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Differential modulation of Quorum-Sensing signaling through QslA in *Pseudomonas*

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

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5 **Running title:** Differential QS modulation by QslA

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26

27 Abstract

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

48

49 Importance

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

59 Introduction

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

69 QS is a cell density-based interbacterial communication system that involves the
70 diffusion of small autoinducer molecules that are used to coordinate gene expression (4–6).
71 Once a crucial threshold concentration of signal molecules has been reached, QS target genes
72 can be either activated or repressed. In *P. aeruginosa* there are two QS systems based on acyl
73 homoserine lactones (HSL) signaling: the LasR/3-oxo-C₁₂-HSL and RhlR/C₄-HSL systems.
74 The *las* system is placed above the *rhl* system in the HSL-dependent QS hierarchy, since
75 LasR/3-oxo-C₁₂-HSL activates both *rhlR* and *rhlI* (7, 8). These two QS systems control the
76 expression of about 6% of genes encoded on *P. aeruginosa* genome (9–11). Among them, QS
77 activates the expression of many genes encoding virulence factors, like the elastase LasB
78 secreted by the Type II Secretion System (T2SS) (12, 13), two Type VI Secretion Systems
79 (T6SS) named H2- and H3-T6SS (14–16), the pyocyanin (17) and biofilm maturation (18). In
80 addition to the HSL-mediated QS, *P. aeruginosa* also produces the *Pseudomonas* quinolone
81 signal (PQS: 2-heptyl-3-hydroxy-4-quinolone) (19). The quinolone signaling system is
82 intertwined in a hierarchical manner to the HSL-based QS systems of *P. aeruginosa*, as LasR
83 and RhlR respectively positively and negatively control the levels of PQS by binding the

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

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

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

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

129

130 **Results**

131 **Global comparison of PAO1 and PA14 RNAseq transcriptomes**

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

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

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

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

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

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

180

181 ***qsIA* expression level drives gene expression of HSL-based QS regulators**

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

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

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

224

225 **H2-T6SS expression is driven by QsIA levels**

226 The H2-T6SS machinery is a virulence factor of *P. aeruginosa* that is known to be
227 activated by QS in both PA14 and PAO1 strains (14, 15). Our transcriptomic data indicates a
228 1.5-fold increased expression of the first gene of the H2-T6SS operon (PA1656) in PA14
229 (Table 1). *P. aeruginosa* utilizes H2-T6SS to invade epithelial cells by manipulating the
230 microtubular network and host kinase pathways (44–46) and to promote autophagy (44, 47–
231 49). H2-T6SS also mediates antibacterial activity (44, 47–49), making it a trans-kingdom cell
232 targeting machinery (48, 50). We used a *lacZ* transcriptional fusion of H2-T6SS promoter

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

239

240 **Production of XcpP depends on *qslA* expression levels**

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

255

256 **Elastase secretion is dependent on *qslA* expression level**

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

272 These data were also confirmed by monitoring the LasB protease activity by observing
273 the formation of a protein degradation halos on skim-milk plates (Figure 6B). We noticed an
274 earlier halo in PA14 strain compared to PAO1 (Figure 6B, compare first and fourth lines). In
275 addition, the delay of halo formation in PAO1 can be visualized in a *qslA* mutant strain (Figure
276 6B, compare first and second lines). As negative controls, we used mutants in T2SS Xcp
277 machinery for both strains (Figure 6B, third and fifth lines). As shown, the halo was weak after
278 35 hours.

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

282

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

284 *P. aeruginosa* ability to form biofilm is a crucial virulence determinant, mainly
285 regulated by QS (18). To assess if QslA has a role during biofilm formation, bacterial adherence
286 to abiotic surface of various strains was visualized (Fig 7A) and quantified using crystal violet
287 staining (Fig 7B). The production of exopolysaccharides, a main biofilm component, was also
288 visualized on Congo red-containing plates (Fig 7C). As expected from the literature, the ability
289 of the PA14 strain to form biofilm was higher than for PAO1, the exopolysaccharide staining
290 (Fig 7C, the PA14 strain is red on Congo-red plates) being consistent with adherence assay
291 (Fig 7A and 7B). A clear difference in the adherence capacities was observable after 8h (Fig
292 7A, compare line 1 with line 3 at 8h) and was even greater at 48h (Fig 7A, compare line 1 with
293 line 3). However, profiles of the PAO1 strains, WT or $\Delta qslA$, mutant and of the PA14 strains,
294 WT or overproducing QslA, were the same, suggesting that QslA is dispensable for biofilm
295 formation (Fig. 7A and 7B). Therefore, these results tend to suggest that QslA is not implicated
296 in controlling biofilm formation of *P. aeruginosa*.

297

298 Cytotoxicity towards macrophages is not dependent on QslA

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

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

309

310 **Discussion**

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

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

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

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

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

357 and LasB, suggests that the modulation of QS between two strains is mostly due to the
358 differential expression of *qsIA*. One could ask what is the molecular mechanism underlining
359 this differential expression of *qsIA*? We performed a simple BLASTn comparing the upstream
360 500 bp sequence of *qsIA* in PAO1 and PA14, but there was only one nucleotide difference,
361 hardly explaining the differential expression of *qsIA* in both strains. Further studies will be
362 needed to elucidate the exact mechanism of *qsIA* expression in these two strains. Altogether,
363 we propose that differential expression of *qsIA* in PAO1 and PA14 leads to modulation of QS
364 signaling. Since QS is a major regulator of virulence factors in *P. aeruginosa*, it is tempting to
365 speculate that QsIA could be a key player in the hyper-virulence phenotype of PA14, along
366 with LadS (34), PAPI-1 and PAPI-2 (30).

