Molecular features underlying *Pseudomonas aeruginosa* persistence in human plasma

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Abstract

Persistence of bacterial pathogens is a main cause of treatment failure and establishment of chronic bacterial infection. *Pseudomonas aeruginosa*, an opportunistic Gram-negative pathogen is a leading cause of bacteremia associated with high mortality. In a previous study, we found that *P. aeruginosa* forms a persister-like sub-population of evaders in human plasma and blood. However, the molecular mechanisms underlying the emergence and biology of evaders remained unknown. Here, using a gain-of-function genetic screen, we examined the molecular determinants of *P. aeruginosa* persistence in plasma. In addition to known virulence factors (long O-specific antigens and exopolysaccharides), we found that ATP and biotin availability greatly impact bacterial survival in plasma. Mutants in genes of purine and biotin pathways displayed higher tolerance and persistence, respectively. Moreover, we identified a novel small protein named SrgA whose expression leads to 100-fold higher survival in plasma. The analysis of different steps of complement activity and the use of an outer-membrane impermeable drug, nisin, suggests that the mutants impede MAC activity *per se*. Through this work we highlight the multifactorial origin of bacterial resilience to plasma and pave the way to a more comprehensive picture of the complex interplay between *P. aeruginosa* and the complement system in the blood.

Introduction

Human bacterial pathogens employ sophisticated strategies to escape from the immune system and to resist or tolerate antibiotic treatments. Bacterial persistence towards antibiotics is a major obstacle in the treatment of life-threatening infections and is frequently associated with the establishment of chronic infections, as it allows the survival of a sub-population that tolerates otherwise lethal antibiotic stress (Fauvart, De Groote, and Michiels 2011; Bartell et al. 2020). Unlike antibiotic resistance, persistence is a non-heritable, fully reversible trait, characterized by a biphasic killing curve with the rapid killing of the bulk population and the survival of a sub-population over a long period of time. Among different factors proposed to lead to antibiotic persistence (Balaban *et al.*, 2019), low intracellular levels of ATP (Shan *et al.*, 2017; Manuse *et al.*, 2021) and production of the "alarmone" signaling molecule guanosine (penta) tetraphosphate, (p)ppGpp (Korch, Henderson and Hill, 2003; Pacios *et al.*, 2020) are the most cited. Upon external antibiotic stress, the stochastic or responsive activation of the stringent response leads to accumulation of (p)ppGpp, which in turn activates toxin-antitoxin modules and degradation of the antitoxins through a regulation cascade, leading to inhibition of cell growth (Gaca, Colomer-Winter and Lemos, 2015; Harms, Maisonneuve and Gerdes, 2016). The host immune system also triggers persistence in different bacterial species. Vacuolar acidification after *Salmonella* internalization by macrophages induces persister cell formation and could contribute to the establishment of a bacterial reservoir for infection relapse (Helaine *et al.*, 2014). In a similar manner, human serum triggers the formation of antibiotic persisters as well as so-called "viable but non-culturable" forms in *Vibrio vulnificus* (Ayrapetyan *et al.*, 2015). Putrinš *et al.*, 2015 also highlighted *Escherichia coli* phenotypic heterogeneity leading to evasion from serum and antibiotic killing. We

74 previously demonstrated that a persister-like sub-population of evaders forms when
75 globally sensitive bacteria are incubated in human blood or human plasma. The
76 evaders were documented in several human pathogens including *Pseudomonas*
77 *aeruginosa* (Pont *et al.*, 2020).

78 *P. aeruginosa* is a leading nosocomial pathogen which is extremely difficult to
79 eradicate due to its high adaptability to different environments and its intrinsic and
80 acquired antibiotic resistance (Moradali, Ghods and Rehm, 2017). Bloodstream
81 infections (BSI) due to *P. aeruginosa* strains hold the highest mortality rate with about
82 40% (Kang *et al.*, 2003; Vitkauskienė *et al.*, 2010). Interestingly, not all BSI isolates
83 are resistant to plasma. Although the complement system (CS) is the major immune
84 component responsible for *P. aeruginosa* killing in human blood and is globally efficient
85 towards sensitive strains, a tiny fraction of the initial bacterial population persists over
86 prolonged incubation in blood and plasma (Pont *et al.*, 2020). Kinetics of *P. aeruginosa*
87 killing by human plasma displays a classical persister-type biphasic profile (Balaban
88 *et al.*, 2019), with a rapid elimination of more than 99.9% of the population reaching a
89 plateau at an average 0.1% after a 2h-incubation. We found that out of eleven *P.*
90 *aeruginosa* isolates tested, three were plasma-resistant, three displayed a tolerant
91 phenotype and five were able to form evaders, suggesting that the evaders may
92 represent a common strategy for *P. aeruginosa* to escape membrane attack complex
93 (MAC)-mediated bactericidal activity in plasma. The plasma evaders differed from
94 antibiotic persisters since a stationary phase population did not give rise to a higher
95 population of evaders as was shown for antibiotic persisters (Keren *et al.*, 2004). The
96 mechanisms permitting the evaders to resist the MAC-induced lysis remained
97 unknown. In this work, we referred to resistance, tolerance and persistence as defined
98 in a context of antibiotics (Balaban *et al.*, 2019). Briefly, resistance is due to the

99 heritable carriage of a resistance factor allowing survival to a higher stress conditions.
100 As opposed to resistance, tolerance is a phenomenon that allows survival of the
101 population to an otherwise lethal stress for a longer period of time without any
102 resistance mechanism. Persistence is a capacity of a tolerant sub-population to survive
103 while the rest of the population is eliminated. Finally, we used the term resilience to
104 describe several of these phenomena.

105 In this work, we employed a genome-wide approach to get first insights into molecular
106 mechanism(s) of *P. aeruginosa* persistence in human plasma. A screen based on
107 transposon-insertion sequencing (Tn-seq) was applied to a recent clinical isolate that
108 showed sensitivity to plasma and displayed between 0.01 and 0.1% of evaders
109 following incubation in plasma of 6h. We hypothesized that the transposon mutants
110 displaying an increased percentage of evaders or those with global increased plasma
111 tolerance/resistance could reveal novel determinants of bacterial resilience to plasma
112 and pathogen immune escape. In addition to known *P. aeruginosa* factors involved in
113 plasma resistance, such as O-specific antigen (OSA) length and exopolysaccharides,
114 we identified an uncharacterized small periplasmic protein whose expression
115 increased complement tolerance up to 100 fold. Moreover, our screen and phenotypic
116 characterization of mutants revealed that the levels of biotin and ATP modulate
117 pathogen sensitivity to MAC-induced lysis.

118 Results

119 Gain-of-function screen in plasma

120 In our previous study we found that the urinary isolate IHMA879472/AZPAE15042
121 (IHMA87) persisted in human blood and plasma. While the global population was
122 sensible to complement killing with 99.9% of the population eliminated within 2 hours,

123 approximately 0.1% evaders survived prolonged incubation in plasma (Pont *et al.*,
124 2020 and this work). In order to examine molecular determinants leading to persistence
125 in plasma, we searched for hyper-evaders using a genome-wide approach. A
126 transposon library of about 300,000 mutants was constructed using the Himar-1
127 transposon and insertion mutants with increased levels of survival in plasma were
128 selected using the phenotypic screen schematically presented in Fig. 1A. The
129 transposon library was challenged in native human plasma (output) or heat inactivated
130 plasma (HIP, input) for three hours and recovered. The bacterial survival was
131 determined by colony forming unit (CFU) counts for the whole library before and after
132 the challenge, showing that the global survival increased by about 2-log in the mutant
133 library compared to the parental strain. Thus, the screen set-up selects for mutants
134 with global increased survival, and the discrimination between resistant, hyper-evaders
135 and tolerant phenotypes can be appreciated by performing killing kinetics over a long
136 incubation period in plasma.

137 Seven mutants were isolated directly during the screen and their survival was assayed
138 in plasma killing assays. They survived 10- to 200-fold better than the parental strain
139 (Fig. 1B), validating the screen. Among the isolated mutants, two mutants with hyper
140 mucoid phenotypes reflecting alginate overproduction, carried insertions within *mucD*
141 and in the intergenic region upstream of *mucA*, two negative regulators of alginate
142 synthesis (reviewed in Damron and Goldberg, 2012). We also isolated a mutant with
143 the transposon inserted in the intergenic region upstream of *mucE*, a positive regulator
144 of alginate production (Qiu *et al.*, 2007; Yin *et al.*, 2013). The orientation of the
145 transposon suggested that this mutant could overexpress *mucE,* which also led to a
146 mucoid phenotype. The alginate indeed may interfere with the CS by decreasing C3b-
147 dependent opsonization and by interfering with CS activation when acetylated

148 (Pedersen *et al.*, 1990; Pier *et al.*, 2001). A transposon insertion in *ladS*, encoding the
149 histidine kinase positively affecting GacS/GacA-RsmA pathway (Ventre *et al.*, 2006;
150 Broder, Jaeger and Jenal, 2017), resulted in increased mutant survival, by yet
151 unknown downstream effector. To note, the bioinformatics analysis of the sequencing
152 data highlighted significant enrichment of transposon insertions in *retS*, a gene
153 encoding for a LadS antagonist, whose inactivation leads to increased synthesis of
154 polysaccharides Psl and Pel (Table 1). Indeed, Psl was shown to improve bacterial
155 survival in serum in a mucoid strain (Jones and Wozniak, 2017). The role of Psl in
156 complement resistance might be isolate-dependent, as it was initially shown using the
157 reference strain PAO1, that Psl reduces bacterial opsonization without affecting C9
158 insertion and survival in serum (Mishra *et al.*, 2012). Moreover, insertion in the
159 promoter of *pprB,* encoding the response regulator acting on type IVb pili/Tad, BapA
160 adhesin and CupE fimbriae expression (Giraud *et al.*, 2011; de Bentzmann *et al.*, 2012)
161 also led to increased survival in plasma. As shown in *Vibrio cholerae* and *Neisseria*
162 *gonorrheae*, type IV pili contribute to serum resistance and recruitment of host negative
163 complement regulator C4BP (Chiang *et al.*, 1995; Blom *et al.*, 2001). Our data
164 suggests that either type IVb pili or CupE-fimbriae could mediate plasma resilience in
165 *P. aeruginosa*. Random picking of colonies did not allow to obtain any LPS-related
166 mutants. However, the importance of LPS and more specifically O-specific antigens
167 biosyntheses in the interaction with plasma was highlighted by the screen and is in
168 accordance with the literature (Goldberg and Pler, 1996; Priebe *et al.*, 2004; Kintz *et*
169 *al.*, 2008; Osawa *et al.*, 2013). Finaly, two isolated clones with increased survival
170 carried the transposon in *bioA* and *bioB*, encoding enzymes of biotin biosynthesis; to
171 our knowledge, not previously linked to resilience to plasma or complement.

172 Analysis of sequencing data was performed for both the insertions in coding regions
173 and insertions in intergenic regions, as the transposon used possesses an outward
174 *tac* promoter, possibly modulating the neighboring gene expression. In both analysis,
175 transposon insertions strongly increasing bacterial survival were associated with three
176 main pathways; *i.e.* biosynthetic pathways of purine and biotin, and biosynthesis,
177 production and regulation of the exopolysaccharide alginate (Fig. 1C and Table 1,
178 complete results Table 1S).

