysaccharides, a main biofilm component, was also visualized on Congo red-containing plates (Fig 7C). As expected from the literature, the ability of the PA14 strain to form biofilm was higher than for PAO1, the exopolysaccharide staining (Fig 7C, the PA14 strain is red on Congo-red plates) being consistent with adherence assay (Fig 7A and 7B). A clear difference in the adherence capacities was observable after 8h (Fig 7A, compare line 1 with line 3 at 8h) and was even greater at 48h (Fig 7A, compare line 1 with line 3). However, profiles of the PAO1 strains, WT or Δ*qslA*, mutant and of the PA14 strains, WT or overproducing QslA, were the same, suggesting that QslA is dispensable for biofilm formation (Fig. 7A and 7B). Therefore, these results tend to suggest that QslA is not implicated in controlling biofilm formation of *P. aeruginosa*.

Cytotoxicity towards macrophages is not dependent on QslA

Finally, we tested the cytotoxicity of the various strains on J774 macrophages. Indeed while PA14 is highly cytotoxic due to the T3SS effector ExoU (31), PAO1 is considered an invasive and poorly cytotoxic *P. aeruginosa* strain. Nevertheless both strains display T3SS-mediated antiphagocytic functions and the T3SS machinery and effector encoding genes are regulated by HSL-mediated QS(56) and PQS. Due to the hyper-cytotoxic phenotype of the PA14 strain, we used two infection conditions according to the two backgrounds (PAO1 and PA14) (Fig. 8). As expected even with longer infection times, the PAO1 strain is poorly cytotoxic compared to PA14 (Fig. 8, compare line 1 and 3). However, the cytotoxicity levels

of the PAO1 strains, WT or Δ*qslA* mutant and of the PA14 strains, WT or overproducing QslA, were the same suggesting that the cytotoxicity of *P. aeruginosa* is not dependent on QslA.

Discussion

PAO1 and PA14 are two stains of *P. aeruginosa* that are broadly studied throughout the *Pseudomonas* research community. Interestingly, the PA14 strain is more virulent than the PAO1 strain in different model infection systems (28, 33, 57, 58). Several studies have been conducted to try to decipher why PA14 strain exhibits higher pathogenicity compared to PAO1. While previous groups have found that pathogenicity island-I and II are present only in the PA14 genome and encodes virulence factors (33), and the *ladS* gene is mutated in PA14 strain, making T3SS and T6SSs more effective (34, 37), we speculated that other determinants might be involved in the differential in virulence between these two strains.

As a result, we decided to use a global transcriptome RNA sequencing approach to highlight new factors that might explain the virulence divergence between these two strains. The transcriptomic data revealed lower expression of the QS regulator *qslA* in PA14 strain than in PAO1, which we further confirmed at a protein level. Given the fact that QslA is highly produced in PAO1 (Figure 1B) and plays a crucial role in determining the activation threshold of QS (27), we hypothesized that QS regulon expression will occur earlier and would be higher in PA14 in comparison to PAO1. To test this hypothesis, we constructed a deletion mutant of *qslA* in PAO1 as well as a plasmid allowing overexpression of *qslA* from a P_{BAD} promoter in PA14 strain (Table 2). This allowed us to investigate whether deletion of *qslA* in PAO1 can mimic the higher QS activation found in PA14, and conversely, if overproduction of QslA in PA14 will lead to the lower QS activation observed in PAO1. We then focused our study on well-known QS-regulated virulence factors. We were able to confirm that the expression level of genes encoding QS system as well as QS gene targets are differentially regulated depending

on the expression level of *qslA* in both strains, except for biofilm and cytotoxicity phenotypes. Considering that a large set of genes encoding virulence factors are regulated by QS in *P. aeruginosa*, and that their expression levels are increased in PA14 compared to PAO1, this could partially explain the hyper-virulent phenotype of PA14. Moreover, a *qslA* mutant in PAO1 strain is more virulent than the wild-type PAO1 strain in a *Caenorhabditis elegans* model (27). These results combined with our findings suggest that the high expression of *qslA* in PAO1 strain is one of the major factors of its decreased virulence compared to PA14, and vice versa for the PA14 strain.

From our results, we questioned whether such mechanisms could be conserved amongst other isolates of *P. aeruginosa*. To that end, a previous study showed significant differences in QS regulon expression between seven clinical and environmental strains of *P. aeruginosa*, this supporting the notion that different isolates of *P. aeruginosa* have defined regulation networks (59). These findings indicate a role for QS in the extension of the range of habitats in which a species can thrive, including the host. Based on this, one can ask whether the differential regulation of QS between the different strains of *P. aeruginosa* is causing different levels of virulence that can be observed between different isolates (33). If so, it would be interesting to investigate *qslA* expression levels in different clinical isolates of *P. aeruginosa*. However, our data concerning biofilm formation and cytotoxicity towards macrophages suggest that the QslA-regulon does not overlap completely the QS-regulon. One explanation could be that another QS regulator is important for controlling biofilm and cytotoxicity.

Interestingly, the expression of other known QS regulator genes such as *qscR*, *rsaL*, *mvaT*, *cdpR* and *qteE* was respectively 1.2, 2.1, 1.6, 3.9, and 1.9-fold increased in PA14 (Table 1), which is not comparable to the 55.8 fold increased expression of *qslA* in PAO1. This, along with our data concerning the differential modulation of QS, H2-T6SS, pyocyanin, Xcp-T2SS,

and LasB, suggests that the modulation of QS between two strains is mostly due to the differential expression of *qslA*. One could ask what is the molecular mechanism underlining this differential expression of *qslA*? We performed a simple BLASTn comparing the upstream 500 bp sequence of *qslA* in PAO1 and PA14, but there was only one nucleotide difference, hardly explaining the differential expression of *qslA* in both strains. Further studies will be needed to elucidate the exact mechanism of *qslA* expression in these two strains. Altogether, we propose that differential expression of *qslA* in PAO1 and PA14 leads to modulation of QS signaling. Since QS is a major regulator of virulence factors in *P. aeruginosa*, it is tempting to speculate that QslA could be a key player in the hyper-virulence phenotype of PA14, along with LadS (34), PAPI-1 and PAPI-2 (30).