367

368 **Material and Methods**

369 **Bacterial strains, culture conditions, plasmids and oligonucleotides**

370 The bacterial strains, plasmids and oligonucleotides used in this study are described in Table
371 2. LB (Lysogeny broth), PIA (*Pseudomonas* isolation agar), and TSB (Tryptic soy broth) broths
372 and agar were used for the growth of *P. aeruginosa* and *Escherichia coli* strains at 37°C.
373 Cultures were inoculated at an optical density at 600 nm (OD₆₀₀) of 0.1 with overnight cultures,
374 and strains were grown at 37°C with aeration in TSB. Recombinant plasmids were introduced
375 into *P. aeruginosa* strain PAO1 and PA14 by conjugation using pRK2013 mobilization
376 properties, as described previously (60). The antibiotic concentrations were as follows: for *E.*
377 *coli*, ampicillin (50 µg ml⁻¹), kanamycin (25 µg ml⁻¹), tetracycline (15 µg ml⁻¹), gentamicin
378 (10 µg ml⁻¹), streptomycin (100µg ml⁻¹); for *P. aeruginosa*, tetracycline (200 µg ml⁻¹ for
379 plates or 50 mg ml⁻¹ for liquid growth), gentamicin (50 µg ml⁻¹), streptomycin (1000µg ml⁻¹
380 1). Expression of the *qsIA* gene under the control of a P_{BAD} promoter in pTS51 was induced or

381 repressed by addition of arabinose or glucose respectively at a final concentration of 0.5% after
382 1h30 of growth.

383

384 **Transcriptional analysis of *Pseudomonas aeruginosa* PA14 and PAO1**

385 To determine if virulence factor genes were differentially expressed between the PAO1 and
386 PA14 *P. aeruginosa* strains, we performed RNAseq on mRNA extracted from cultures of both
387 strains grown in rich media (LB) to early stationary phase. Total RNA was isolated from cell
388 pellets equivalent to 2 OD₆₀₀ units of bacterial culture using SV total RNA isolation system
389 (Promega) following the manufacturer's instructions. Once isolated and prior to the library
390 preparation, the integrity of RNA samples was assessed with a Bioanalyzer system. Barcoded,
391 strand-specific, cDNA libraries were constructed, pooled and sequenced in an Illumina HiSeq
392 2000, single-end 50 bp reads by BGI-Hong Kong. Illumina reads were mapped to the *P.*
393 *aeruginosa* genome PAO1 (AE004091.2 (61)) and PA14 (NC_008463.1 (33)) by Bowtie
394 (version Bowtie1 v0.12.9 (62)), indicating strand-specific sequencing. Quantification of gene
395 expression was determined by the HTSeq package (63) using the GeneBank *P. aeruginosa*
396 PA14 and PAO1 annotations files and discarding multimapped reads. Data were normalized
397 by RPKM and filtered to the 5,263 orthologous genes conserved between the *P. aeruginosa*
398 strains PA14 and PAO1. Two biological replicates were performed per condition. Differential
399 expression analysis was analyzed using the Bioconductor package NOISeq version 2.22.1 (64),
400 a non-parametric approach suitable for lowly replicated data, and using a q-value of 0.99 for
401 strong control of false positives.

402

403 **cDNA library preparation**

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

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

411

412 **Construction of the $\Delta qslA$ mutant**

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

422

423 **Construction of the chromosomal *qslA*_{V5} recombinant gene**

424 PAO1 strains chromosomally encoding QslA_{V5} translational fusion was engineered by
425 exchanging the *qslA* stop codon with the sequence encoding the V5 tag followed by a stop
426 codon. This was achieved by overlapping PCR of the 500 bp upstream and downstream regions
427 of the native stop codon of *qslA* with the High Fidelity DNA polymerase (ROCHE) using
428 primers TSO103, TSO104, TSO105 and TSO106 (Table 2). The PCR product was cloned in
429 pCR2.1 (TA-cloning KIT, Invitrogen), which was then sequenced (GATC) and subcloned in
430 pKNG101 suicide vector giving the pTS52. pTS52, maintained in the *E. coli* CC118 λ pir strain,

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

434

435 ***lacZ* reporter fusion and β -galactosidase assay**

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

445

446 **Pyocyanin production**

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

454

455 **SDS-PAGE and immunoblotting**

456 Proteins from the extracellular medium were loaded at an equivalent of 1.0 OD₆₀₀ unit, while
457 proteins from cellular extracts were loaded at an equivalent of 0.1 OD₆₀₀ unit. Proteins were
458 then separated on SDS gels containing 11 or 15% acrylamide depending on the size of the
459 proteins being further detected.

460 For Western blotting, proteins were transferred from gels onto nitrocellulose membranes. After
461 30 min to overnight saturation in Tris-buffered saline (TBS) (0.1 M Tris, 0.1 M NaCl, pH7.5),
462 0.05% (v/v) Tween 20 and 5% (w/v) skim milk, the membrane was incubated for 1 h with anti-
463 V5 (diluted 1:2500), anti-XcpP (1/2000) or anti-DsbA (1:25000); washed three times with
464 TBS, 0.05% Tween 20; incubated for 45 min with goat anti-rabbit immunoglobulin G (IgG)
465 antibodies (Sigma) diluted 1:5000; washed three times with TBS, 0.05% Tween 20; and then
466 revealed with a Super Signal Chemiluminescence system (Pierce).