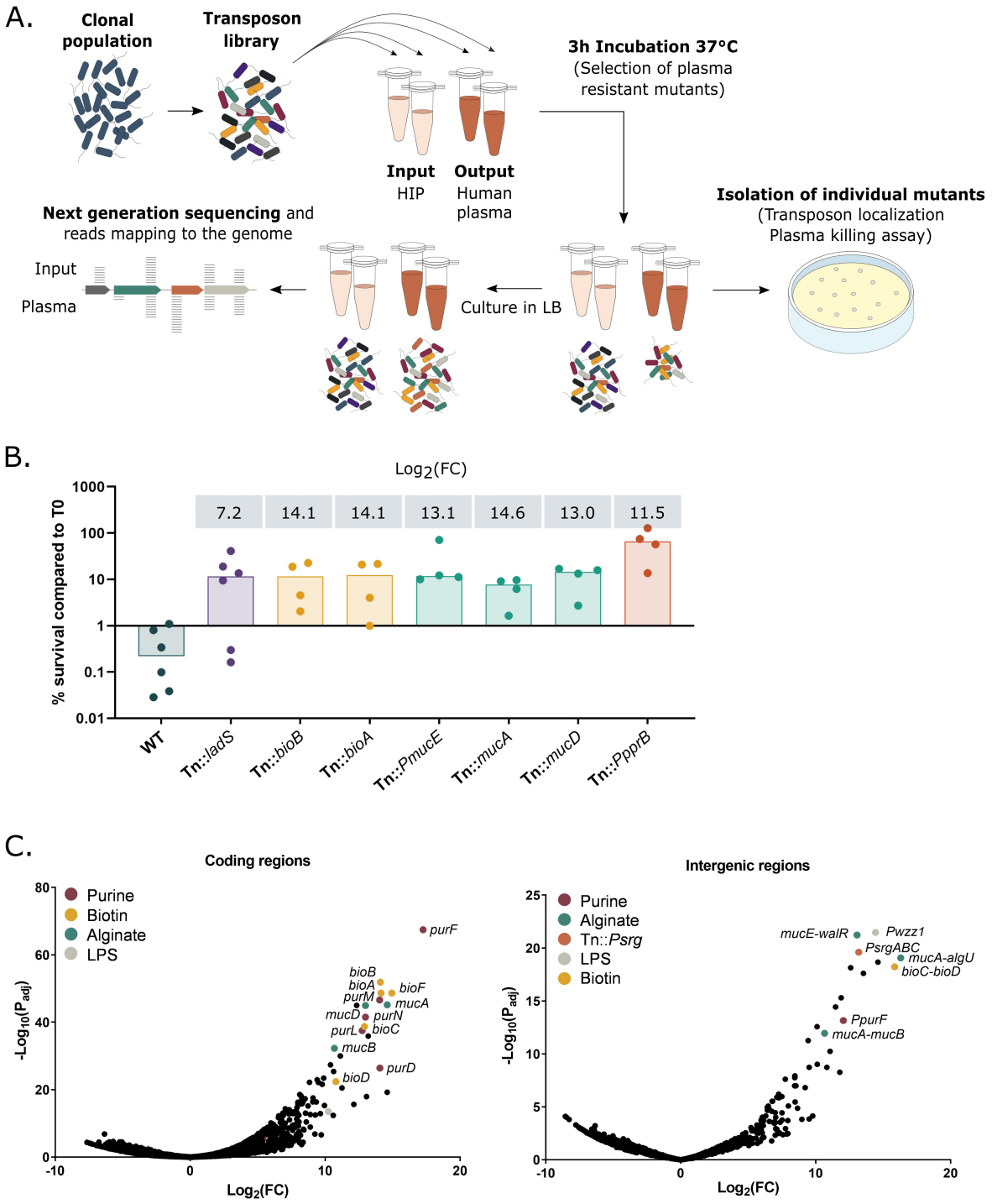


Figure 1. Gain-of-function Tn-seq reveals common as well as novel pathways involved in *P. aeruginosa* survival in plasma. A. Schematic representation of the screen. A transposon mutant library was generated in the plasma-sensitive clinical isolate *P. aeruginosa* IHMA87 and incubated either in human plasma or in HIP (input) for 3h. Aliquots were plated on LB and individual transposon mutants were stored for further analysis. Transposon-insertion sequencing was performed by Illumina-based next generation sequencing. **B.** Survival of isolated transposon mutants in plasma following 3h-incubation. The survival was calculated using CFUs measurements. A

188 log-transformation was performed on the dataset and all mutants were shown
189 significantly different from the wild-type strain (p-value < 0.005). Log_2(Fold-
190 Change(FC)) obtained from the Tn-seq analysis for each gene or intergenic region is
191 indicated above. **C.** Bioinformatics analysis presented as Volcano plots. Insertions in
192 coding regions (left) and intergenic regions (right) were analyzed separately.
193 Significant hits in genes of the same pathway are in the same color.
194
195

IHMA87 ID	PAO1 ID	Name/prediction	Log_2(FC)	P_{adj}
Purine biosynthetic pathway				
IHMA87_01837	*PA3108*	*purF*	17.26	3.64E-68
IHMA87_05343	*PA4855*	*purD*	14.06	3.85E-27
IHMA87_04292	*PA0944*	*purN*	12.99	3.14E-42
IHMA87_04291	*PA0945*	*purM*	14.04	2.51E-47
IHMA87_01191	*PA3763*	*purL*	12.75	3.02E-38
IHMA87_04214	*PA1013*	*purC*	7.67	1.23E-5
IHMA87_02487	*PA2629*	*purB*	5.35	7.23E-4
Biotin biosynthetic pathway				
IHMA87_00400	*PA0420*	*bioA*	14.15	2.29E-49
IHMA87_00477	*PA0501*	*bioF*	14.94	2.29E-49
IHMA87_00476	*PA0500*	*bioB*	14.10	1.39E-52
IHMA87_00479	*PA0503*	*bioC*	12.92	1.90E-39
IHMA87_00480	*PA0504*	*bioD*	10.80	4.23E-23
Alginate biosynthetic/regulation genes				
IHMA87_04494	*PA0762*	*algU*	11.25	3.01E-21
IHMA87_04493	*PA0763*	*mucA*	14.60	6.76E-46
IHMA87_04492	*PA0764*	*mucB*	10.69	5.56E-33
IHMA87_04490	*PA0766*	*mucD*	13.00	1.10E-45
IHMA87_00895	*PA4033*	P_{mucE}	13.07	5.88 E-22
IHMA87_05847	*PA5322*	*algC*	2.66	4.76 E-2
Other top hits				
IHMA87_02894		-	14.60	6.18E-20
IHMA87_01786_IG	*PA3161_IG*	P_{wzz1}	14.44	3.42E-22
IHMA87_02666	*PA2484*	-/transcriptional regulator	13.20	1.33E-36
IHMA87_06317		-	12.34	1.02E-45
IHMA87_02554	*PA2573*	-/chemotaxis transducer	11.13	1.02E-30
IHMA87_06033	*PA5490*	*cc4*	10.63	4.10E-26

IHMA87_04274		*slyX*	10.60	4.67E-13
IHMA87_04133	*PA1078*	*flgC*	10.40	4.59E-28
IHMA87_04779	*PA4457*	*kdsD*	10.24	3.15E-14
IHMA87_04779	*PA4457*	-/arabinose-5-phosphate isomerase	10.24	3.15E-14
IHMA87_00896	*PA4032*	*walR*	9.89	3.67E-24
IHMA87_00319	*PA0341*	*lgt*	9.95	5.21E-16
IHMA87_03447	*PA1758*	*pabB*	9.79	2.55E-22
IHMA87_03022	*PA2122*	-/lipid biosynthetic process	9.65	1.27E-13
IHMA87_01755		-	9.51	7.37E-23
IHMA87_04617	*PA4315*	*mvaT*	9.41	1.21E-23
IHMA87_02603		-	9.34	4.46E-17
IHMA87_02570		-	9.28	3.29E-13
IHMA87_05344	*PA4856*	*retS*	9.11	2.94E-18

Table 1. Insertion mutants enriched in plasma compared to HIP in the three main pathways. The genes and pathways showing the most significant (P_{adj}) fold change (Log_2(FC)) in the Tn-seq screen. IHMA87 gene identity (ID) (NCBI accession numbers CP041354 and CP04135), for the chromosome and plasmid sequences respectively (Trouillon *et al.*, 2020) are indicated with PAO1 equivalents where applicable. For each gene, the Log_2(FC) in plasma compared to HIP (input) condition is indicated together with the corresponding adjusted *p*-value (P_{adj}), obtained by the DeSeq2 program. The genes are sorted according to their predicted pathway taken from the KEGG database and the *Pseudomonas* genome database (Kanehisa *et al.*, 2008; Winsor *et al.*, 2011). IG is added when insertions were detected in the intergenic region between two genes and the intergenic region is attributed to the upstream gene, independently on the genes orientation.

In several mutants showing >9 Log_2(FC) in survival, the transposon was inserted in uncharacterized genes, representing potentially novel factors of CS evasion (Table 1). Together, those results confirmed the role of LPS, alginates and Psl in resistance to the CS, which validated the Tn-seq approach and pointed toward several novel determinants to explore.

216 **Deficiency in purine and biotin pathways leads to increased persistence**

217 We further focused on hits in genes and intergenic regions of biotin and purine
218 biosynthesis. Mutants with insertions in loci of the entire biotin biosynthesis, the
219 *bioBFHCD* operon and the *bioA* gene, were significantly overrepresented in plasma
220 (Table 1, Fig. 2A), suggesting that the absence of biotin leads to better survival in
221 plasma. This finding was surprising, as the biotin pathway was found essential for
222 survival of several pathogens in human serum including *Klebsiella pneumoniae* and
223 *Mycobacterium tuberculosis* (Woong Park *et al.*, 2011; Carfrae *et al.*, 2020; Weber *et*
224 *al.*, 2020). In addition to the mutants isolated from the screen (Tn::*bioA* and Tn::*bioB*),
225 and in order to confirm this result, we engineered a *bioB* deletion mutant (Δ*bioB*) and
226 examined its behavior in plasma over a 6h-challenge (Fig. 2B). Compared to the wild-
227 type strain, the fraction of Δ*bioB* that survived in plasma was over 10% compared to
228 about 0.01% of parental strain. Moreover, the survival of IHMA87Δ*bioB* displayed a
229 biphasic killing curve, suggesting that the low level of biotin triggered the persistence
230 in human plasma.

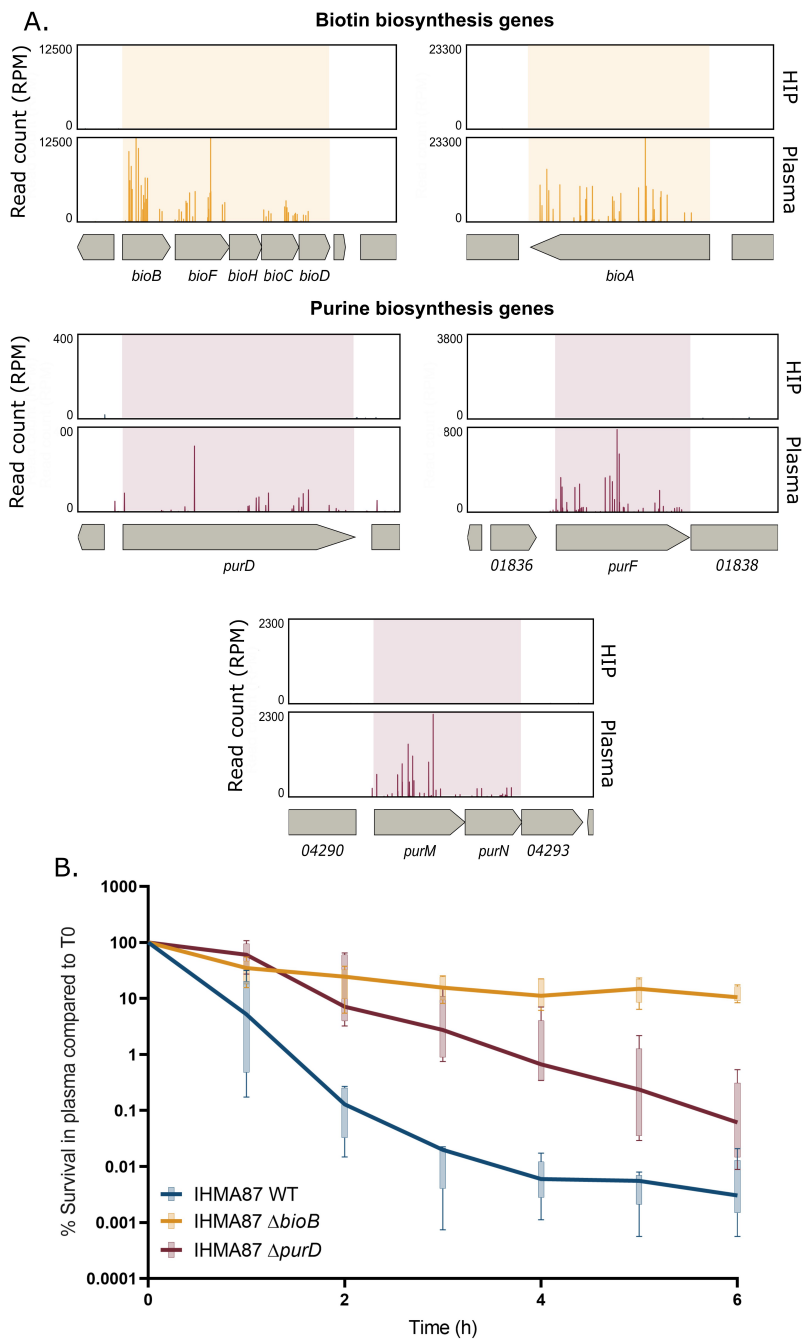


Figure 2. Inactivation of biotin and purine biosynthetic pathways leads to higher survival rates of IHMA87 in plasma. A. Zoom on Tn-seq profiles of *bio* and *pur* genes and operons, showing number of normalized reads in input (HIP) and output (plasma) samples. **B.** Survival kinetics of IHMA87 wild-type strain, Δ*bioB* and Δ*purD* mutants in plasma over 6h incubation measured by CFU counting (n=5). Note the biphasic killing curves for the parental strain and Δ*bioB* mutant, indicating increased persistence of Δ*bioB.* The Δ*purD* mutant displayed increased tolerance compared to the parental strain.