Material and Methods

Bacterial strains, culture conditions, plasmids and oligonucleotides

The bacterial strains, plasmids and oligonucleotides used in this study are described in Table 2. LB (Lysogeny broth), PIA (*Pseudomonas* isolation agar), and TSB (Tryptic soy broth) broths and agar were used for the growth of *P. aeruginosa* and *Escherichia coli* strains at 37°C. Cultures were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 with overnight cultures, and strains were grown at 37°C with aeration in TSB. Recombinant plasmids were introduced into *P. aeruginosa* strain PAO1 and PA14 by conjugation using pRK2013 mobilization properties, as described previously (60). The antibiotic concentrations were as follows: for *E. coli*, ampicillin (50 µg ml–1), kanamycin (25 µg ml–1), tetracycline (15 µg ml–1), gentamicin (10 µg ml–1), streptomycin (100µg ml-1); for *P. aeruginosa*, tetracycline (200 µg ml–1 for plates or 50 mg ml–1 for liquid growth), gentamicin (50 µg ml–1), streptomycin (1000µg ml-1). Expression of the *qslA* gene under the control of a P_{BAD} promoter in pTS51 was induced or

repressed by addition of arabinose or glucose respectively at a final concentration of 0.5% after 1h30 of growth.

Transcriptional analysis of *Pseudomonas aeruginosa* PA14 and PAO1

To determine if virulence factor genes were differentially expressed between the PAO1 and PA14 *P. aeruginosa* strains, we performed RNAseq on mRNA extracted from cultures of both strains grown in rich media (LB) to early stationary phase. Total RNA was isolated from cell pellets equivalent to 2 OD_{600} units of bacterial culture using SV total RNA isolation system (Promega) following the manufacturer's instructions. Once isolated and prior to the library preparation, the integrity of RNA samples was assessed with a Bioanalyzer system. Barcoded, strand-specific, cDNA libraries were constructed, pooled and sequenced in an Illumina HiSeq 2000, single-end 50 bp reads by BGI-Hong Kong. Illumina reads were mapped to the *P. aeruginosa* genome PAO1 (AE004091.2 (61)) and PA14 (NC_008463.1 (33)) by Bowtie (version Bowtie1 v0.12.9 (62)), indicating strand-specific sequencing. Quantification of gene expression was determined by the HTSeq package (63) using the GeneBank *P. aeruginosa* PA14 and PAO1 annotations files and discarding multimapped reads. Data were normalized by RPKM and filtered to the 5,263 orthologous genes conserved between the *P. aeruginosa* strains PA14 and PAO1. Two biological replicates were performed per condition. Differential expression analysis was analyzed using the Bioconductor package NOISeq version 2.22.1 (64), a non-parametric approach suitable for lowly replicated data, and using a q-value of 0.99 for strong control of false positives.

cDNA library preparation

The RNA was fragmented with RNase III. Then the 5'PPP structures were removed from the RNA samples using RNA 5' polyphosphatase (Epicentre) and subsequently, the RNA was poly

(A)-tailed using poly (A) polymerase. Then an RNA adapter was ligated to the 5′-phosphate of the RNA fragments. First-strand cDNA synthesis was performed using an oligo (dT)-adapter primer and M-MLV reverse transcriptase. The resulting cDNA was PCR-amplified to about 30 ng/µl using a high fidelity DNA polymerase. The cDNA was purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics).

Construction of the Δ*qslA* mutant

To generate *qslA* deletion strain, 500 bp upstream and 500 bp downstream of the *qslA* gene were amplified by overlapping PCR with High Fidelity DNA polymerase (Roche Applied Science) using TSO108, TSO109, TSO110 and TSO111 primers (see Table 2). The PCR product was cloned in pCR2.1 (TA cloning kit; Invitrogen) giving pTS48, which was then sequenced (GATC) and subcloned in pKNG101 suicide vector giving the mutator pTS50. pTS50, maintained in the *E. coli* CC118 λpir was further conjugated in *P. aeruginosa* strain PAO1 using protocol previously described (60). The mutant, in which the double recombination events occurred and resulted in the nonpolar deletion of *qslA* gene were verified by PCR using external primers TSO112–TSO113.

Construction of the chromosomic *qslA*$_{V5}$ recombinant gene

PAO1 strains chromosomally encoding QslA$_{V5}$ translational fusion was engineered by exchanging the *qslA* stop codon with the sequence encoding the V5 tag followed by a stop codon. This was achieved by overlapping PCR of the 500 bp upstream and downstream regions of the native stop codon of *qslA* with the High Fidelity DNA polymerase (ROCHE) using primers TSO103, TSO104, TSO105 and TSO106 (Table 2). The PCR product was cloned in pCR2.1 (TA-cloning KIT, Invitrogen), which was then sequenced (GATC) and subcloned in pKNG101 suicide vector giving the pTS52. pTS52, maintained in the *E. coli* CC118λpir strain,

was mobilized in the wild type *P. aeruginosa* strain PAO1 or PA14. The strains, in which the double recombination events occurred and resulted in the chromosomic tagging of *qslA* with V5, were verified by PCR using primers TSO58 and TSO107.

lacZ reporter fusion and β-galactosidase assay

For $P_{H2\text{-}T6SS}$-'*lacZ*, the promoter fragment was integrated at the CTX phage attachment site in PAO1 or PA14 and isogenic mutants using established protocols (65). For P_{rhlR}-'*lacZ*, P_{rhlI}-'*lacZ*, P_{lasR}-'*lacZ*, plasmids carrying promoter fragment were conjugated in PAO1 or PA14 and isogenic mutants using established protocol (60). Overnight culture, grown in TSB supplemented with tetracyclin, was diluted in TSB to OD_{600}= 0.1. Growth and ß-galactosidase activity were monitored by harvesting samples at different time intervals. ß-galactosidase activity was measured according to Miller, as described previously (16), and based on onitrophenyl-b-D-galactopyranoside hydrolysis. ß-galactosidase activities were expressed in Miller units.

Pyocyanin production

Pyocyanin was extracted from the extracellular medium by adding an equal volume of chloroform ($CHCl_3$) and vigorous vortexing as previously described (66). The lower pyocyanin-containing organic layer was then taken and vortexed with an equal volume of 0.2 M HCl. The pink pyocyanin-containing aqueous layer resulting from the previous step was then taken, and its absorbance at 520 nm (OD_{520}) was read. Concentrations expressed as micrograms of pyocyanin produced per ml of culture supernatant were determined by multiplying the OD_{520} by 17.072.

SDS-PAGE and immunoblotting

Proteins from the extracellular medium were loaded at an equivalent of 1.0 OD_{600} unit, while proteins from cellular extracts were loaded at an equivalent of 0.1 OD_{600} unit. Proteins were then separated on SDS gels containing 11 or 15% acrylamide depending on the size of the proteins being further detected.