467

468 **Adherence assay on inert surfaces and exopolysaccharide production**

469 The adherence assay was performed as previously described in (67) with some modifications.
470 Cultures inoculated at OD₆₀₀=0.05 with O/N cultures were grown at 37°C with aeration in LB
471 with appropriate antibiotics. Induction of the promoter arabinose (P_{BAD}) was done at OD₆₀₀=0.6
472 with 0.2% arabinose for 1.5 hours. 24-well polystyrene microtitre plates were then inoculated
473 at OD₆₀₀=0.2 in 1 ml of MM63 medium supplemented with casamino acids 0.5%, 1 mM
474 MgSO₄ and glucose or arabinose 0.2% for 2 to 48 hours at 30°C. Bacteria were stained with
475 0.1% crystal violet for a period of 10 min and washed twice with water. The stain was then
476 dissolved in ethanol and absorbance was measured at 595 nm.

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

479

480 **Cytotoxicity towards macrophages**

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

487

488 **Statistical analysis**

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

492

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504

505 **Author contributions**

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

511

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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	
<i>qslA</i>	QS regulator	55.8	/	
<i>qscR</i>	QS regulator	/	1.2	
<i>rsaL</i>	QS regulator	/	2.1	
<i>mvaT</i>	QS regulator	/	1.6	
<i>cpdR</i>	QS regulator	/	3.9	
<i>qteE</i>	QS regulator	/	1.9	
<i>rhIR</i>	Rhl QS system	2.5	/	X
<i>rhII</i>	Rhl QS system	1.8	/	X
<i>lasR</i>	Las QS system	1.9	/	X
<i>lasI</i>	Las QS system	1.5	/	X
<i>PA1656</i>	H2-T6SS machinery	1.5	/	X
<i>lasB</i>	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
<i>E. coli</i> strains		
TG1	<i>supE, hsdΔR, thiΔ(lac-proAB), F' (traD36, proAB+, lacIq, lacZΔM15)</i>	Laboratory collection
CC118(λpir)	<i>(λpir) Δ (ara-leu), araD, ΔlacX74, galE, galK, phoA-20, thi-1, rpsE, rpoB, Arg(Am), recA1, Rfr (λpir)</i>	Laboratory collection
TOP10F'	<i>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ</i>	Laboratory collection
<i>P. aeruginosa</i> strains		
PAO1	Wild-type, prototroph, <i>chl-2</i>	B. Holloway collection
PAO1 <i>qslA-v5</i>	Chromosomally encoded QslA _{v5} translational fusion in PAO1	This work
PAO1 <i>ΔqslA</i>	<i>qslA</i> deletion mutant in PAO1	This work
PAO1TS2	<i>P_{H2-T6SS}</i> integrated at <i>att</i> site in PAO1	(15)
PAO1TS2 <i>ΔqslA</i>	<i>P_{H2-T6SS}</i> integrated at <i>att</i> site in PAO1 <i>ΔqslA</i>	This work
PA14	Clinical isolate	F. Ausubel collection
PA14 <i>qslA-v5</i>	Chromosomally encoded QslA _{v5} translational fusion in PA14	This work
PA14TS2	<i>P_{H2-T6SS}</i> integrated at <i>att</i> site in PA14	This work
D40ZQ	<i>xcp</i> locus deletion in PAO1	(68)
PA14 <i>ΔxcpT</i>	<i>xcpT</i> deletion mutant in PA14	(69)
Plasmids		
pCR2.1	TA cloning, <i>lacZα</i> , ColE1, fl ori, Ap ^R Km ^R	Invitrogen
pMini-CTX:: <i>lacZ</i>	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, <i>araC</i> -pBAD	Laboratory collection
pTS2	722 bp upstream region of <i>H2-T6SS</i> in pMini-CTX:: <i>lacZ</i>	(15)
pTS48	500 bp upstream and 500 bp downstream <i>qslA</i> in pCR2.1	This work
pTS49	<i>qslA</i> gene in pCR2.1	This work
pTS50	500 bp upstream and 500 bp downstream <i>qslA</i> in pKNG101	This work

pTS51	<i>qslA</i> gene in pJN105	This work
pTS52	<i>qslA_{v5}</i> in pCR2.1	This work
pTS53	<i>qslA_{v5}</i> in pKNG101	This work
pMAL.R	<i>PlasR-lacZ</i> transcriptional fusion in pMP220	(7)
pMAL.V	<i>PrhlR-lacZ</i> transcriptional fusion in pMP220	(7)
pMAL.I	<i>PrhlI-lacZ</i> transcriptional fusion in pMP220	(7)

Oligonucleotides

TSO58	5'-CCTATCCCTAACCCCTCCTCGGT-3'	(45)
TSO103	5'-CGACCGCAGTTTTCCAACCTGCGGGCCTTCATGGCGG-3'	This work
TSO104	5'TCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT TACCACCGGAACGTCGAGCGGCTACCAGGCGCTGCTGC-3'	This work
TSO105	5'GGTAAGCCTATCCCTAACCCCTCCTCGGTCTCGATTCTACG TGACCCGGCCATGGCGAATGACGCCGGTGCGTCG-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