241 *De novo* purine biosynthesis, together with the pyrimidine pathway, are essential for
242 bacterial nucleotide metabolism and is important for bacterial growth and survival in
243 plasma and serum (Samant *et al.*, 2008; Andersen-Civil *et al.*, 2018; Poulsen *et al.*,
244 2019; Zhu *et al.*, 2021). Similarly to biotin synthesis, in our model, mutants in the purine
245 biosynthetic pathway clearly displayed better survival in plasma (Fig. 2A). Transposon-
246 insertion mutants in three main operons encoding enzymes of purine biogenesis were
247 found overrepresented in the screen. Notably, mutants in *purF*, encoding an amido-
248 phospho-ribosyltransferase were significantly enriched with a Log_2(FC) of 17. To
249 further study the link between purine biosynthesis and increased plasma resistance,
250 we designed a *purD* deletion mutant (Δ*purD*) and investigated its killing kinetics over
251 6-hours in plasma. In accordance with Hill *et al.*, 2021 in an antibiotic context, a Δ*purD*
252 mutant displayed a tolerant phenotype in plasma (Fig. 2B). To note, no genes were
253 found to be enriched to the same level in the pyrimidine pathway, suggesting that the
254 specific product of the purine pathway and not the global nucleotide metabolism,
255 contributed to increased survival in human plasma.

256

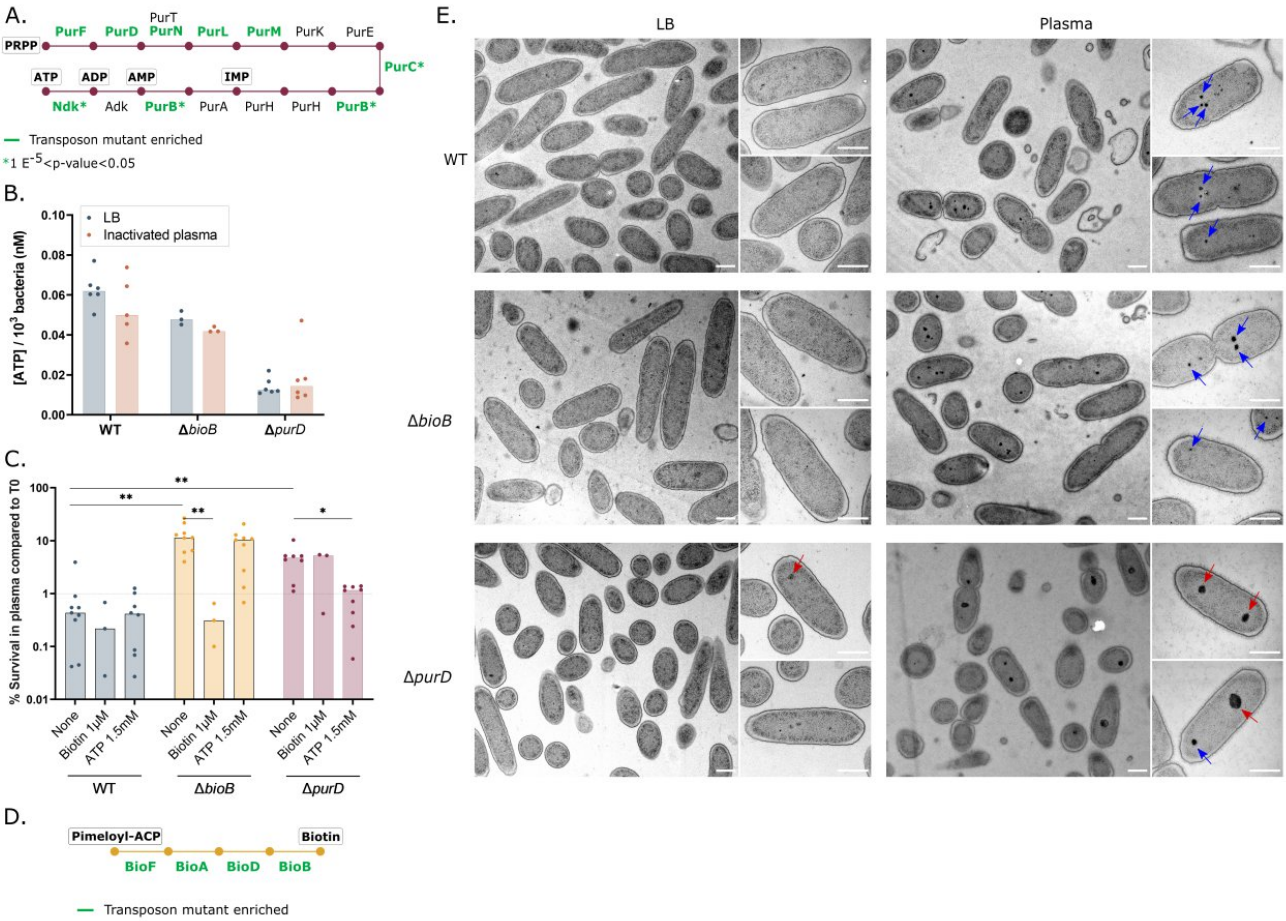
257 **ATP levels influence *P. aeruginosa* tolerance to plasma**

258 Recent studies showed that low ATP levels resulted in increased antibiotic persistence
259 (Shan *et al.*, 2017; Manuse *et al.*, 2021). In the same line, bacteria from the stationary
260 phase of growth have lower levels of intracellular ATP and form a higher proportion of
261 persisters when treated with antibiotics (Conlon *et al.*, 2016). In our hands, growth
262 phase did not contribute significantly to evaders' population suggesting that the initial
263 metabolic status of bacteria had no important role in persistence (Pont *et al.*, 2020).
264 However, because ATP is a final product of the purine pathway (Fig. 3A), we
265 hypothesized that it could also play a role in evaders' formation.

266 To tackle this question, we first measured intracellular ATP levels in Δ*purD* both in rich
267 medium and after incubation in HIP. As expected, Δ*purD* exhibited 5 times lower ATP
268 concentration than the parental strain (Fig. 3B), and no difference could be observed
269 between HIP and LB. To confirm that the low ATP concentration was indeed
270 responsible for higher resilience to human plasma, we modulated ATP levels by
271 supplementing the plasma with exogenous ATP during the plasma killing assay (Fig.
272 3C). Indeed, the addition of ATP during the incubation in plasma restored a wild-type-
273 like sensitivity to plasma, further demonstrating the ATP-dependent phenotype of *pur*
274 mutants. To further characterize the Δ*purD* mutant, we investigated potential envelope
275 changes due to the ATP depletion that could account for resistance to MAC killing at
276 the cellular level. We imaged and compared the different strains grown in LB medium
277 or challenged in plasma by transmission electron microscopy (TEM) (Fig. 3E). When
278 grown in LB, the wild-type strain and the Δ*bioB* and Δ*purD* mutants displayed similar
279 size, shape, and apparent envelope thickness. However, the Δ*purD* mutant cells
280 occasionally harbored small electron dense granules (Fig. 3E, blue arrow). As
281 expected from its higher sensitivity to plasma, more cell debris was visible on the
282 samples of the wild-type strain incubated in plasma. Interestingly, when Δ*purD* cells
283 were challenged by human plasma, the size and number of the electron dense
284 granules increased (Fig. 3E, red arrows). In wild-type and Δ*bioB* backgrounds, plasma-
285 challenged bacteria displayed small granules, similar to the ones present in the Δ*purD*
286 mutant grown in LB (Fig. 3E, blue arrows). Bibliography mining indicated that these
287 objects may be polyphosphate (Poly-P) storage granules as suggested in Racki *et al.*,
288 2017, whose formation is accelerated in stress and starvation conditions.

289 The biotin biosynthesis pathway (Fig. 3D) had no apparent link to ATP synthesis.
290 However, as biotin impacts many biological processes, we tested whether a Δ*bioB*

291 mutant could be rescued by ATP. As shown in Figure 3C, biotin but not ATP was able
292 to restore a wild-type-like sensitivity of Δ*bioB* suggesting a distinct molecular
293 mechanism for evader formation in this mutant.



294

295 **Figure 3. ATP and biotin influence bacterial resistance to plasma. A.** Schematic
296 view of the purine pathway (adapted from KEGG database (Kanehisa *et al.*, 2008)).
297 Significantly enriched insertions in corresponding genes of the Tn-seq screen are
298 indicated**. B.** Measurement of intra-bacterial ATP levels in LB and after 2h-incubation
299 in HIP normalized to the CFU counts. **C.** Trans-complementation of Δ*bioB* and Δ*purD*
300 phenotype by exogenous biotin or ATP, respectively. Biotin (1 μM) and ATP (1.5 mM)
301 were added at the beginning of the incubation of bacteria in plasma. The survival was
302 estimated by CFU counting. **D.** The biotin biosynthetic pathway. Significantly enriched
303 hits in Tn-seq are highlighted in green. **E.** Transmission electron microscopy images
304 of indicated strains after growth in LB (left) or 1h-incubation in human plasma (right).
305 Red and blue arrow show big- and small-sized granules respectively. Scale bar = 500
306 nm.