For Western blotting, proteins were transferred from gels onto nitrocellulose membranes. After 30 min to overnight saturation in Tris-buffered saline (TBS) (0.1 M Tris, 0.1 M NaCl, pH7.5), 0.05% (v/v) Tween 20 and 5% (w/v) skim milk, the membrane was incubated for 1 h with anti-V5 (diluted 1:2500), anti-XcpP (1/2000) or anti-DsbA (1:25000); washed three times with TBS, 0.05% Tween 20; incubated for 45 min with goat anti-rabbit immunoglobulin G (IgG) antibodies (Sigma) diluted 1:5000; washed three times with TBS, 0.05% Tween 20; and then revealed with a Super Signal Chemiluminescence system (Pierce).

Adherence assay on inert surfaces and exopolysaccharide production

The adherence assay was performed as previously described in (67) with some modifications. Cultures inoculated at $OD6_{00}$=0.05 with O/N cultures were grown at 37°C with aeration in LB with appropriate antibiotics. Induction of the promoter arabinose (P_{BAD}) was done at OD_{600}=0.6 with 0.2% arabinose for 1.5 hours. 24-well polystyrene microtitre plates were then inoculated at OD_{600}=0.2 in 1 ml of MM63 medium supplemented with casamino acids 0.5%, 1 mM $MgSO_4$ and glucose or arabinose 0.2% for 2 to 48 hours at 30°C. Bacteria were stained with 0.1% crystal violet for a period of 10 min and washed twice with water. The stain was then dissolved in ethanol and absorbance was measured at 595 nm.

Congo red assay was performed as previously described to measure the production of exopolysaccharides (68).

Cytotoxicity towards macrophages

The cytotoxicity of *P. aeruginosa* strains grown to mid-log phase in LB broth was assayed using J774 macrophages as described in (69) except that macrophages were infected for 45 min or 2.5 hr at a multiplicity of infection (MOI) of 20 for the PAO1 and PA14 strains respectively. The percentage of LDH release was calculated relatively to that of the uninfected control, which was set at 0% LDH release, and that of uninfected cells lysed with Triton X-100, which was set at 100% LDH release.

Statistical analysis

For multigroup comparisons, a main P value was calculated by ANOVA (Stat Plus software) and unpaired Student's t tests were performed using Excel software (Microsoft) for two-group comparisons. On the figures, asterisks indicate statistical significance.

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Author contributions

T.G.S. and S.B. designed and conceived the experiments. T.G.S., R.L., M.R.G., A.L., C.S., C.C., and B.I. performed the experiments. T.G.S. and S.B. supervised the execution of the experiments. T.G.S., R.L., A.C., B.I. and S.B. analyzed the data and discussed with M.R.G., A.L and R.V. T.G.S. and S.B. wrote the paper with contribution from B.I. and reading from R.L., A.C., M.R.G. and R.V.

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716 **Table 1:** Highlights from Table S1 of genes of interest

Gene name	Function	Ratio expression		Activated by QS?
		PA14/PAO1	PAO1/PA14	
qslA	QS regulator	55.8	/	
qscR	QS regulator	/	1.2	
rsaL	QS regulator	/	2.1	
mvaT	QS regulator	/	1.6	
cpdR	QS regulator	/	3.9	
qteE	QS regulator	/	1.9	
rhlR	Rhl QS system	2.5	/	X
rhlI	Rhl QS system	1.8	/	X
lasR	Las QS system	1.9	/	X
lasI	Las QS system	1.5	/	X
PA1656	H2-T6SS machinery	1.5	/	X
lasB	LasB Elastase	7.7	/	X

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718 **Table 1:** Highlights from Table S1 of genes of interest. Presentation of genes of interests regarding
719 their ratio of expression between strains PAO1 and PA14.

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728 **Table 2 Strains, plasmids and oligonucleotides used in this study**

Strain, plasmid or oligonucleotide	Genotype, description or sequence	Source and/or reference
***E. coli* strains**		
TG1	*supE, hsd*Δ*R, thi*Δ*(lac-proAB),* F' (*traD36, proAB*+*, lacI*q*, lacZ*Δ*M15)*	Laboratory collection
CC118(λpir)	(λ*pir*) Δ (*ara-leu*), *araD,* Δ*lacX74, galE, galK, phoA-20, thi-1, rpsE, rpoB, Arg*(Am), *recA1,* Rfr (λ*pir*)	Laboratory collection
TOP10F'	F- *mcrA* Δ*(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*(Str^R) *endA1* λ$^-$	Laboratory collection
P. aeruginosa strains		
PAO1	Wild-type, prototroph, *chl*-2	B. Holloway collection
PAO1 *qslA-v5*	Chromosomally encoded QslA$_{V5}$ translational fusion in PAO1	This work
PAO1 Δ*qslA*	*qslA* deletion mutant in PAO1	This work
PAO1TS2	P$_{H2\text{-}T6SS}$ integrated at *att* site in PAO1	(15)
PAO1TS2 Δ*qslA*	P$_{H2\text{-}T6SS}$ integrated at *att* site in PAO1Δ*qslA*	This work
PA14	Clinical isolate	F. Ausubel collection
PA14 *qslA-v5*	Chromosomally encoded QslA$_{V5}$ translational fusion in PA14	This work
PA14TS2	P$_{H2\text{-}T6SS}$ integrated at *att* site in PA14	This work
D40ZQ	*xcp* locus deletion in PAO1	(68)
PA14 Δ*xcpT*	*xcpT* deletion mutant in PA14	(69)
Plasmids		
pCR2.1	TA cloning, *lacZ*α, ColE1, f1 ori, Ap^R Km^R	Invitrogen
pMini-CTX::*lacZ*	Ω-FRT-attP-MCS, ori, int, oriT, Tc^R	(63)
pRK2013	Tra+, Mob+, ColE1, Km^R	(70)
pKNG101	oriR6K, mobRK2, sacBR$^+$, Sm^R (suicide vector)	(71)
pJN105	GmR, *araC*-pBAD	Laboratory collection
pTS2	722 bp upstream region of *H2-T6SS* in pMini-CTX::*lacZ*	(15)
pTS48	500 bp upstream and 500 bp downstream *qslA* in pCR2.1	This work
pTS49	*qslA* gene in pCR2.1	This work
pTS50	500 bp upstream and 500 bp downstream *qslA* in pKNG101	This work