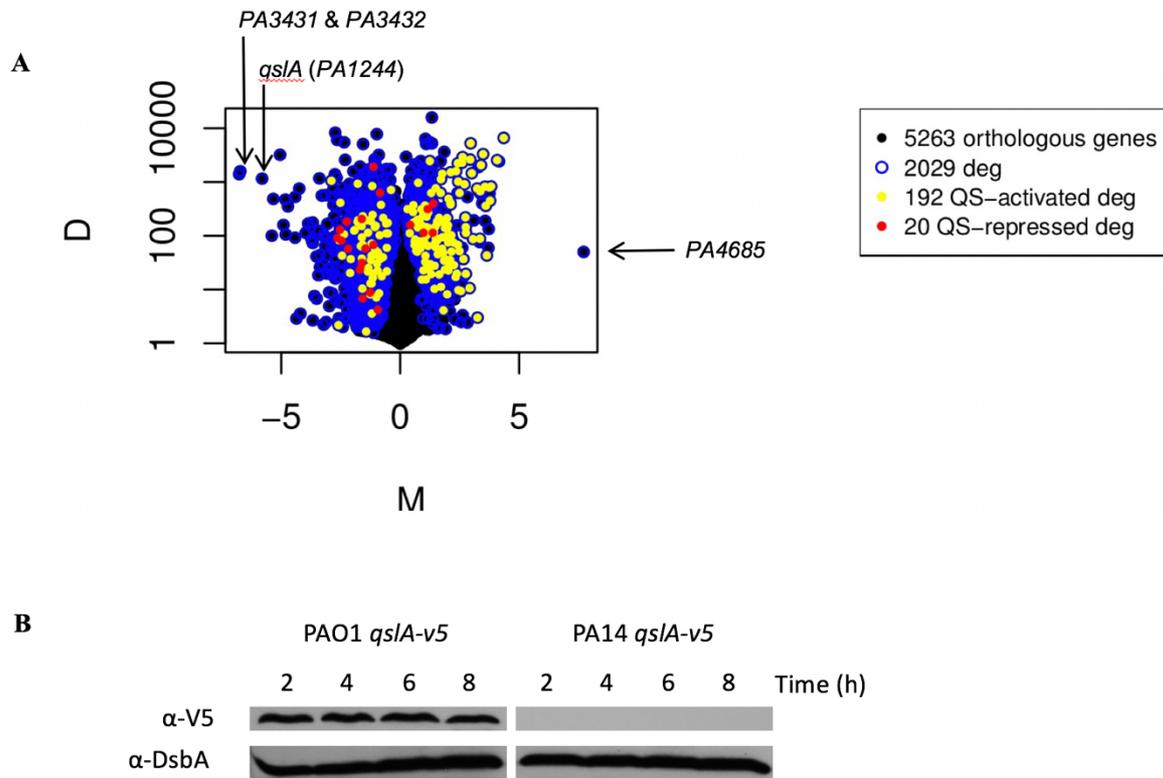
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Figure 1



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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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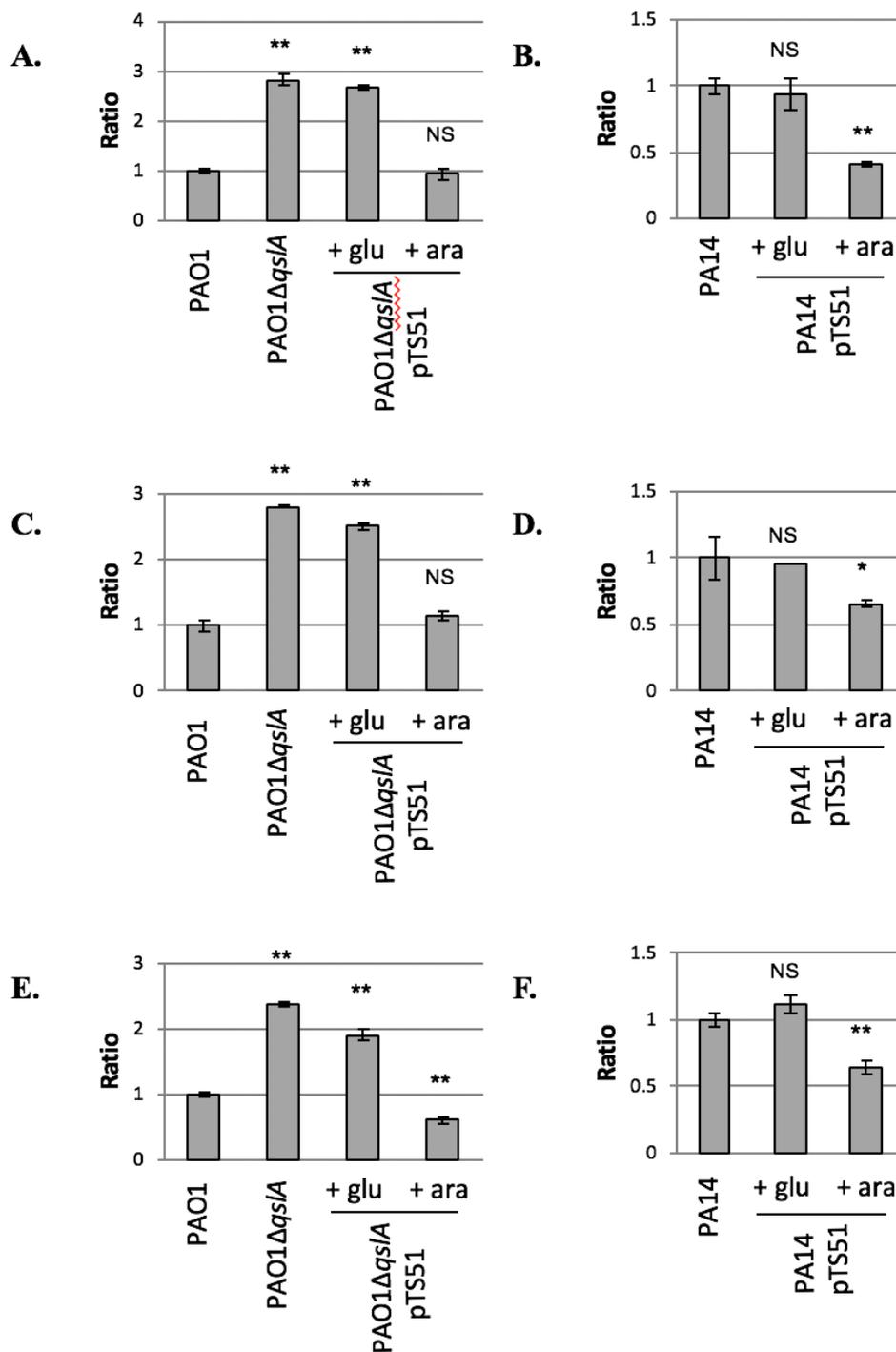
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Figure 2