307

308 **Expression of the three-gene operon *srgABC* leads to better survival in plasma**

309 One mutant isolated during the screen was significantly enriched in plasma with a
310 Log_2(FC) of 13.2 (Fig. 4A). The Sanger sequencing determined the exact transposon
311 insertion within the intergenic region between an uncharacterized predicted three-
312 gene operon *IHMA87_01573-IHMA87_01571* (predicted in PA14 by Wurtzel *et al.*,
313 2012, corresponding to *PA3369-PA3371* in PAO1) and *panM*
314 (*IHMA87_01574/PA3368*), encoding a shorter version of a probable acetyltransferase
315 (Fig. 4B). The isolated mutant displayed a tolerant phenotype over a 6-hour period
316 (Fig. S1) and a 100 fold increase in survival compared to the parental strain after 3h
317 of incubation (Fig. 4C). The position of the transposon and the RT-qPCR analysis did
318 not allow us to assess the effect of the insertion. In addition, strains carrying deletions
319 either in *panM* or in *IHMA87_01573-IHMA87_01571* in the IHMA87 background
320 showed no difference in survival in plasma compared to the wild-type strain.
321 Therefore, to globally apprehend the transposon-induced changes in the mutant, we
322 determined the transcriptomic profile of the mutant and compared it to the parental
323 strain in LB. The RNA-seq data showed the overexpression of the predicted operon
324 *IHMA87_01573-IHMA87_01571*, named *srgABC* for serum resistance genes, with
325 about a 20-fold change compared to the parental strain. Thus, the transposon insertion
326 mutant will be referred to as Tn::P_{srg}. All together 90 genes were differentially
327 expressed (|FC|>2, $p_{adj}<1x10^{-10}$) between Tn::P_{srg} and the wild-type strain (Table 2S),
328 including *algU* encoding the alternative sigma factor AlgU and five other alginate genes
329 (*algR* and *mucABCD* operon, Fig. 4D). Consistent with the observed upregulation of
330 *algU*, colonies displayed characteristic mucoïd phenotype on solid medium. The *srg*
331 operon encodes three small putative proteins of 9.9 (SrgA), 5.3 (SrgB) and 6.5 (SrgC)
332 kDa. Based on structural predictions, SrgA is a periplasmic or secreted protein

333 presenting the signal peptide cleavage site at position Gly27. Both SrgB and SrgC are
334 predicted membrane proteins with one and two transmembrane alpha-helices for SrgB
335 and SrgC, respectively. The *srg* genes are highly conserved over the 232 complete *P.*
336 *aeruginosa* genomes available on *Pseudomonas* Genome database (Winsor *et al.*,
337 2016) with 98-100% amino acid sequence identity.

338 The individual contributions of the *srg* genes in *P. aeruginosa* tolerance to plasma
339 were evaluated by testing single, double and triple deletion mutants directly in the
340 Tn::P_{srg} background strain. Plasma killing assay of the mutants showed that SrgA was
341 required and sufficient for increased survival in plasma (Fig. 4C).

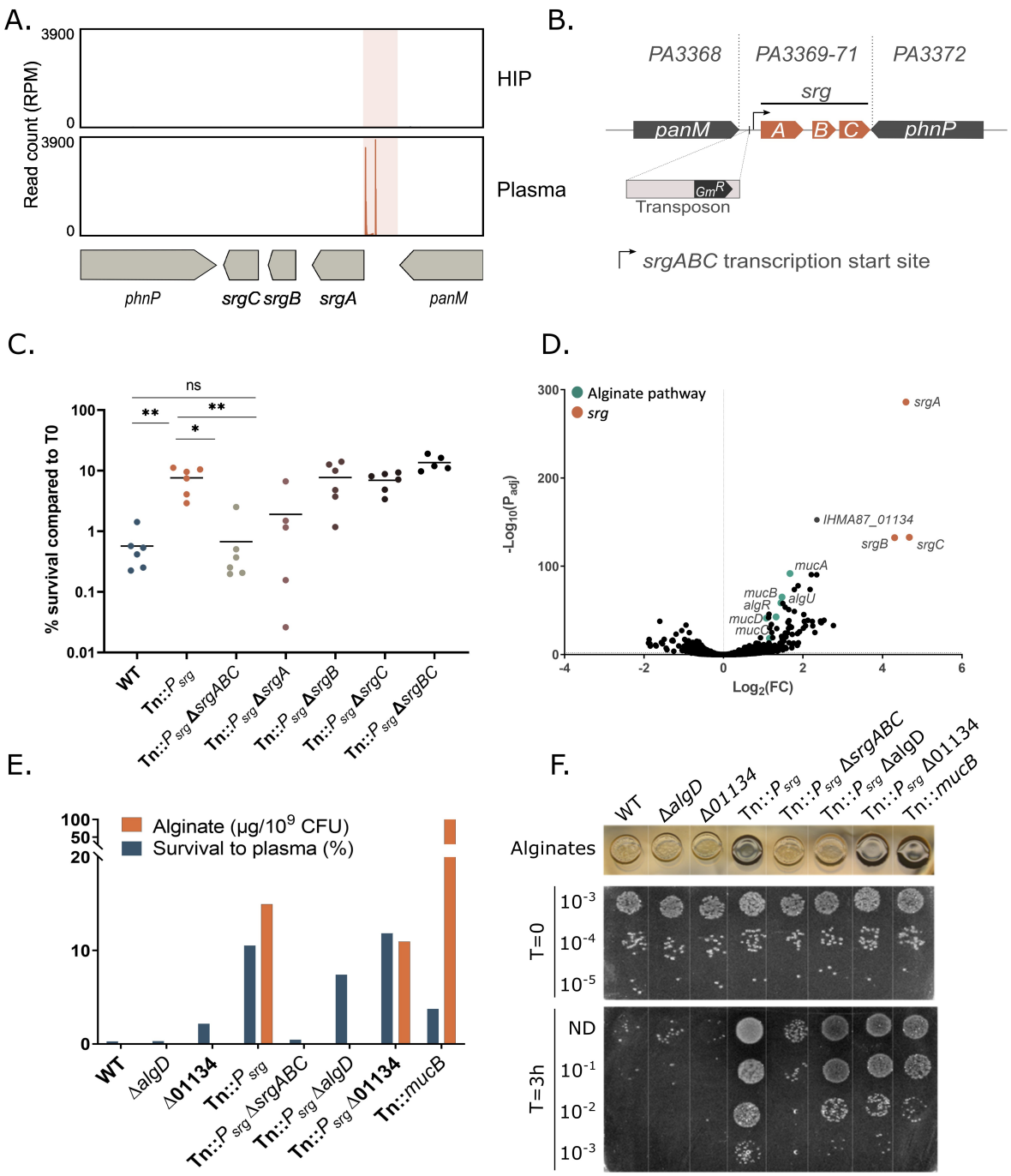


Figure 4. Overexpression of *srgA* leads to a 100-fold increase in survival. A. Zoom into the Tn-seq profiles in the region around the *srg* operon showing number of normalized reads in input (HIP) and output (plasma). **B.** Schematic representation of the predicted *srgABC* operon and position of the transposon in the isolated Tn::P_{srg} mutant as revealed by Sanger sequencing. Corresponding genes from PAO1 are indicated. Note that the transposon is inserted 13 bp upstream of the TSS **C.** Role of individual *srg* genes in the plasma resilience phenotype of the Tn::P_{srg} strain. Note that the deletion of *srgA* restores the sensitivity to plasma. **D.** Differential gene expression between Tn::P_{srg} and the parental IHMA87 strain represented in a volcano plot

352 showing the overexpression of *srgABC* and alginate related genes. RNA was obtained
353 from LB-grown cultures and the whole transcriptomes were determined by RNA-seq
354 pipeline **E-F.** Survival in plasma and alginate production of indicated strains. Alginate
355 synthesis was visualized from colony morphology and quantified by carbazole assay,
356 as described in Materials and Methods. Note that alginate overproduction appears as
357 darker bacterial spot (**F**). The data are from one representative experiment, performed
358 in biological triplicates.

359

360 **Contribution of *srgABC* and alginates in tolerance to complement killing**

361 To establish the relationship between the *srg* operon, alginate production and bacterial
362 tolerance to complement, we inactivated *algD*, encoding GDP-mannose 6-
363 dehydrogenase, a checkpoint in alginate biosynthesis (Deretic, Gill and Chakrabarty,
364 1987; Berry, DeVault and Chakrabarty, 1989; Wood and Ohman, 2012) both in the
365 parental strain and in Tn::P_{srg}, and investigated relevant phenotypes. In the assay, we
366 included alginate-overproducing mutant Tn::*mucB* isolated during the screen. We also
367 designed a deletion mutant in the gene *IHMA87_01134* (*PA3819*), an uncharacterized
368 gene that was overexpressed (Log_2(FC)=2.35, p_{adj}=2x10^{-153}) in the Tn::P_{srg} strain. For
369 each strain, we established the survival rate by CFU counting, the quantities of
370 alginates using carbazole assay and assessed the colony morphology on plates (Fig.
371 4E-F). The mutant Tn::P_{srg} displayed a 100-fold higher survival in human plasma
372 compared to the parental strain, along with an increased alginate production. Although
373 alginate overproduction can lead to higher resistance to human plasma, as seen for
374 the mutant Tn::*mucB*, the alginates in Tn::P_{srg} did not account significantly for its
375 tolerance to plasma. Finally, the deletion of *IHMA87_01134* had no impact neither on
376 alginate production nor on plasma resistance. Overall, these results demonstrate that
377 increased plasma tolerance in a *srg* overexpressing mutant is alginate-independent,
378 and that SrgA alone modulates plasma tolerance.

379 **The specificity of mutants toward MAC-induced killing**

380 In order to examine the possible mechanisms leading to increased resistance of
381 selected mutants to bactericidal activity of the MAC in plasma, we set up experiments
382 to survey different steps of the complement cascade. The complement activation of
383 both classical and alternative pathways by bacteria was examined by measuring the
384 residual complement activity toward sensitized sheep or rabbit erythrocytes
385 respectively, as described in Dumestre-Pérard *et al.*, 2008. Plasma incubated with the
386 different mutants exhibited the same level of residual complement capacity to lyse red
387 blood cells as that incubated with the parental strain (Fig. 5A-B), suggesting that
388 complement activation was not altered by the mutants. To investigate whether the
389 mutants are impaired in the formation of C3b and MAC (C5b-9) complexes, we first
390 assessed the deposition of CS components at the bacterial surface by flow cytometry
391 (Fig. 5C-H). As shown on Figure 5C-D, an increase in C3b deposition was observed
392 at 15 min for the the Δ*bioB* mutant. When we exposed bacteria to pooled human serum
393 supplemented with fluorescently labelled C9 (Figure 5E-F), we observed similar
394 deposition of C9, with an apparent increase for the Tn::P_{srg} mutant. Finally, we
395 investigated MAC polymerisation using an anti-C5b-9 antibody and flow cytometry
396 (Fig. G-H). No significant variation in the quantity of the C5b-9 complex was detected
397 between the different mutants, suggesting that globally, the mutants had similar
398 amount of MAC at their surface. To assess the functionality of the inserted MAC pore,
399 we compared serum survival of the different bacteria in the presence of nisin. Normally,
400 nisin cannot act on Gram-negative bacteria when they have an intact outer membrane
401 (Heesterbeek *et al.*, 2019). However, MAC-induced damage to the outer-membrane
402 allows the influx of nisin resulting in bacterial killing. As previously shown for plasma,
403 the wild-type displays high sensitivity to killing in serum, with a very efficient bacterial

404 elimination (Fig. 5I). In contrast, survival of all mutants was increased. Interestingly,
405 nisin further killed most of the evaders' population. In a similar manner, the Tn::P_{srg}
406 mutant showed a reduction in numbers in presence of nisin, indicating MAC-induced
407 outer membrane damage. This suggests that outer membrane damage by the MAC
408 itself was not sufficient to kill this mutant or the evaders' population. In contrast, the
409 Δ*bioB* and Δ*purD* strain showed no decreased survival upon nisin addition to the
410 serum. All bacteria could be rescued by addition of C5 inhibitor OmCI, confirming that
411 the killing is complement-driven. Taken together, these data suggest that while the
412 Δ*bioB*, Δ*purD*, and Tn::P_{srg} mutants display increased survival to serum compared to
413 the wild-type, the Tn::P_{srg} mutant interferes at a different stage of MAC-dependent
414 killing than the Δ*bioB* and Δ*purD* mutants.