pTS51	*qslA* gene in pJN105	This work
pTS52	$qslA_{V5}$ in pCR2.1	This work
pTS53	$qslA_{V5}$ in pKNG101	This work
pMAL.R	P*lasR-lacZ* transcriptional fusion in pMP220	(7)
pMAL.V	P*rhlR-lacZ* transcriptional fusion in pMP220	(7)
pMAL.I	P*rhlI-lacZ* transcriptional fusion in pMP220	(7)
Oligonucleotides		
TSO58	5'-CCTATCCCTAACCCTCTCCTCGGT-'3	(45)
TSO103	5'-CGACCGCAGTTTTCCAACTGCGGGCCTTCATGGCGG-'3	This work
TSO104	5'TCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT TACCACCGGAACGTCGAGCGGCTACCAGGCGCTGCTGC-'3	This work
TSO105	5'GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG TGACCCGGCCATGGCGAATGACGCCGGTGGCGTCG-'3	This work
TSO106	5'-ATGCTCGGCGCGTAGGCATCGTTGTACAGGGCGACG-'3	This work
TSO107	5'-TGCTTGTCGCCGATGCTCGGC-'3	This work
TSO108	5'-CAGCGCCCTCTTCGAAGAAGC-'3	This work
TSO109	5'-ATGGCCGGGTCACACATGACCTGCCGCCTTCGC-'3	This work
TSO110	5'-GCAGGTCATGTGTGACCCGGCCATGGCGAATGACG-'3	This work
TSO111	5'-TGCTTGTCGCCGATGCTCGGCGC-'3	This work
TSO112	5'-AGAAAGGGTTATATCCTTATGC-'3	This work
TSO113	5'-GGTTCGAGGTCATCCCACAGC-'3	This work
TSO114	5'-CTCCATCGATTGACAGCGAAGG-'3	This work
TSO115	5'-TCAACCGGAACGTCGAGAGGC-'3	This work
TSO116	5'-TCCTCGCACCAGGACCAGTACC-'3	This work
TSO117	5'-GCGTCTTCCTCGCTCTCTTCGG-'3	This work

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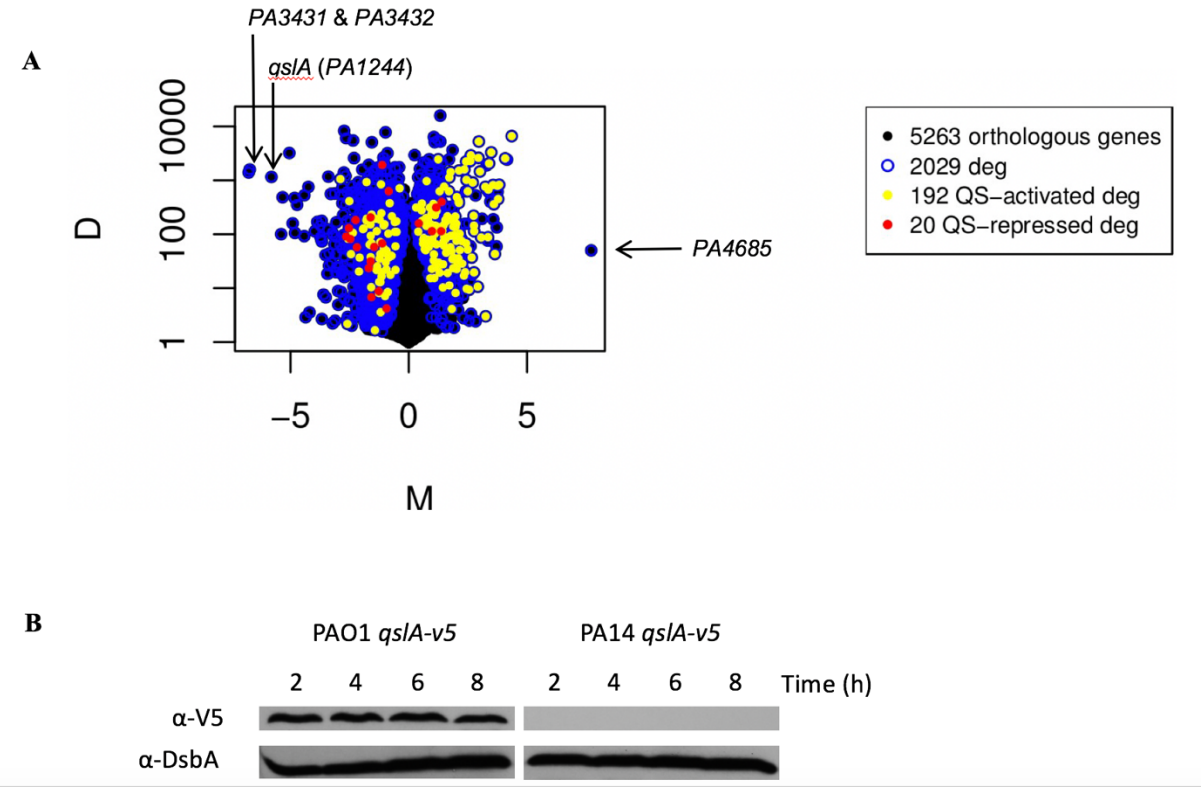
Figure 1



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734 **Figure 1: Transcriptome of PAO1 and PA14 reveals differential QS target genes and *qslA***
735 **expression. (A) M&D plot representing differentially expressed genes between PA14 and**
736 **PAO1 strains of *P. aeruginosa*.** QS target genes are more expressed in PA14 . **(B) QslA is**
737 **only produced in the PAO1 strain.** $QslA_{V5}$ protein is immunodetected using V5 antibody in
738 cellular extracts obtained after 2, 4, 6 and 8 hours of growth in TSB at 37°C with aeration.
739 DsbA immunodetection is used as a loading control.