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749 **Figure 2: β -galactosidase activities of transcriptional fusions P_{lasR} - $lacZ$ (A,B), P_{rhlI} - $lacZ$** 750 **(C,D), et P_{rhlR} - $lacZ$ (E,F). Ratio of expression in parental PAO1 (A,C,E) or PA14 (B,D,F)**751 **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 *qsIA*. **, P <0.01, *, P <0.05 and NS, not significant.

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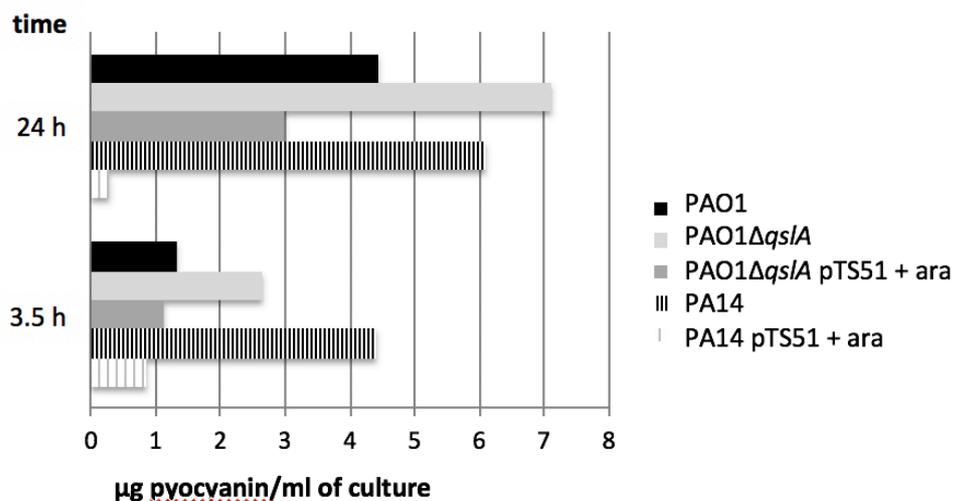
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Figure 3



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

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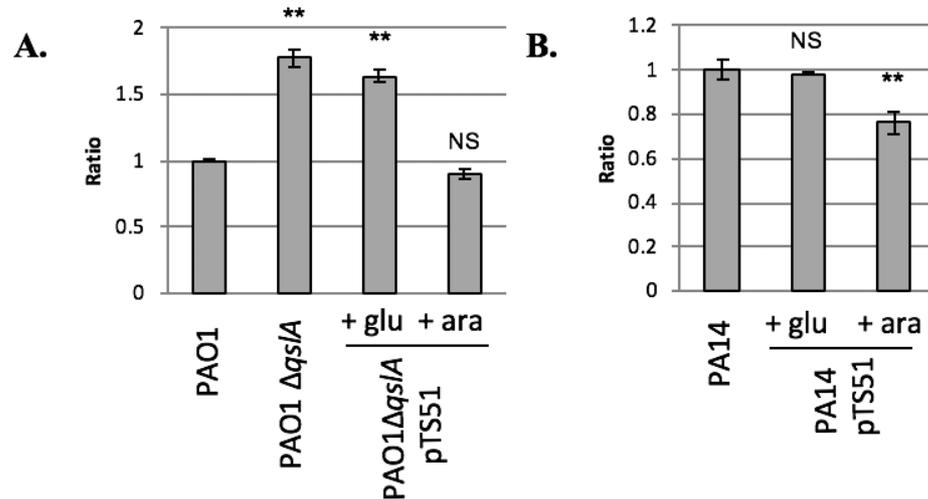
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Figure 4



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797 **Figure 4: β -galactosidase activities of transcriptional fusions $P_{H2-T6SS}$ - $lacZ$.** Ratio of
 798 expression in parental PAO1 (A) or PA14 (B) strain versus expression in referenced strain.

799 Results were obtained after 4 hours of growth at 37°C with aeration. Glucose and arabinose
 800 were added in the culture medium after 1h30 of growth at a final concentration of 0.5%

801 allowing respectively repression or induction of the P_{BAD} promoter of pTS51 encoding *qsIA*.

802 **, $P < 0.01$ and NS, not significant.