415

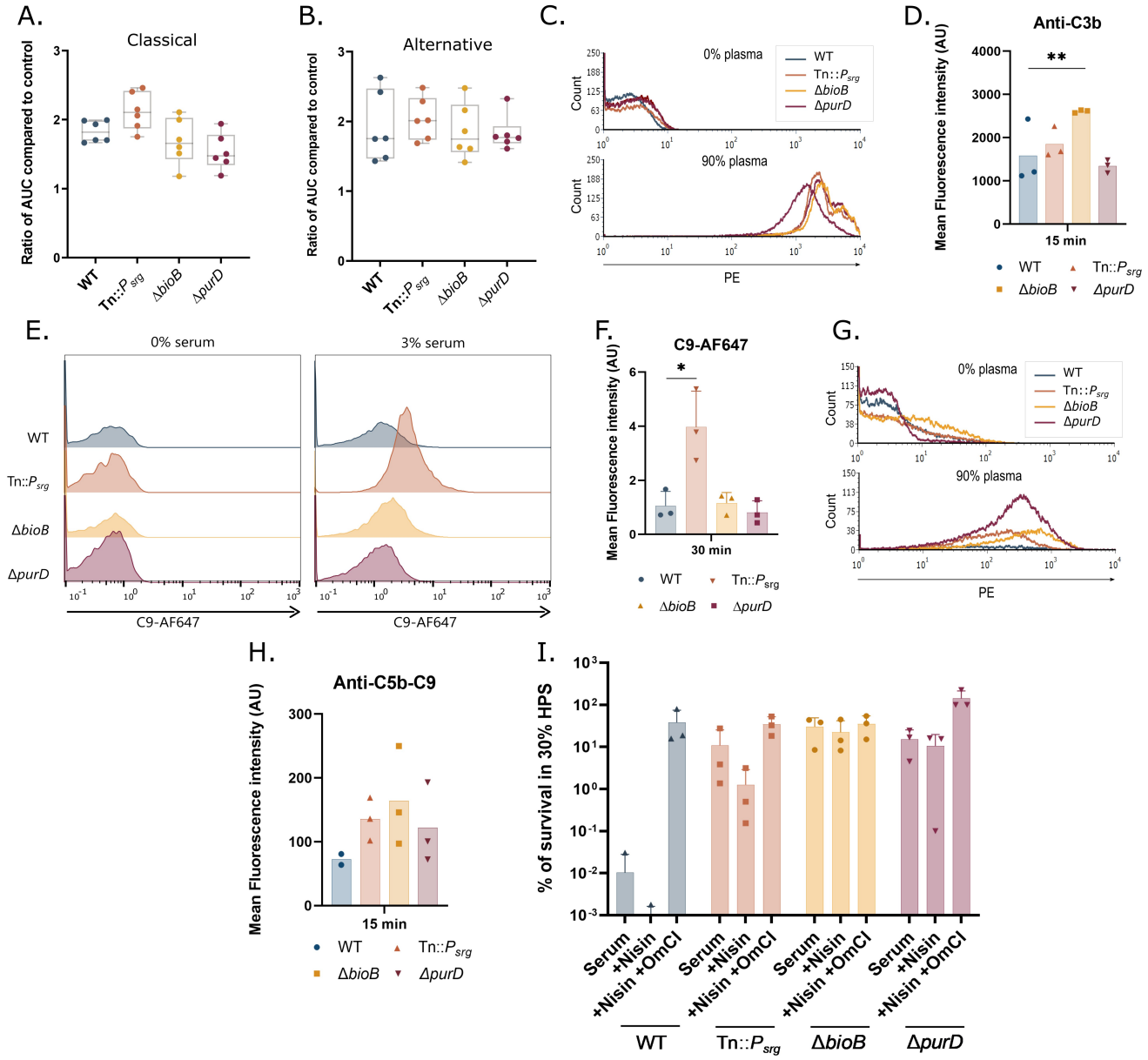


Figure 5. Mutants response to MAC-dependent killing. Residual complement activity of the classical (**A.**) and alternative (**B.**) complement pathways was measured after bacterial challenge in plasma. n=3. **C.** C3b deposition on bacterial surface after 15 min of incubation in 0% or 90% plasma determined by FACS and **D.** plotted mean fluorescence intensities. n=3. *p*-value < 0.001 when indicated by **. **E.** C9-AF647 insertion into bacterial membrane after 30 min in 0% or 3% human pooled serum and **F.** plotted mean fluorescence intensities p-value < 0.05 when indicated by *. **G.** Bacterial C5b-9 deposition on bacterial surface after 15min of incubation in 0% and 90% plasma determined by FACS and plotted mean fluorescence intensities (**H.**). n=3. **I.** Bacterial survival in human pooled serum, with nisin or in presence of nisin and OmCl complement inhibitor.

429 **Discussion**

430 The variability of *P. aeruginosa* strains concerning their survival in human plasma is
431 high, ranging from fully resistant to sensitive strains. The majority of strains displaying
432 sensitivity at the whole population level form a persistent sub-population with still
433 uncharacterized features (Pont *et al.*, 2020). In this work, we identified ATP, biotin and
434 a small periplasmic protein, named SrgA, as novel molecular determinants of *P.*
435 *aeruginosa* resilience to the CS in human plasma. In accordance with genome-wide
436 screens performed in complement-resistant strains of different pathogens in human
437 serum (Phan *et al.*, 2013; Sanchez-Larrayoz *et al.*, 2017; McCarthy, Stabler and
438 Taylor, 2018; Poulsen *et al.*, 2019; Short *et al.*, 2020), our screen confirmed several
439 previously identified factors, such as long OSA and exopolysaccharides, as being
440 important for the survival of *P. aeruginosa* in human plasma. Also, in agreement with
441 genetic screens that identified bacterial persistence determinants facing antibiotic
442 treatment (Molina-Quiroz *et al.*, 2016; Cameron *et al.*, 2018), we found that persistence
443 in plasma is a multifactorial and probably a stochastic phenomenon. The mutant
444 depleted in intracellular ATP (by a mutation in the purine pathway) showed a higher
445 tolerance profile in plasma and became more MAC-sensitive upon the external
446 addition of ATP. In the context of higher tolerance/persistence to antibiotics, it has
447 been proposed that the ATP-depleted conditions could decrease the global protein
448 synthesis and availability of antibiotic targets (Shan *et al.*, 2017). Similarly, the slow
449 growth *per se*, due to any restricted conditions triggers a persistent state (Pontes and
450 Groisman, 2019). Although incapable of growing, Δ*bioB* and Δ*purD* mutants were able
451 to survive and persist in HIP for more than 24 hours. Whereas, *in vivo* mutations in the
452 purine and biotin pathways are unlikely to occur, bacteria could use regulatory
453 mechanisms to modulate purine and biotin synthesis and enter in an antibiotic or

454 complement tolerant state. Several reports highlighted the potential of targeting biotin
455 biosynthesis as an alternative antibacterial strategy (Carfrae *et al.*, 2020). Our results
456 showing that the mutants in the biotin biosynthetic pathway are more resistant to
457 microbicidal activity of CS call for more careful assessment of the role of biotin in BSI.

458 The number of plasma evaders following the 3h incubation varies between
459 experiments, strains and origin of plasma (Pont *et al.*, 2020); and this work). Therefore,
460 it is possible that the initial incubation in plasma, which is considered as a poor nutritive
461 and stressful milieu for bacteria may stochastically trigger formation of evaders.
462 However, our preliminary results show that pre-incubation of bacteria in HIP does not
463 modify the level of evaders, suggesting that the hostile nutritional environment of HIP
464 is not sufficient to increase the persistence. The adaptation of bacteria to human serum
465 includes upregulation of hundreds of genes, depending on the species. Notably,
466 human blood and serum upregulate quorum sensing genes in plasma-resistant *P.*
467 *aeruginosa* strain PAO1, partly through the global virulence regulator Vfr (Kruczek *et*
468 *al.*, 2014, 2016; Beasley *et al.*, 2020). However, the serum factors and downstream
469 effectors are still unknown.

470 In our search for mechanisms underlying escape of here-identified mutants from the
471 complement attack, we found that Tn::P_{srg}, Δ*bioB* and Δ*purD* mutants do not impede
472 complement activation, but probably modulate the function of MAC itself. MAC is a
473 multiprotein pore whose oligomerization occurs by sequential addition of C5b, C6, C7,
474 C8 and finally C9 which forms an oligomeric ring of 18 units inserted into bacterial
475 membranes (Bayly-Jones, Bubeck and Dunstone, 2017). Although the composition of
476 MAC has been known for years, its contribution to bacterial cell death and the way the
477 bacteria die upon MAC insertion is still a debate (Doorduijn, Rooijakkers and
478 Heesterbeek, 2019), and recent work suggested that complement-resistant bacteria

479 can specifically block polymerization of C9 (Doorduijn *et al.*, 2021). SrgA is a protein
480 of 7.1 kDa after predicted signal peptide cleavage, probably localized in the bacterial
481 periplasm and/or exported to the bacterial surface. We can speculate that SrgA directly
482 interferes with MAC assembly. Alternatively, SrgA may act on peptidoglycan or other
483 components of the Gram-negative envelope, which impede MAC function. Our efforts
484 to detect SrgA using protein tags in *P. aeruginosa* were until now unsuccessful, but
485 preliminary visualization of the protein in *E. coli* confirms that the *srgA* gene product is
486 of a peptide nature. However, the nature of *srgB* and *srgC* gene products is completely
487 speculative. The role of ATP in MAC activity is intriguing. No data are available on the
488 requirement of energy in MAC assembly, but we can speculate that it may be required
489 for one of the steps during pore formation. Interestingly, the mutants displayed
490 resistance to an outer-membrane-impermeable polycyclic antibacterial peptide nisin
491 (Heesterbeek *et al.*, 2019), while the majority of the evaders were sensitive. This
492 indicates that, in these mutants, the MAC pore did not allow the nisin incorporation
493 through the permeabilized outer membrane, either due to defective pore insertion or
494 to more resistant inner membranes. On the contrary, the evaders' outer membrane
495 seems to have been damaged by the MAC, suggesting that evaders possess a more
496 resistant envelope or have a quicker ability to repair envelope damage. Further
497 characterization of evaders' biology compared to the bulk population is needed to
498 identify molecular mechanisms triggering the evader state.

499 Taking into account the genetic and phenotypic diversity of *P. aeruginosa* strains
500 (Hilker *et al.*, 2015; Freschi *et al.*, 2018) and their variable levels of survival in human
501 plasma (Pont *et al.*, 2020), we can expect both common and strain-specific
502 determinants that counteract the MAC-dependent killing. Only parallel screens on

503 several representative bacterial strains may deliver the global picture on *P. aeruginosa*
504 plasma and blood resistome/persistome.

505 In an era where bacterial resistance to antibiotics has become the global health
506 concern, any alternative for pathogen elimination could be interesting to explore. The
507 use of a complement system or MAC may be one of those, in combination with
508 classical antibiotics, phages, or antibodies that enhance MAC formation (Abd El-Aziz,
509 Elgaml and Ali, 2019; Cruz *et al.*, 2019; Gulati *et al.*, 2019). However, we still have a
510 very limited knowledge on different pathogen's strategies to escape the complement.
511 Better understanding of molecular mechanisms involved in pathogen-complement
512 interplay should help designing strategies to fight multi-drug resistant bacteria.
513

514 Materials and methods

515 Bacterial strains and genetic manipulations

516 Bacterial strains, plasmids and primers are listed in Tables 3S and 4S. *E. coli* and *P.*
517 *aeruginosa* were grown in Lysogeny Broth (LB) at 37°C with shaking (300 rpm). *P.*
518 *aeruginosa* was selected on LB plates containing 25 µg/mL irgasan. Antibiotics
519 concentrations were: 75 µg/mL gentamicin, 75 µg/mL tetracycline and 300 µg/mL
520 carbenicillin for *P. aeruginosa* and 50 µg/mL gentamicin 10 µg/mL tetracycline, 100
521 µg/mL ampicillin for *E. coli*. To create deletion mutants, approximately 500 bp
522 upstream and downstream flanking regions were amplified from genomic DNA by PCR
523 using appropriate primer pairs (sF1/sR1, sF2/sR2). Overlapping fragments were
524 cloned into *Sma*I-digested pEXG2, pEX100T or pEX18Tc by Sequence- and Ligation-
525 Independent Cloning (SLIC, Li and Elledge, 2007). Plasmids were transformed into *E.*
526 *coli* Top10 competent cells and their sequences were verified (Eurofins). Allelic

527 exchange vectors derived from pEXG2, pEX100T or pEX18Tc were introduced into *P.*
528 *aeruginosa* by triparental mating, using pRK600 as a helper plasmid. Merodiploids,
529 resulting from homologous recombinations, were selected on LB agar plate containing
530 irgasan and the appropriate antibiotic. Single merodiploid colonies were streaked on
531 NaCl-free LB plates with 10 % sucrose (w/v), selecting for plasmid loss. Resulting
532 sucrose resistant clones were screened for antibiotic sensitivity and gene deletion by
533 PCR with appropriate primer pairs (F0/R0).