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Figure 2

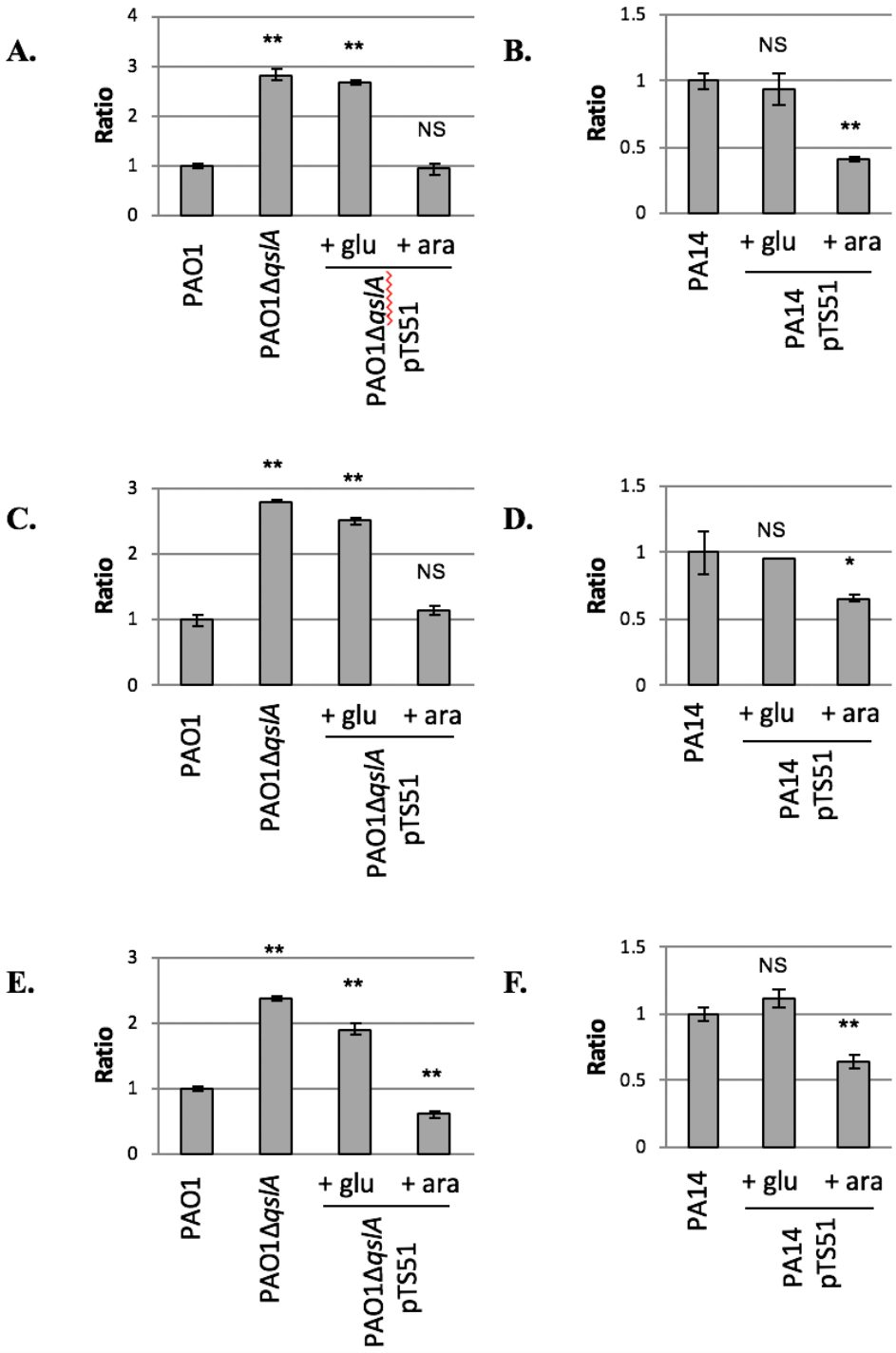

Figure 2: β-galactosidase activities of transcriptional fusions P_{lasR}-'*lacZ* (A,B), P_{rhlI}-'*lacZ* (C,D), et P_{rhlR}-'*lacZ* (E,F). Ratio of expression in parental PAO1 (A,C,E) or PA14 (B,D,F) strain versus expression in referenced strain. Results were obtained after 6 hours of growth at

752 37°C with aeration. Glucose and arabinose were added in the culture medium after 1h30 of
753 growth at a final concentration of 0.5% respectively allowing repression or induction of the
754 P_{BAD} promoter of pTS51 encoding *qslA*. **, P <0.01, *, P <0.05 and NS, not significant.
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Figure 3

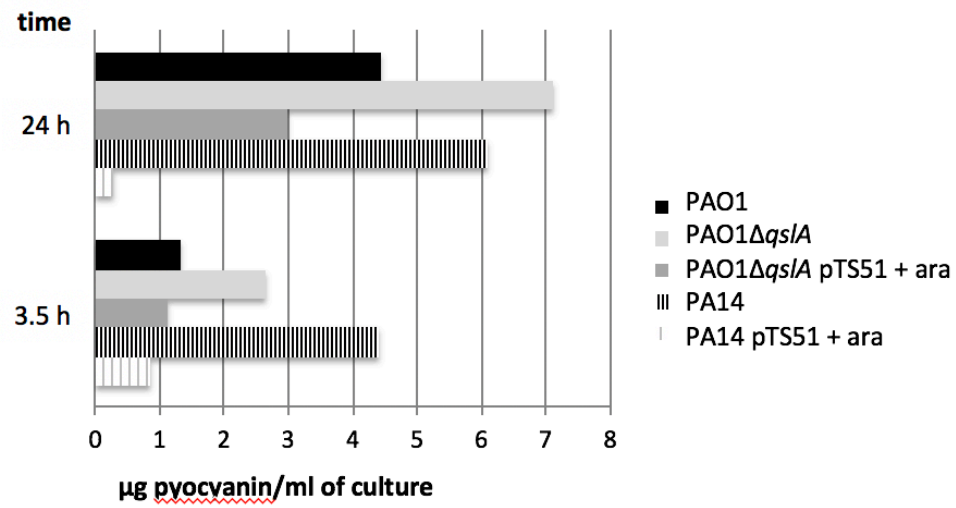

Figure 3: Pyocyanin quantification in the extracellular medium. Pyocyanin was extracted from culture medium as described in the experimental section after 3.5 and 24 hours of growth at 37°C in LB with aeration. Arabinose was added in the culture medium after 1h30 of growth at a final concentration of 0.5% to induce the P_{BAD} promoter of pTS51 encoding *qslA*. Pyocyanin concentrations are reported as mg of pyocyanin produced per ml of culture. Data of a typical experiment are shown. The experiment has been done in triplicate.