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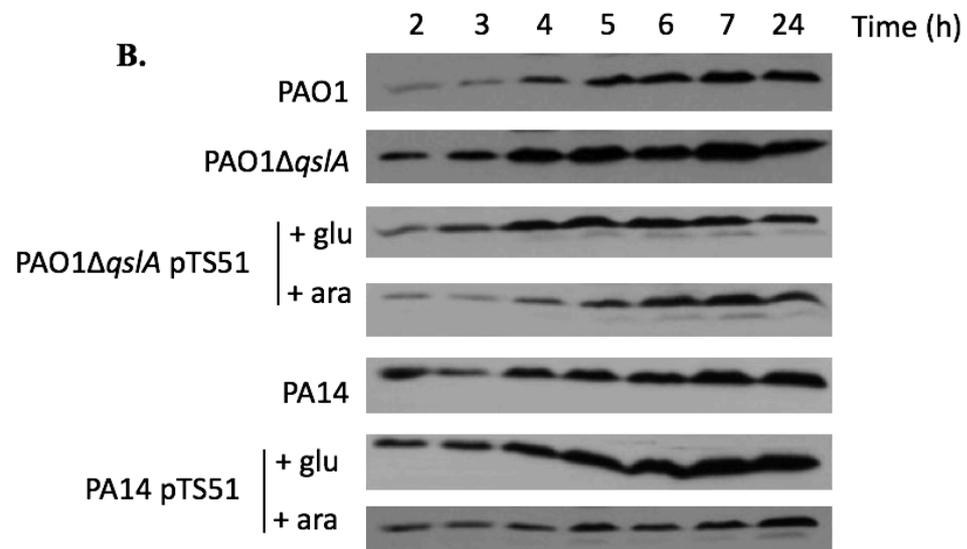
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Figure 5



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

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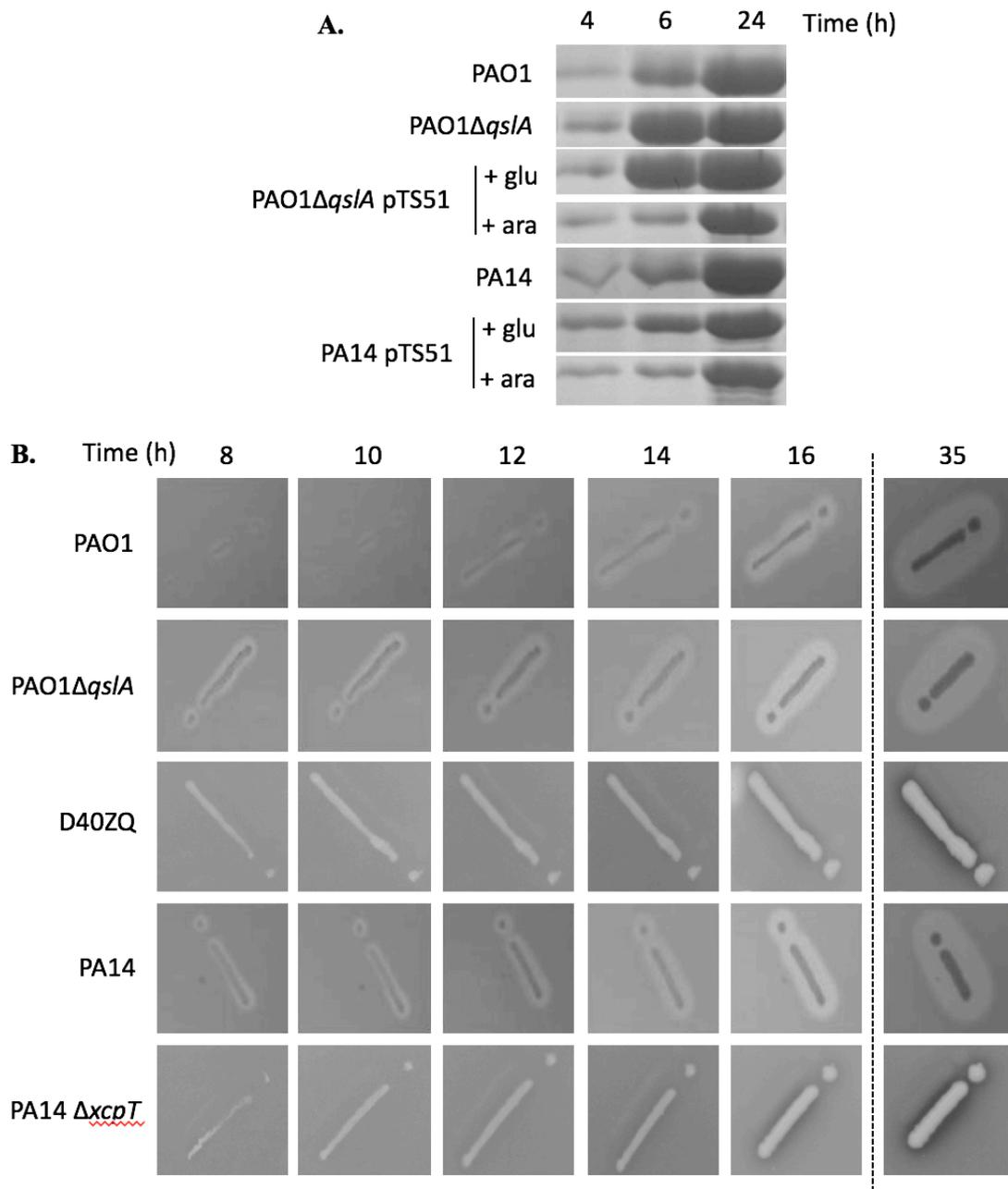
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Figure 6



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826 **Figure 6: (A) Detection of elastase in the extracellular medium. Commassie blue stained**
827 **gel of extracellular medium of different *P. aeruginosa* strains. LasB (or elastase) was**
828 **observed at 33 kDa after 4, 6 and 24 hours of growth at 37°C with aeration. Glucose and**
829 **arabinose were added in the culture medium after 1h30 of growth at a final concentration of**
830 **0.5% allowing respectively repression or induction of the P_{BAD} promoter of pTS51 encoding**
831 ***qsIA*. (B) Kinetics of proteasic activity of elastase secreted by *P. aeruginosa*. Proteolysis of**

832 proteins contained in skim milk allowed formation of a halo all around the colonies of *P.*
833 *aeruginosa* depending on the quantity of elastase secreted. D40ZQ (PAO1 strain lacking all
834 the *xcp* genes) and PA14 $\Delta xcpT$ were used as controls showing that protease activity observed
835 is mainly dependent on this T2SS effector. Elastase activity was observed after 8, 10, 12, 14,
836 16 and 35 hours of growth at 30°C without agitation.