534 **Generation of the IHMA87 transposon library**

535 The IHMA87 transposon mutant library was constructed essentially as previously
536 described (Skurnik *et al.*, 2013; Trouillon *et al.*, 2020). Briefly, IHMA87, grown
537 overnight at 42°C with shaking, was mixed with two *E. coli* strains carrying pRK2013
538 (Figurski and Helinski, 1979) and pBTK24. The conjugation mixtures containing 100
539 µl of each bacterial culture adjusted to optical densities measured at 600 nm (OD_{600})
540 of 1, were centrifuged, washed with LB, deposited onto pre-warmed LB plates and let
541 at 37°C for 5 h. Approximately 100 conjugation mixtures yielded a library of > 300,000
542 mutants. Bacteria were scraped off into liquid LB and aliquots were plated on LB plates
543 containing irgasan (25 µg/ml) and gentamicin (75 µg/ml). Following growth at 37°C,
544 the colonies were collected directly into LB containing 20% glycerol, aliquoted and
545 stored at -80 °C.

546 **Plasma pools preparation**

547 Pools of plasma were prepared from heparinized human blood from healthy donors
548 provided by the French National Blood Institute (EFS, Grenoble, France). Fresh blood
549 was centrifuged for 10 min at 1,000 x g at room temperature. The supernatants of ten
550 different donors were pooled, filtered with a 0.45 µm membrane filter and aliquoted

551 prior to storage at -80 °C until needed. A pool was prepared using the same procedure
552 from citrated blood from 30 healthy donors. Prior to use, plasma pool aliquots were
553 thawed on ice centrifuged 10 min at 10,000 rpm prior to filtration through 0.22 µm
554 membrane. For the heat inactivated plasma (HIP) condition, plasma was thawed and
555 heat inactivated for 30 min at 56°C and centrifuged 10 min at 10,000 rpm prior to
556 filtration through 0.22 µm membrane.

557 **Screen in plasma**

558 A vial containing the IHMA87 mutant library was thawed on ice, transferred into a flask
559 containing 30 mL of LB and grown 16h under agitation at 27°C to limit bacterial growth.
560 The next day, bacterial suspension was diluted to OD_{600nm} ~0.1 and grown at 37°C
561 under agitation. Meanwhile, plasma and HIP were prepared. At an OD_{600nm} ~1, bacteria
562 were harvested and resuspended in PBS supplemented with calcium and magnesium
563 ions (Thermofisher Scientific) before infecting plasma, HIP or LB samples to a
564 concentration of $2.25x10^7$ CFU/mL (90% final plasma concentration). The precise
565 initial CFU count of each experiment was determined by serial dilution. Samples were
566 incubated at 37°C for 3h on a rotating wheel. At the end of the challenge, part of the
567 sample was transferred to LB and grown overnight, part was spread on LB agar plates
568 to isolate individual mutants. Finally, bacterial survival was calculated by CFU counts
569 of each sample. In parallel, the same protocol was performed with the wild-type strain
570 for survival comparison. The next day, vials were taken from the culture and stored for
571 further mutant isolation. About $1x10^9$ bacteria were harvested for each sample and
572 subjected to DNA extraction.

573

574

575 **Illumina library construction, sequencing and data analysis**

576 **Genomic DNA.** Bacterial pellets from the screen in plasma with either the HIP samples
577 (Input) or the plasma samples (Output) were used for gDNA preparations. Classical
578 protocol using SDS-NaCl lysis (2% SDS, 0.15M NaCl, 0.1M EDTA pH8, 0.6M Sodium
579 perchlorate) step followed by two successive phenol-chloroform extractions was
580 carried out. The gDNA was precipitated with 100% cold ethanol and diluted in a 200µl
581 TE buffer (10mM Tris-HCl pH8, 1mM EDTA) to final concentration of 50-100 ng/µl.
582 Mechanical DNA shearing was done at 4°C with a QSonica sonicator (Q700) during
583 20 min with 15 second pulses. Fragments were concentrated on columns (Monarch
584 PCR & DNA Cleanup Kit, NEB). The size of generated DNA fragments (150-400 bp)
585 was controlled on 2% agarose gel.

586 **Library construction.** Fragmented gDNA (1 µg) were end-repaired using an End-
587 repair module (NEB#E6050) followed by a dA-tailing with Klenow (NEB#6053). Short
588 and Long adaptors (Table 1S) were annealed in 10 mM $MgCl_2$ in thermocycler with a
589 program allowing decrease of 1°C/cycle, from 95°C to 20°C. Ligation of gDNA
590 fragments with annealed adaptors was achieved overnight at 16°C with T4 DNA ligase
591 (NEB#M0202). A size selection was performed on a 2% agarose gel by extracting the
592 bands from 200 to 400 bp (Monarch DNA Gel Extraction Kit, NEB#T1020L).
593 Amplification of purified adaptor-DNA fragments was carried out with Phusion
594 polymerase (NEB#M0530) and specific primers (PCR1 Tn-spe direct and PCR1
595 Adaptor comp.). A second amplification step was performed with a primer carrying a
596 P5 illumina sequence and Indexed-P7 illumina primers (NEB#E7335S). Primer
597 sequences are available on request. After each step, quantification was performed
598 using the Qubit dsDNA HS Assay kit (Q33230). The quality of the libraries was
599 assessed on the Agilent Bioanalyzer 2100 using high-sensitivity DNA chips (Ref

600 #5067-4626). Sequencing was performed on an Illumina NextSeq High (I2BC, Saclay
601 Paris).

602 **Data analysis.** Sequencing reads were trimmed and aligned to the genome of IHMA87
603 using Bowtie2 (Langmead and Salzberg, 2012). Htseq-count (Anders, Pyl and Huber,
604 2015) was then used to obtain read counts per feature. Differential representation of
605 insertion mutants between the plasma and HIP sample was performed using (Love,
606 Huber and Anders, 2014). For the analysis on insertions in intergenic regions, an
607 annotation file was created with intergenic regions attributed to the upstream gene
608 independently of its orientation. The same analysis as above was performed using
609 Htseq-count and DESeq2. Tn-seq was performed in biological duplicates.

610 **Plasma killing assay**

611 Killing assay was performed as described previously (Pont *et al.*, 2020). Unless stated
612 otherwise, bacterial pre-cultures were diluted in LB to OD_{600nm} = 0.1 and grown at 37°C
613 under agitation until OD_{600nm} ~ 1. Bacteria were resuspended into PBS +/+
614 (Thermofisher Scientific) and incubated in plasma to a concentration of $2.25x10^7$
615 CFU/mL (90 % final plasma concentration). The precise initial CFU count of each
616 experiment was determined by serial dilution. Bacterial survival was calculated by CFU
617 counts at the indicated time(s) and compared to the initial count. ATP, biotin and purine
618 were added to the plasma prior to bacterial addition at the indicated concentrations.

619 **Transcriptomics**

620 **RNA extraction.** Bacterial cultures were diluted to an OD_{600nm} of 0.1 and grown at
621 37°C under agitation until they reached an OD_{600nm} of 1. RNA isolation was performed
622 using the hot phenol-chloroform method. Briefly, bacteria were lysed in a hot phenol

623 lysis solution (2 mM EDTA, 1 % SDS, 40mM sodium acetate in acid phenol (Invitrogen
624 #15594-047)). RNA was then isolated by successive steps of phenol-chloroform
625 extraction, followed by one cold chloroform extraction and ethanol precipitations. An
626 additional Turbo DNAse (Invitrogen #AM1907) treatment was performed to remove
627 genomic DNA contamination. Total RNA quantity and quality were assessed using a
628 Agilent Bioanalyzer.

629 **RNA-seq libraries construction.** Following RNA extraction, ribosomal RNAs (rRNAs)
630 depletion was performed using ribominus kit (Invitrogen) according to manufacturer's
631 instructions. Depletion quality was verified on an Agilent Bioanalyzer using RNA pico
632 chips and RNA was concentrated using ethanol precipitation.

633 NEBNext Ultra II directional RNA library prep kit for illumina (NEB) was used to build
634 cDNA libraries for sequencing starting from 50ng of rRNA depleted RNA according to
635 manufacturer's instructions. cDNA libraries quality and concentration were assessed
636 using an Agilent Bioanalyzer High sensitivity DNA chips.

637 **Sequencing and data analysis.** Sequencing was performed by the Institute for
638 Integrative Biology of the Cell (I2BC http://www.i2bc.paris-saclay.fr) high-throughput
639 sequencing facility using an illumina NextSeq500 instrument. More than 12 millions
640 75-bp single-end reads were obtained per sample. Processed reads were mapped
641 onto the IHMA87 genome (available on NCBI CP041354 and CP041355 accession
642 numbers, for the chromosome and plasmid sequences respectively (Trouillon *et al.*,
643 2020)) using Bowtie2 (Langmead and Salzberg, 2012) and the readcount per feature
644 was calculated using Ht-seq count (Anders, Pyl and Huber, 2015). Finally, differential
645 gene expression between the Tn::P_{srg} and the wild-type strains was performed using

646 DESeq2 (Love, Huber and Anders, 2014). RNA-seq experiment was performed on
647 biological duplicates.

648 **Alginate dosage**

649 Equivalent amounts of OD_{600nm} adjusted bacterial cultures were spread onto
650 *Pseudomonas* isolation agar (PIA) plates and incubated for 24h at 37°C. Bacterial
651 lawns were flushed with saline solution (0.9% NaCl) and scraped off the plates.
652 Bacteria were pelleted and supernatant was stored in a separated tube. Bacterial pellet
653 was resuspended in LB and incubated on a rotating wheel for 1-2 hours to avoid
654 bacterial aggregates. Bacterial titer was calculated by serial dilutions and subsequent
655 CFU counting. The alginate dosage was done as described in Knutson and Jeanes,
656 1968 and May and Chakrabarty, 1994. Briefly, 600µL of ice cold borate working
657 solution (borate stock solution (4 M BO_3^{3-}) diluted to a final concentration of 100 mM
658 in H_2S0_4) was added to 70 µL of the bacteria supernatant, saline solution for a blank,
659 and to commercial sodium alginate solution to obtain a standard curve. Keeping on
660 ice, 20 µL of carbazole solution (0.1% carbazole (Sigma #442506) in 100% ethanol)
661 was added, and samples were incubated at 55°C for 30 min after mixing. The
662 absorbance was measured at 530 nm.

663 **Quantitative measurement of intracellular bacterial ATP**

664 For ATP measurement in LB, about 1 x 10^8 bacteria were pelleted and resuspended
665 in PBS. HIP was inoculated with a final concentration of 2.025 x 10^7 bacterial/mL in
666 PBS (90% final HIP concentration). Samples were incubated for 2h at 37°C on a
667 rotating wheel. At the end of the incubation, samples were centrifuged and
668 resuspended in PBS prior to a gentle sonication step using Qsonica sonicator (Q700)
669 for 45 sec with 10% intensity and 5 sec ON-5 sec OFF intervals in order to dissociate

670 the bacterial aggregates. For the two conditions, 10-fold serial dilutions were prepared
671 in 96-well plates and precise bacterial counts were determined by counting CFUs. The
672 concentration of ATP was determined using the BacTiter-Glo kit (Promega, #G8230).
673 Briefly, equal volumes of bacterial suspension and BacTiter-Glo reagent were mixed
674 in white 96-well Lumitrac plates (BioRad #655075) and incubated for 5 min with
675 shaking. An ATP standard curve (0.1 to 100 nM) was established for each experiment.
676 Bioluminescence was measured on a plate reader (Spark, TECAN). ATP
677 concentration was then reported to the CFU counts. Experiments were performed in
678 more than 3 biological replicates.