Figure 4

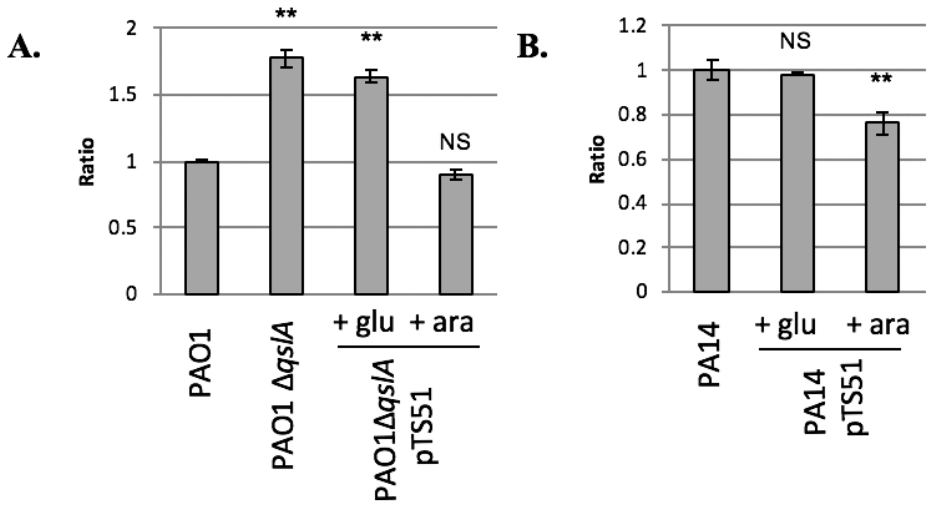

Figure 4: β-galactosidase activities of transcriptional fusions $P_{H2\text{-}T6SS}$-'*lacZ*. Ratio of expression in parental PAO1 (A) or PA14 (B) strain versus expression in referenced strain. Results were obtained after 4 hours of growth at 37°C with aeration. Glucose and arabinose were added in the culture medium after 1h30 of growth at a final concentration of 0.5% allowing respectively repression or induction of the P_{BAD} promoter of pTS51 encoding *qslA*. **, P <0.01 and NS, not significant.

Figure 5

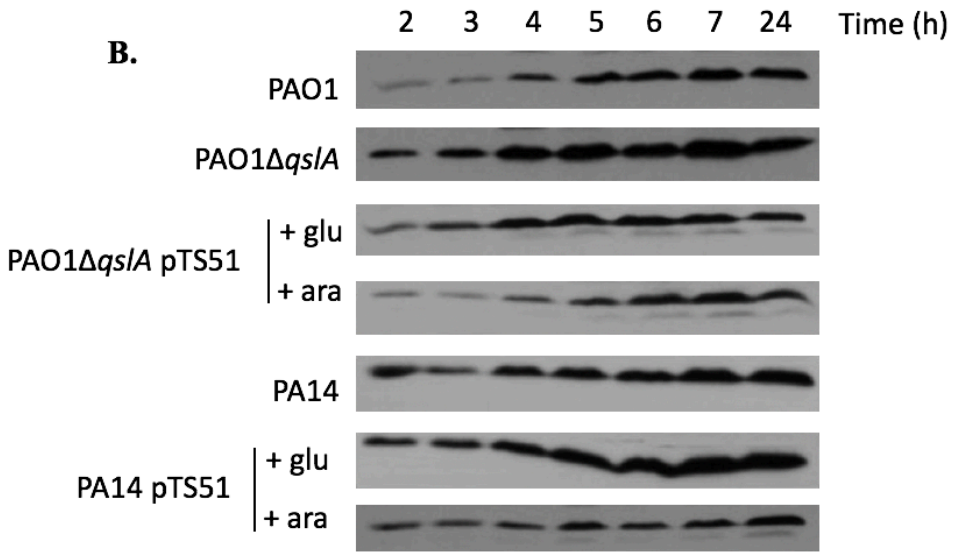

Figure 5: Immunodetection of the T2SS Xcp machinery component XcpP in cellular extracts. Immunodetection of XcpP at 25 kDa after 2, 3, 4, 5, 6, 7 and 24 hours of growth of indicated strains at 37°C with aeration. Glucose and arabinose were added in the culture medium after 1h30 of growth at a final concentration of 0.5% allowing respectively repression or induction of the P_{BAD} promoter of pTS51 encoding *qslA*.