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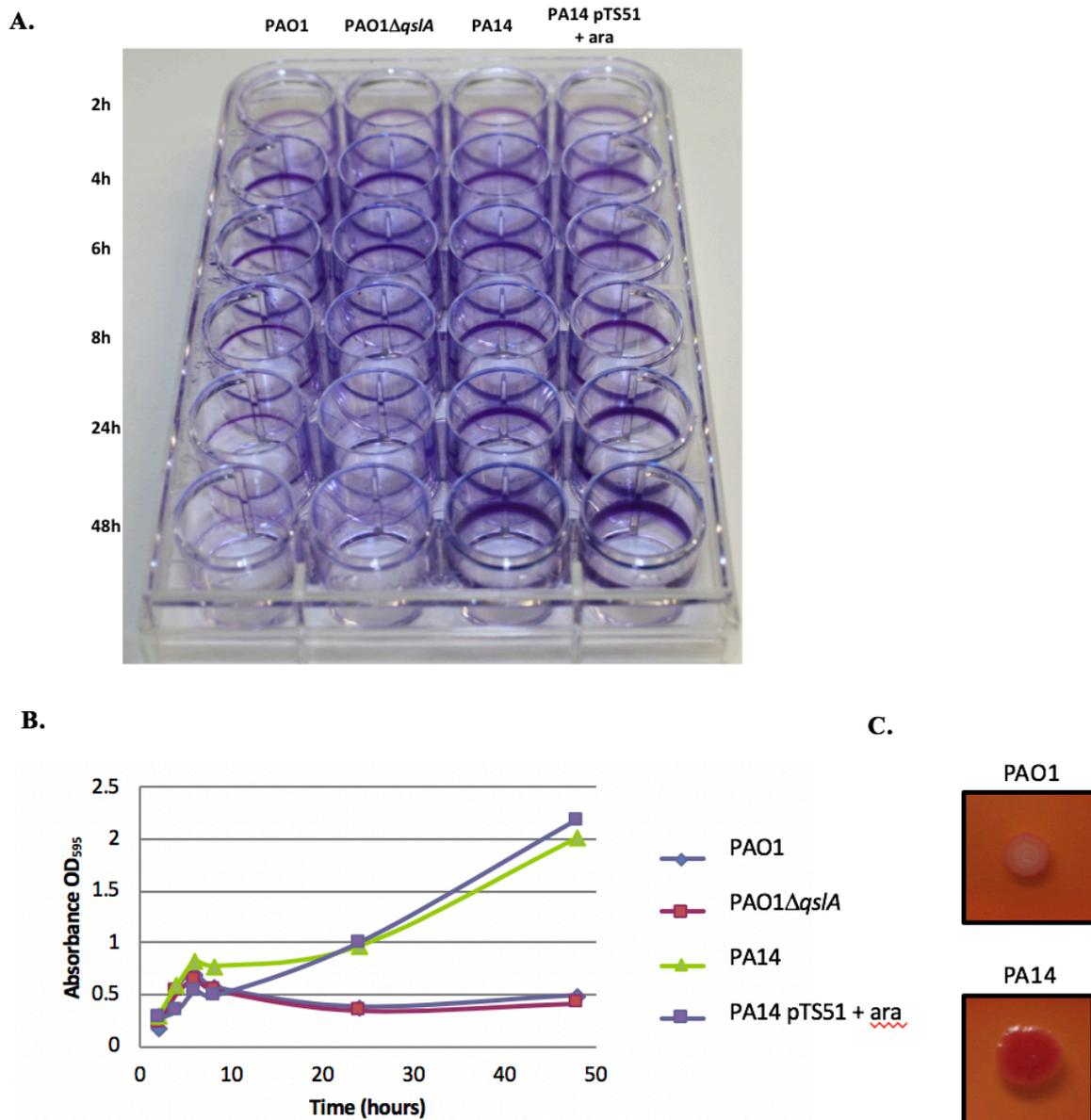
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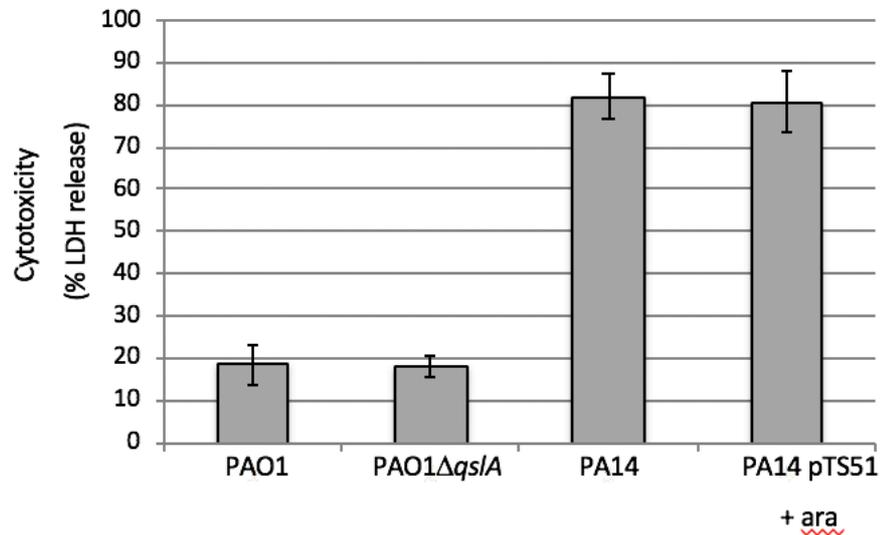
Figure 7