679 **Residual complement activity**

680 **Sample preparation.** Bacteria grown to $0D_{600nm}$ ~ 1 were resuspended into PBS +/+
681 (Thermofisher Scientific) and incubated in plasma to a concentration of $2.25x10^9$
682 CFU/mL (90% final plasma concentration) or PBS only for the reference. Samples
683 were incubated for 3 hours at 37°C on a rotating wheel to allow complement activation
684 by bacteria. The initial CFU count was determined by serial dilution. At the end of the
685 incubation, samples were centrifuged and supernatant was filtered through 0.22 µm
686 membrane to remove any remaining bacteria. Samples were aliquoted and frozen at
687 -80°C until use. The remaining complement activity in the bacterial/plasma supernatant
688 was measured using a total hemolytic assay for classical and alternative pathways, as
689 described by (Dumestre-Pérard *et al.*, 2008).

690 **Classical complement hemolytic activity.** Briefly, sheep erythrocytes (Orgentec)
691 were sensitized using anti-erythrocytes antibodies (1/40000 - Hemolysin, Orgentec)
692 for 15min at 37°C. Bacterial/plasma supernatant (25 µL) was mixed with 3 mL of
693 sensitized erythrocytes ($4x10^6$ cells/mL) diluted in $DGVB^{2+}$ (2.5% glucose, 0.05%

694 gelatin, 2.5 mM Veronal, 72.5 mM NaCl, 0.15 mM Ca^{2+}, 0.5 mM Mg^{2+}) and lysis kinetic
695 was monitored by OD_{660nm} measurement using a spectrophotometer (safas UVmc2).
696 Area under the curve was measured for each series and reported to the 90% plasma
697 pool control.

698 **Alternative complement hemolytic activity.** Briefly, rabbit erythrocytes (Ecole
699 Nationale Vétérinaire de Toulouse) were washed 3 times using DGVB-Mg-EGTA
700 (2.5% glucose, 0.05% gelatin, 2.5 mM Veronal, 72.5 mM NaCl, 5 mM Mg^{2+}, 3 mM
701 EGTA). Bacterial/plasma supernatant (150 µL) was added to 3 mL of rabbit
702 erythrocytes suspension (diluted in DGVB-Mg-EGTA at 1/250) at 37°C and lysis kinetic
703 was measured similarly to classical complement activity.

704 **C3b and C5b-9 binding assays**

705 Forty million bacteria were incubated for 15 min in a 90% heparinized plasma pool at
706 37°C. Incubation was stopped by dilution of bacteria suspension in ten volumes of
707 PBS containing 20 mM of EDTA pH8. C3b binding was detected with PE-conjugated
708 mouse monoclonal anti-C3b/iC3b (5 µg/mL, BioLegend #846103) and C5b-9
709 membrane deposition/insertion was detected with mouse monoclonal anti-C5b-9
710 antibody (8 µg/mL, Abcam #ab66768) next revealed with PE-conjugated goat anti-
711 mouse antibodies (2.5 µg/mL, Abcam #ab97041). Bacteria in 0% plasma were used
712 as negative control. Samples were analyzed in a FACSCalibur instrument (Becton
713 Dickinson) and flow cytometry analysis were performed using the FCSExpress6
714 Software.

715

716

717 **C9 binding**

718 *P. aeruginosa* strains expressing green fluorescent protein (GFP) under the control of
719 the constitutive P_{X2} promoter were grown overnight in LB supplemented with 10 µg/ml
720 tetracycline at 37°C shaking. Bacteria were subcultured 1:30 in LB containing the
721 antibiotic and let grow to a mid-log OD_{600nm} of 0.5 at 37°C with shaking. Bacteria were
722 pelleted by centrifugation, washed, and resuspended to a final OD_{600nm} of 0.05 in RPMI
723 + 0.05% human serum albumin (HSA) for the experiments. Bacteria were then
724 exposed to pooled human serum supplemented with 100 nM C9-AF647 and incubated
725 for 30 minutes at 37°C shaking. For CFU counting, serial bacterial dilutions were made
726 in MilliQ water, spotted on LB plates, and incubated overnight at 37°C. For flow
727 cytometry, bacteria were diluted 10-fold in RPMI-HSA and analyzed in a MACSquant
728 VYB (Miltenyi Biotech) flow cytometer. Bacteria were gated on GFP-expression and
729 subsequently analyzed for AF647 fluorescence. Flow cytometry data was analyzed in
730 FlowJo V.10.

731 **Nisin assay**

732 Bacteria were grown overnight in LB at 37°C shaking, subcultured 1:30 in LB to a mid-
733 log OD_{600nm} of 0.5 at 37°C shaking, pelleted by centrifugation, washed, and
734 resuspended to a final OD_{600nm} of 0.05 in RPMI + 0.05% HSA for the experiments.
735 Bacteria were then exposed to human pooled supplemented with buffer, 3 µg/ml nisin,
736 or 3 µg/ml nisin and 10 µg/ml OmCI, and incubated for 3 hours at 37°C. Serial dilutions
737 were made in MilliQ water, and spotted on LB plates for incubation overnight at 37°C
738 for determination of CFU the next day.

739

740 **Transmission electron microscopy**

741 Bacterial pre-cultures were diluted to an OD_{600nm} of 0.1 in flasks containing 30 mL of
742 LB and incubated at 37°C under agitation. Plasma was then inoculated with bacterial
743 suspension to reach a bacterial concentration of 1.125 $x10^9$ bacteria/mL and incubated
744 on a rotating wheel for 1 hour at 37°C. At the end of the challenge, bacteria were
745 pelleted by centrifugation and resuspended in LB prior to fixation. At the same time,
746 bacteria grown in LB to an OD_{600nm} of 1 were pelleted by centrifugation and
747 resuspended in LB prior to fixation.

748 Sample preparation was treated as described in (Mohamed *et al.*, 2021). Briefly, the
749 cell pellet was spread on the 200-µm side of a 3-mm type A gold plate (Leica
750 Microsystems) covered with the flat side of a 3-mm type B aluminium plate (Leica
751 Microsystems) and vitrified by high-pressure freezing using an HPM100 system (Leica
752 Microsystems). After freezing at -90°C in acetone with 1% OsO4, the samples were
753 slowly warmed to -60°C, stored for 12 hours, then the temperature was increased to -
754 30°C and the samples stored for a further 12 hours before a step at 0°C for 1 hour
755 before being cooled again to -30°C (AFS2; Leica Microsystems). The samples were
756 rinsed 4 times in pure acetone before being infiltrated with progressively increasing
757 concentrations of resin (Epoxy embedding medium, Sigma) in acetone while
758 increasing the temperature to 20°C. Pure resin was added at room temperature. After
759 polymerisation, 70 nm thin sections were obtained using a UC7 ultramicrotome (Leica
760 Microsystems) and collected on 100 mesh copper grids coated with Formvar carbon.
761 The thin sections were post-stained for 5 minutes with 2% uranyl acetate, rinsed with
762 water and incubated for 2 minutes with lead citrate. Samples were observed using a
763 Tecnai G2 Spirit BioTwin microscope (FEI) operating at 120 kV with an Orius SC1000B
764 CCD camera (Gatan).

765 **Bioinformatics analysis and protein conservation**

766 Protein sequence files from all *P. aeruginosa* with a closed assembled genome were
767 retrieved from Pseudomonas genome database and *srg* conservation was investigated
768 using Blast Reciprocal Best Hits program (Cock *et al.*, 2015) computed with a 90%
769 coverage and 50% identity.

770 Signal peptide prediction was perfomed using SignalP website (Almagro Armenteros
771 *et al.*, 2019).

772 **Statistical analysis**

773 Sigmaplot software wasused to perform statistical analysis. Multiple groups
774 comparison was performed using one-way ANOVA or Kruskal-Wallis tests depending
775 on data normality followed by a Student-Newman-Keuls pairwise comparison. Non-
776 normally distributed data were converted to normally-distributed dataset by log_{10}-
777 transformation where indicated. Figures were designed using Graphpad Prism9.

778 **Data availability**

779 The Tn-seq and RNA-seq data generated in this work is available at GEO accession
780 numbers GSExxxxxx and GSExxxxxx respectively.

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801

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Supplementary material

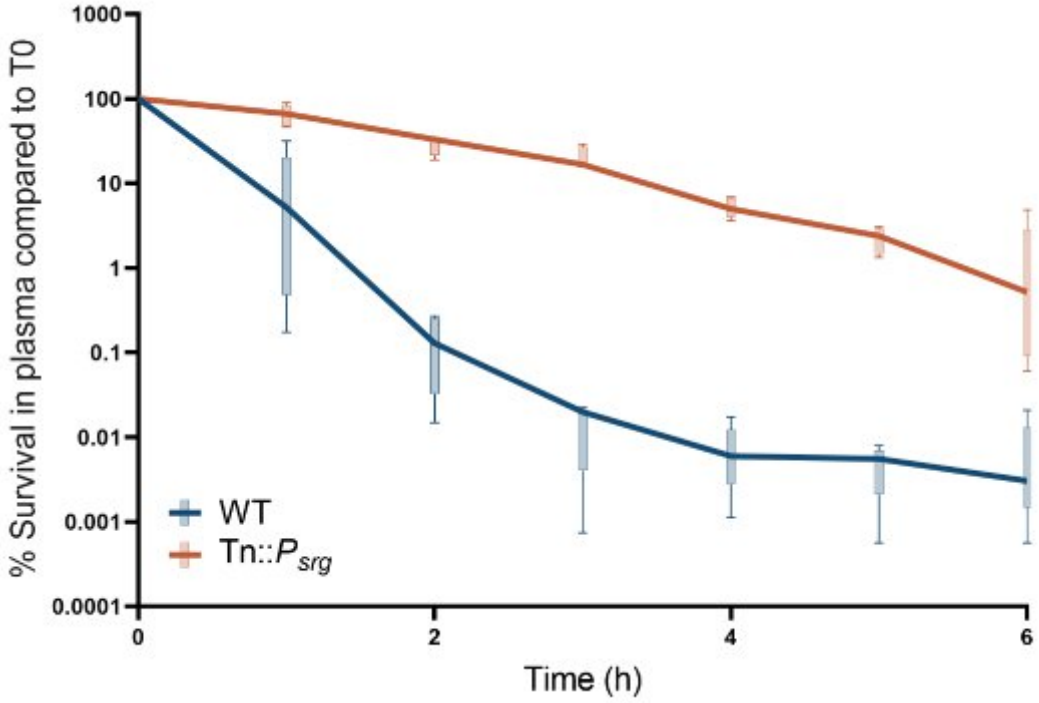

Figure S1. Overexpression of *srg* operon leads to a population tolerant to plasma killing. Survival kinetics of IHMA87 wild-type strain (dame data as presented in Fig. 2B) and of Tn::P_{srg} in plasma over 6h incubation measured by CFU counting (n=5).