Figure 6

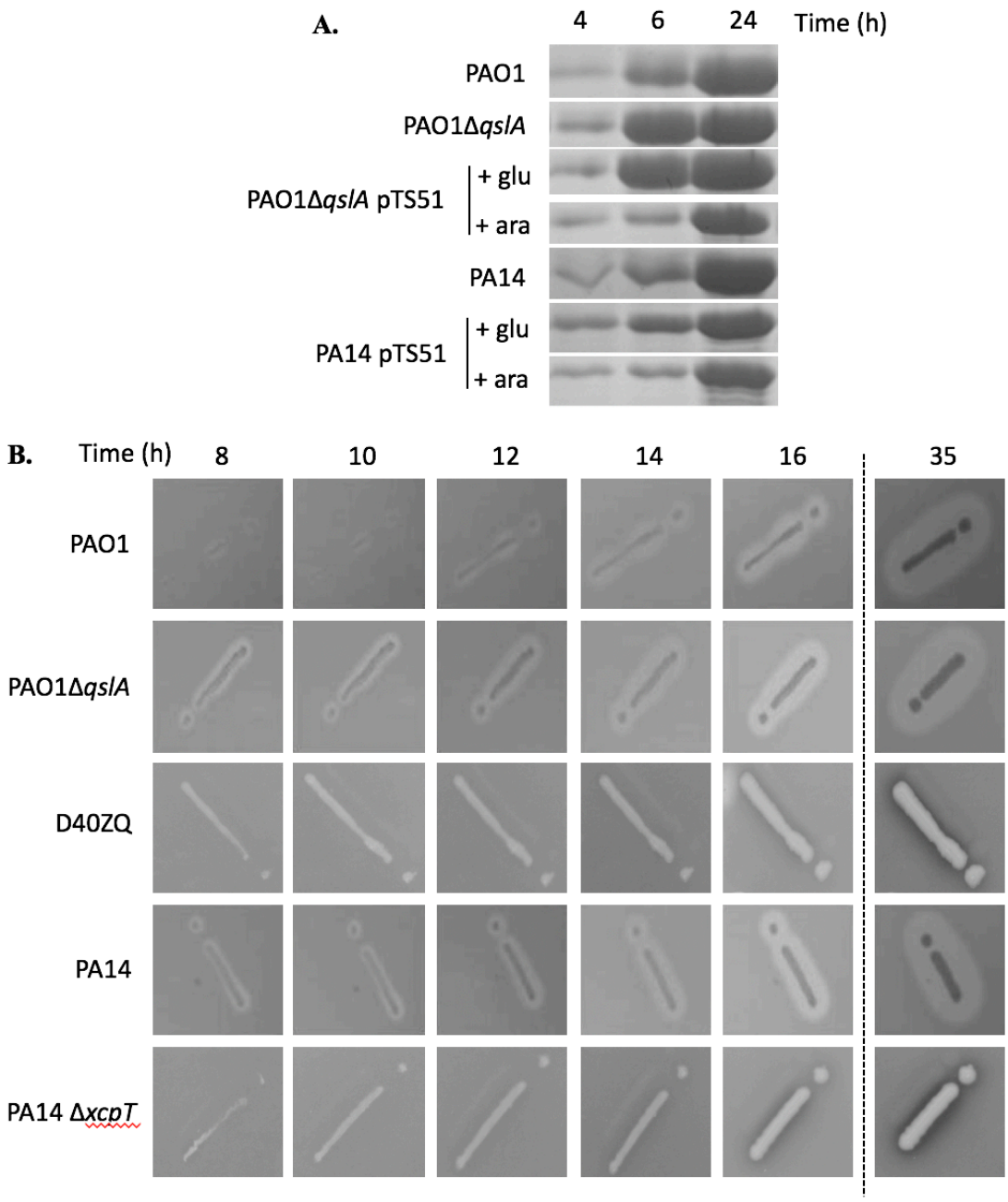

Figure 6: (A) Detection of elastase in the extracellular medium. Commassie blue stained gel of extracellular medium of different *P. aeruginosa* strains. LasB (or elastase) was observed at 33 kDa after 4, 6 and 24 hours of growth at 37°C with aeration. Glucose and arabinose were added in the culture medium after 1h30 of growth at a final concentration of 0.5% allowing respectively repression or induction of the P_{BAD} promoter of pTS51 encoding *qslA*. **(B) Kinetics of proteasic activity of elastase secreted by *P. aeruginosa*.** Proteolysis of

proteins contained in skim milk allowed formation of a halo all around the colonies of *P. aeruginosa* depending on the quantity of elastase secreted. D40ZQ (PAO1 strain lacking all the *xcp* genes) and PA14 Δ*xcpT* were used as controls showing that protease activity observed is mainly dependent on this T2SS effector. Elastase activity was observed after 8, 10, 12, 14, 16 and 35 hours of growth at 30°C without agitation.

Figure 7

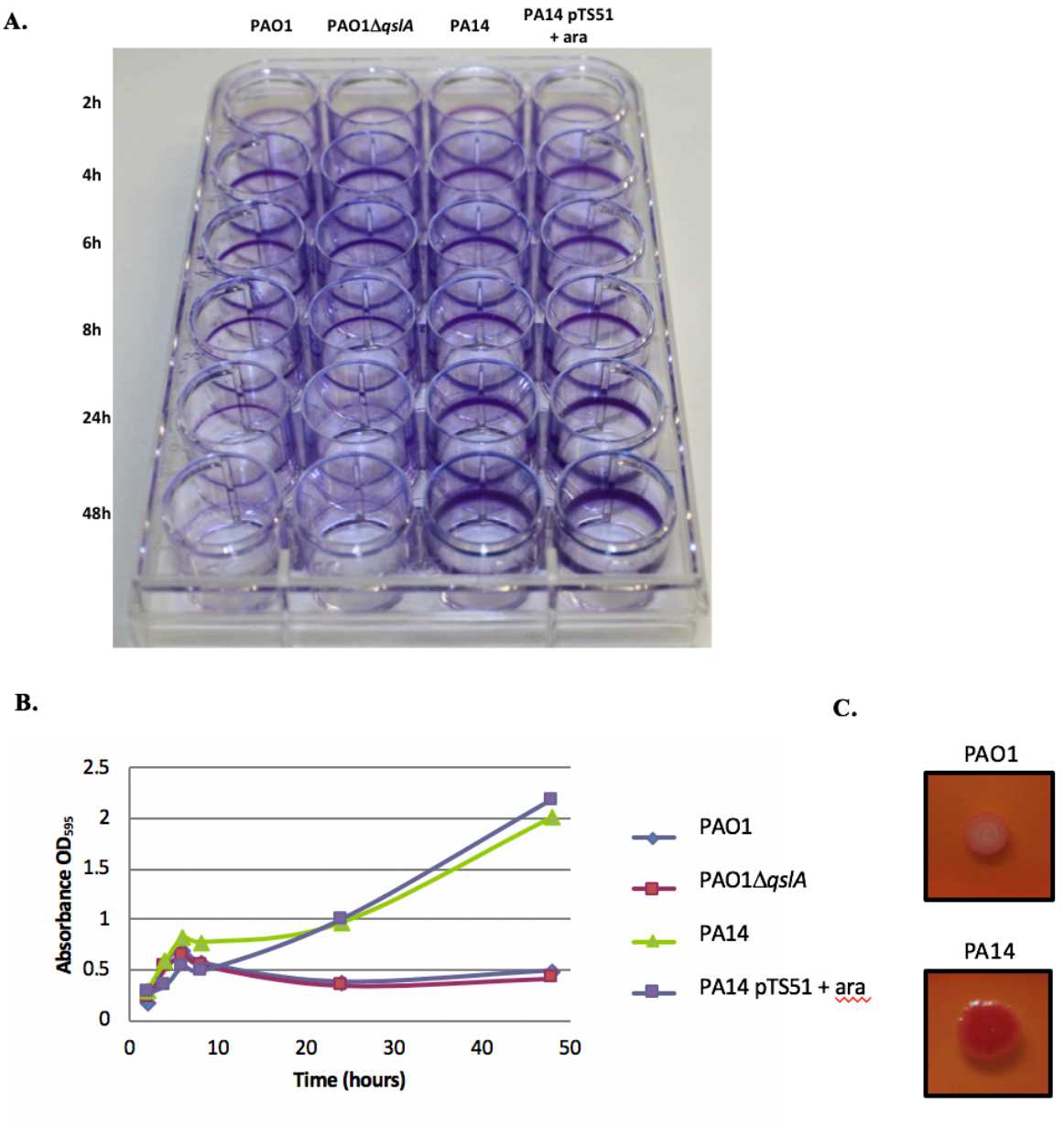

Figure 7: (A) Bacterial adherence to plastic to infer the ability of strains to form biofilm. Adherence assay with indicated *P. aeruginosa* strains grown at 30°C from 2 to 48 hours in minimal medium. Arabinose at 0.5% was added in the culture medium to induce the P_{BAD} promoter of pTS51 encoding *qslA*. **(B) Biofilm quantification.** Crystal violet quantification (OD_{595}) showed over the time from two independent experiments. **(C) Bacterial colony staining on Congo red-containing agar plates.** Red color indicates exopolysaccharide production.