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858 **Figure 7: (A) Bacterial adherence to plastic to infer the ability of strains to form biofilm.**859 Adherence assay with indicated *P. aeruginosa* strains grown at 30°C from 2 to 48 hours in860 minimal medium. Arabinose at 0.5% was added in the culture medium to induce the P_{BAD}861 promoter of pTS51 encoding *qsIA*. **(B) Biofilm quantification.** Crystal violet quantification862 (OD₅₉₅) showed over the time from two independent experiments. **(C) Bacterial colony**863 **staining on Congo red-containing agar plates.** Red color indicates exopolysaccharide

864 production.

Figure 8

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866 **Figure 8: Cytotoxicity towards J774 macrophages** evaluated using a LDH assay after 2
867 hours and 45 min of infection with PAO1 (WT and *qsA* mutant) and PA14 (WT and QsIA
868 overproduction) strains respectively. Arabinose at 0.5% was added in the culture medium after
869 1h30 of growth to induce the P_{BAD} promoter of pTS51 encoding *qsA*. Error bars correspond to
870 standard deviations from three independent experiments done in triplicate. No statistical
871 difference was observed (P values calculated with Student's t test).

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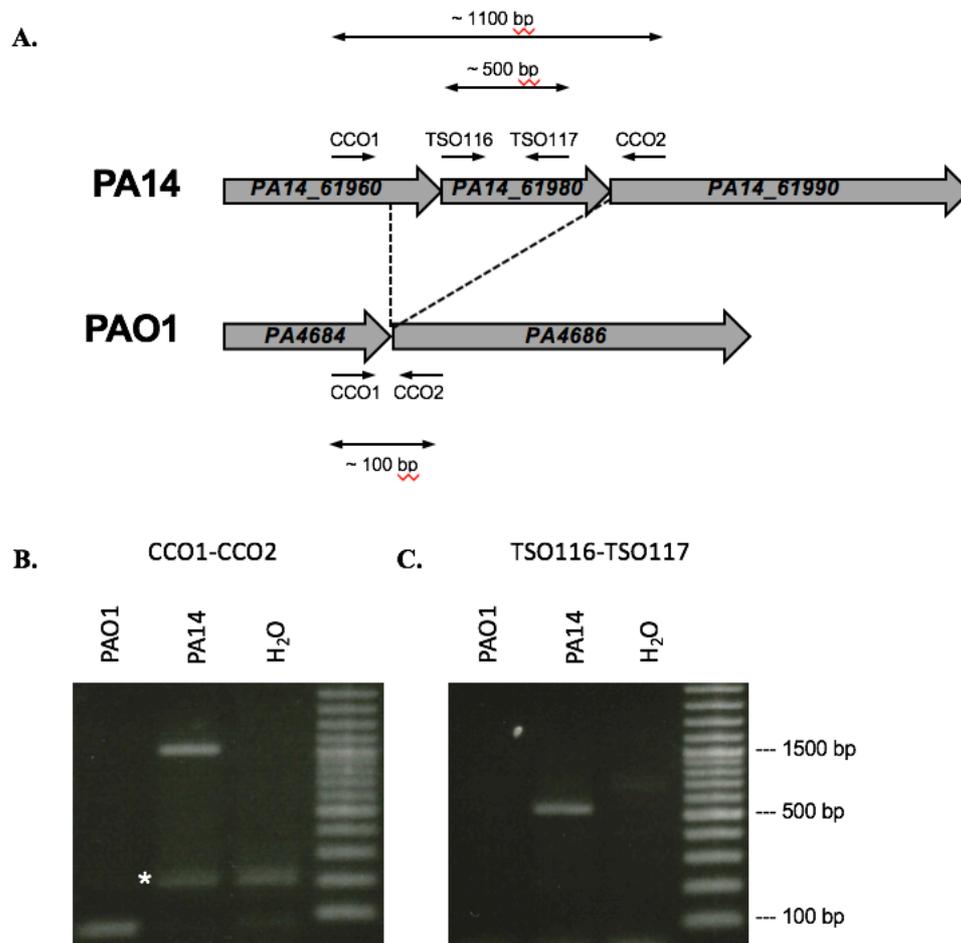
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Figure S1



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

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Figure S2

PA01_QslA : 1-MTLRNGVPSMTKDEKEKTHVDAlIERYKDLMVEIPPADRQPGLSLLWPVPAQPAIDKGVR
PA14_QslA : 1-MTLRNGVPSMTKDEKEKTHVDAlIERYKDLMVEIPPADRQPGLSLLWPVPAQPAIDKGVR
Query : 1-MTLRNGVPSMTKDEKEKTHVDAlIERYKDLMVEIPPADRQPGLSLLWPVPAQPAIDKGVR

PA01_QslA : QAENWLADQIEGQLWTAFAFGRDSLPTPMQKTAFEVAFLLTRLQQRLVAARRSG-131
PA14_QslA : QAENWLADQIEGQLWTAFAFGRDSLPTPMQKTAFEVAFLLTRLQQRLVAARRSG-131
Query : QAENWLADQIEGQLWTAFAFGRDSLPTPMQKTAFEVAFLLTRLQQRLVAARRSG-131

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892 **Figure S2 : BlastP of QslA from PA01 and PA14 strains.**

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