Table 1S. Tn-seq data

Table 2S. RNA-seq differential gene expression data

Table S3. Bacterial strains and plasmids

Bacteria	Features	Reference/origin
Pseudomonas aeruginosa		
IHMA879472/AZPAE15042	Urinary isolate, plasma sensitive	IHMA[1] collection (Kos *et al.*, 2015; Pont *et al.*, 2020; Trouillon *et al.*, 2020) (Kos, Trouillon, Pont)
IHMA87Δ*bioB*	IHMA87 with *bioB* deletion	This work
IHMA87Δ*purD*	IHMA87 with *purD* deletion	This work
IHMA87Δ*algD*	IHMA87 with *algD* deletion	This work
IHMA87Δ*01134*	IHMA87 with *01134* deletion	This work
IHMA87 Tn::P_{srg}	IHMA87 with mariner transposon inserted in the promoter of *srg* operon (Gm^R)	This work
IHMA87 Tn::P_{srg} Δ*srgABC*	IHMA87 Tn::P_{srg} with *srgABC* deletion (Gm^R)	This work

IHMA87 Tn::P_{srg} Δ*srgA*	IHMA87 Tn::P_{srg} with *srgA* deletion (Gm^R)	This work
IHMA87 Tn::P_{srg} Δ*srgB*	IHMA87 Tn::P_{srg} with *srgB* deletion (Gm^R)	This work
IHMA87 Tn::P_{srg} Δ*srgC*	IHMA87 Tn::P_{srg} with *srgC* deletion (Gm^R)	This work
IHMA87 Tn::P_{srg} Δ*srgBC*	IHMA87 Tn::P_{srg} with *srgBC* deletion (Gm^R)	This work
IHMA87 Tn::P_{srg} Δ*algD*	IHMA87 Tn::P_{srg} with *algD* deletion (Gm^R)	This work
IHMA87 Tn::P_{srg} Δ*01134*	IHMA87 Tn::P_{srg} with *01134* deletion (Gm^R)	This work
IHMA87 *ladS*::Tn	IHMA87 with mariner transposon inserted in *ladS* gene (Gm^R)	This work
IHMA87 *bioA*::Tn	IHMA87 with mariner transposon inserted in *bioA* gene (Gm^R)	This work
IHMA87 *bioB*::Tn	IHMA87 with mariner transposon inserted in *bioB* gene (Gm^R)	This work
IHMA87 *mucE*::Tn	IHMA87 with mariner transposon inserted in *mucE* gene (Gm^R)	This work
IHMA87 *mucA*::Tn	IHMA87 with mariner transposon inserted in *mucA* gene (Gm^R)	This work
IHMA87 *mucD*::Tn	IHMA87 with mariner transposon inserted in *mucD* gene (Gm^R)	This work
IHMA87 *pprB*::Tn	IHMA87 with mariner transposon inserted in *pprB* gene (Gm^R)	This work
IHMA87 *mucB*::Tn	IHMA87 with mariner transposon inserted in *mucB* gene (Gm^R)	This work
Escherichia coli		
DH5α	Laboratory strain	Lab collection
TOP10	Cloning strain	Invitrogen
Plasmids		
pBTK24	Plasmid with Himar-1 mariner transposon and C9 transposase (Amp^R, Gm^R)	(Kulasekara *et al.*, 2005)
pRK600	Helper plasmid with conjugative properties (Cm^R)	(Kessler, de Lorenzo and Timmis, 1992)
pEXG2	Allelic exchange vector (Gm^R), *sacB*	(Rietsch *et al.*, 2005)
pEX18Tc	Allelic exchange vector (Tc^R), *sacB*	(Li *et al.*, 2011)
pEX100T	Allelic exchange vector (Cb^R), *sacB*	(Schweizer and Hoang, 1995)
pminiCTX-*pX2*-GFP	pminiCTX harboring the *gfp* gene under the control of a constitutive promoter (*attP* site, Tc^R)	(Thibault *et al.*, 2009)
pminiCTX-*Psrg-lacZ*	pminiCTX-*lacZ* harboring the srg promoter fused to *lacZ* reporter gene (*attP* site, Tc^R)	This work
pEXG2-mut-*bioB*	pEXG2 carrying DNA fragment for *bioB* deletion obtained by SLIC (Gm^R)	This work
pEXG2-mut-*purD*	pEXG2 carrying DNA fragment for *purD* deletion obtained by SLIC (Gm^R)	This work
pUC57-mut-*srgABC*	pUC57 carrying the synthetic DNA fragment for *srgABC* deletion in Tn::P_{srg} (Genewiz)	This work
pEX18Tc-mut-*srgABC*	pEX18Tc carrying DNA fragment for srgABC deletion in Tn::P_{srg} mutant obtained by SLIC (Tc^R)	This work
pEX18Tc-mut-01134	pEX18Tc carrying DNA fragment for IHMA87_01134 deletion obtained by SLIC (Tc^R)	This work
pEX100T-mut-*srgA*	pEX100T carrying DNA fragment for *srgA* deletion obtained by SLIC (Cb^R)	This work
pEX100T-mut-*srgB*	pEX100T carrying DNA fragment for *srgB* deletion obtained by SLIC (Cb^R)	This work

pEX100T-mut-*srgC*	pEX100T carrying DNA fragment for *srgC* deletion obtained by SLIC (Cb^R)	This work
pEX100T-mut-*srgAB*	pEX100T carrying DNA fragment for *srgAB* deletion obtained by SLIC (Cb^R)	This work
pEX100T-mut-*srgBC*	pEX100T carrying DNA fragment for *srgBC* deletion obtained by SLIC (Cb^R)	This work
pEX100T-mut-*algD*	pEX100T carrying DNA fragment for *algD* deletion obtained by SLIC (Cb^R)	This work

[1] International Health Management Association, USA

Table S4. Oligonucleotides used for PCRs

Primers	**Sequence (5'-3')**	
pEXG2-mut-*bioB*-sF1	**GGTCGACTCTAGAGGATCCCC**GTTCGGACCCCTCGAACATCG	*bioB* deletion
pEXG2-mut-*bioB*-sR1	GGTGGCGACGGCTGCGGAT	*bioB* deletion
pEXG2-mut-*bioB*-sF2	**GCGCATCCGCAGCCGTCGCCACC**CAGCTGTTCTATAACGCCGCCT	*bioB* deletion
pEXG2-mut-*bioB*-sR2	**ACCGAATTCGAGCTCGAGCCC**CTTGCCGACCAGCGCGGTGA	*bioB* deletion
F0-mut-*bioB*	GCGAGCAGCCGTTCGGCG	PCR verification for *bioB* deletion
R0-mut-*bioB*	TTGAGGCGGTCCTCCAGCA	PCR verification for *bioB* deletion
pEXG2-mut-*purD*-sF1	**GGTCGACTCTAGAGGATCCCC**CAGGAGATCCACGACCTGATCT	*purD* deletion
pEXG2-mut-*purD*-sR1	ACCGCCGCTGCCGATGATGA	*purD* deletion
pEXG2-mut-*purD*-sF2	**TACTCATCATCGGCAGCGGCGGT**GAGCGCGGCGAGTCCTGAC	*purD* deletion
pEXG2-mut-*purD*-sR2	**ACCGAATTCGAGCTCGAGCCC**TCGCGGACCAGCTGGCCGT	*purD* deletion
F0-mut-*purD*	CTGAAGATCGTCACTCGCCG	PCR verification for *purD* deletion
R0-mut-*purD*	GACTCGCCGGTGTGCTGGT	PCR verification for *purD* deletion
pEX18Tc-mut-*srgABC*-sF1	**GTCGACTCTAGAGGATCCC**CGGATCCCCGACATTCGGCTA	Transfer DNA fragment from pUC57 to pEX18Tc for *srgABC* deletion in Tn::P_{srg}
pEX18Tc-mut-*srgABC*-sR1	**CGAATTCGAGCTCGGTACCC**AAGCTTTGATCTACGTGCAAGC	
F0–mut-*srgABC*	GCCTCGCCGACCTCTACA	PCR verification for *srgA, srgB, srgC* and *srgABC* deletion
RO-Tn::P_{srg}-mut-*srgABC*	GCTTGCTGCCTTCGACCAAG	PCR verification for *srgA, srgB, srgC* and *srgABC* deletion
pEX18Tc-mut-01134-sF1	**GTCGACTCTAGAGGATCCCC**CACCTCGGTGTCCACGCTGC	IHMA87_01134 deletion
pEX18Tc-mut-01134-sR1	CGACGAGCATCGACTTGTTCAC	IHMA87_01134 deletion
pEX18Tc-mut-01134-sF2	**TGAACAAGTCGATGCTCGTC**GACAAGAACGGTCGGCTGGT	IHMA87_01134 deletion
pEX18Tc-mut-01134-sR2	**CGAATTCGAGCTCGGTACCC**ATGTTCCGCAGCCTGGTCGG	IHMA87_01134 deletion
F0-mut-01134	CTGCTGTACGTCGGCTTCCG	PCR verification for IHMA87_01134 deletion
R0-mut-01134	CTGGATGGCACCACCAACTTC	PCR verification for IHMA87_01134 deletion
pEX100T-mut-*srgA*-sF1	**ACCCTGTTATCCCTACCC**GTGATCTACG	*srgA* and *srgAB* deletion in

	TGCAAGCAGA	Tn::P_{srg}
pEX100T-mut-*srgA*-sR1	CAGGTTGCGTGAGCTGCTCA	*srgA* and *srgAB* deletion in Tn::P_{srg}
pEX100T-mut-*srgA*-sF2	**TGAGCAGCTCACGCAACCTGTGA**CCTGGACATTCTGACGAGGTA	*srgA* deletion in Tn::P_{srg}
pEX100T-mut-*srgA*-sR2	**GGATAACAGGGTAATCCC**CACCAGCGCAAGCAGCAGC	*srgA* deletion in Tn::P_{srg}
pEX100T-mut-*srgB*-sF1	**ACCCTGTTATCCCTACCC**ATGGAGGTGAACATGAGCAGC	*srgB* and *srgBC* deletion in Tn::P_{srg}
pEX100T-mut-*srgB*-sR1	AGCCAGAATCCACATGTTTC	*srgB* and *srgBC* deletion in Tn::P_{srg}
pEX100T-mut-*srgB*-sF2	**GAAACATGTGGATTCTGGCTTGA**ACGGAAGCTCGGCGCCACT	*srgB* deletion in Tn::P_{srg}
pEX100T-mut-*srgB*-sR2	**GGATAACAGGGTAATCCC**CGTGTTGACTCACGTCGGAC	*srgB* deletion in Tn::P_{srg}
pEX100T-mut-*srgC*-sF1	**ACCCTGTTATCCCTACCC**CGACGAACAGGCCTGGACAT	*srgC* deletion in Tn::P_{srg}
pEX100T-mut-*srgC*-sR1	AAGAATGACGGCGGATCGTGA	*srgC* deletion in Tn::P_{srg}
pEX100T-mut-*srgC*-sF2	**TCACGATCCGCCGTCATTCTTTGA**GATCCCAGCCTGGACTGATC	*srgC* deletion in Tn::P_{srg}
pEX100T-mut-*srgC*-sR2	**GGATAACAGGGTAATCCCC**CACTCGAAGCCGACATTCG	*srgC* deletion in Tn::P_{srg}
pEX100T-mut-*srgAB*-sF2	**TGAGCAGCTCACGCAACCTGTGA**ACGGAAGCTCGGCGCCACT	*srgAB* deletion in Tn::P_{srg}
pEX100T-mut-*srgAB*-sR2	**GGATAACAGGGTAATCCC**CGTGTTGACTCACGTCGGAC	*srgAB* deletion in Tn::P_{srg}
pEX100T-mut-*srgBC*-sF2	**GAAACATGTGGATTCTGGCTTGA**GATCCCAGCCTGGACTGATC	*srgBC* deletion in Tn::P_{srg}
pEX100T-mut-*srgBC*-sR2	**GGATAACAGGGTAATCCCC**CACTCGAAGCCGACATTCG	*srgBC* deletion in Tn::P_{srg}
pEX100T-mut-*algD*-sF1	**ACCCTGTTATCCCTACCC**CGGAAACGCCATCAAGTTGGTA	*algD* deletion
pEX100T-mut-*algD*-sR1	GCCAGCACATACCGCACCGA	*algD* deletion
pEX100T-mut-*algD*-sF2	**TCGGTGCGGTATGTGCTGGC**CAGGCCGAGGGCATCTGCT	*algD* deletion
pEX100T-mut-*algD*-sR2	**GGATAACAGGGTAATCCC**GCTGTCGATGGCTTCGCGGA	*algD* deletion
F0-mut-*algD*	CTCGTGGCGAATAGGCCTAC	PCR verification for *algD* deletion
R0-mut-*algD*	GCGAAGCGCAGCTTGTGCC	PCR verification for *algD* deletion
Primers for Tn-seq		
Short adpator	TACCACGACCA-NH2	
Long adaptor	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTCGTGGTAT	
PCR1 Tn-specific	CACAGGAAACAGGACTCTAGAGG	
PCR2 adaptor complementary	GTGACTGGAGTTCAGACGTGTG	
P5+ Illumina	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGAGACCGGGGACTTATCAGC	
P7-index	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**	

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