Figure 8

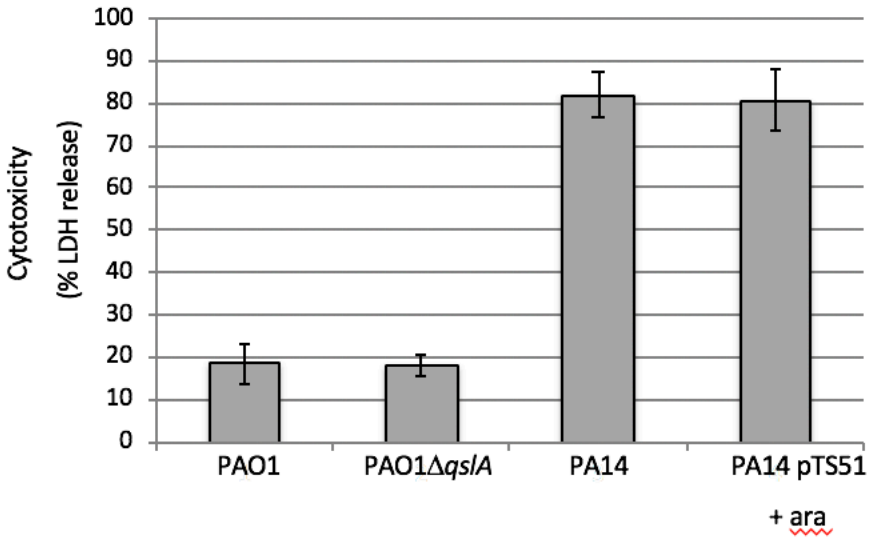

Figure 8: Cytotoxicity towards J774 macrophages evaluated using a LDH assay after 2 hours and 45 min of infection with PAO1 (WT and *qslA* mutant) and PA14 (WT and QslA overproduction) strains respectively. Arabinose at 0.5% was added in the culture medium after 1h30 of growth to induce the P_{BAD} promoter of pTS51 encoding *qslA*. Error bars correspond to standard deviations from three independent experiments done in triplicate. No statistical difference was observed (P values calculated with Student's t test).

Figure S1

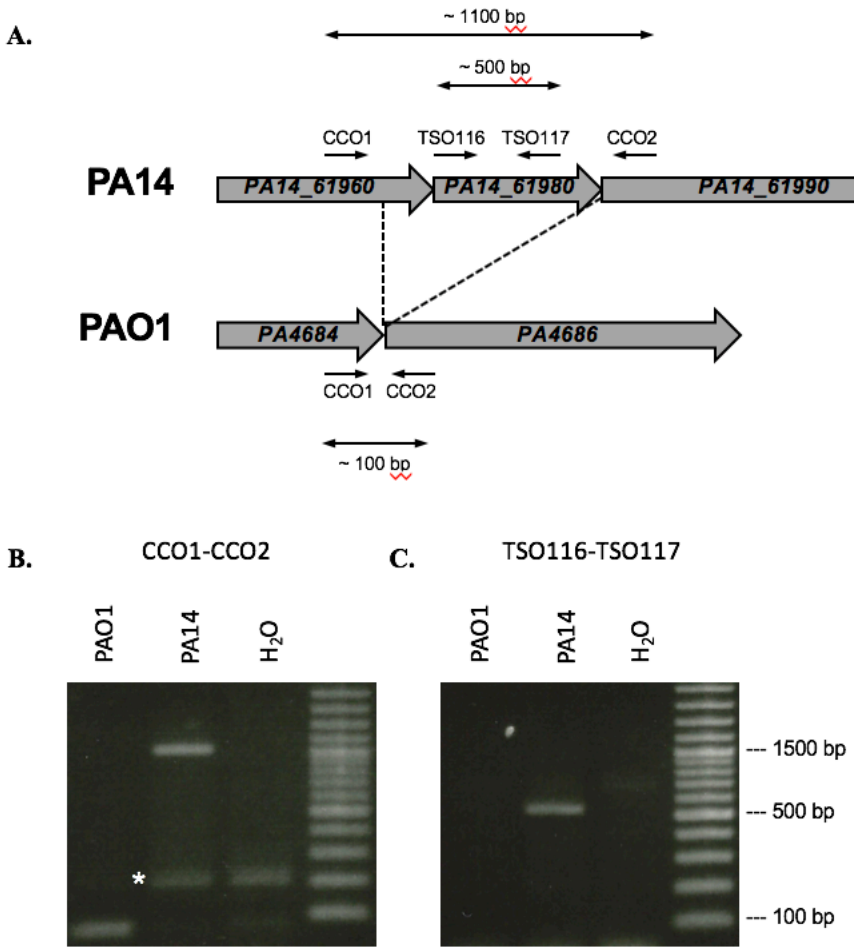

Figure S1: Validation of the absence of PA4685 gene in PAO1 strain by PCR. (A) Loci of PA4685 in PAO1 and its homologous gene PA14_61980 in PA14 strain. CCO1-CCO2 are external oligonucleotides allowing a PCR product around 1100 bp if PA4685 is present and around 100 bp if absent. TSO116-TSO117 are internal oligonucleotides allowing a PCR product of 500 bp only if the gene is present. (B) Agarose gels of resulting PCR products on the genomics DNA of PAO1 and PA14 strains. (*) represents a non-specific product of PCR.

Figure S2

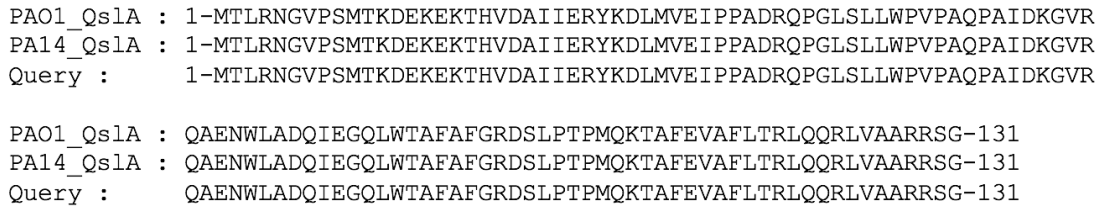

Figure S2 : BlastP of QslA from PAO1 and PA14 strains.