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

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

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

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

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

Results

Gain-of-function screen in plasma

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

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

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

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

log-transformation was performed on the dataset and all mutants were shown significantly different from the wild-type strain (p-value < 0.005). Log₂(Fold-Change(FC)) obtained from the Tn-seq analysis for each gene or intergenic region is indicated above. **C.** Bioinformatics analysis presented as Volcano plots. Insertions in coding regions (left) and intergenic regions (right) were analyzed separately. Significant hits in genes of the same pathway are in the same color.

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

IHMA87_04274		<i>slyX</i>	10.60	4.67E-13
IHMA87_04133	PA1078	<i>flgC</i>	10.40	4.59E-28
IHMA87_04779	PA4457	<i>kdsD</i>	10.24	3.15E-14
IHMA87_04779	PA4457	-/arabinose-5-phosphate isomerase	10.24	3.15E-14
IHMA87_00896	PA4032	<i>walR</i>	9.89	3.67E-24
IHMA87_00319	PA0341	<i>lgt</i>	9.95	5.21E-16
IHMA87_03447	PA1758	<i>pabB</i>	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	<i>mvaT</i>	9.41	1.21E-23
IHMA87_02603		-	9.34	4.46E-17
IHMA87_02570		-	9.28	3.29E-13
IHMA87_05344	PA4856	<i>retS</i>	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.

Deficiency in purine and biotin pathways leads to increased persistence

We further focused on hits in genes and intergenic regions of biotin and purine biosynthesis. Mutants with insertions in loci of the entire biotin biosynthesis, the *bioBFHCD* operon and the *bioA* gene, were significantly overrepresented in plasma (Table 1, Fig. 2A), suggesting that the absence of biotin leads to better survival in plasma. This finding was surprising, as the biotin pathway was found essential for survival of several pathogens in human serum including *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* (Woong Park *et al.*, 2011; Carfrae *et al.*, 2020; Weber *et al.*, 2020). In addition to the mutants isolated from the screen (Tn::*bioA* and Tn::*bioB*), and in order to confirm this result, we engineered a *bioB* deletion mutant ($\Delta bioB$) and examined its behavior in plasma over a 6h-challenge (Fig. 2B). Compared to the wild-type strain, the fraction of $\Delta bioB$ that survived in plasma was over 10% compared to about 0.01% of parental strain. Moreover, the survival of IHMA87 $\Delta bioB$ displayed a biphasic killing curve, suggesting that the low level of biotin triggered the persistence 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, $\Delta bioB$ and $\Delta purD$ mutants in plasma over 6h incubation measured by CFU counting (n=5). Note the biphasic killing curves for the parental strain and $\Delta bioB$ mutant, indicating increased persistence of $\Delta bioB$. The $\Delta purD$ mutant displayed increased tolerance compared to the parental strain.

De novo purine biosynthesis, together with the pyrimidine pathway, are essential for bacterial nucleotide metabolism and is important for bacterial growth and survival in plasma and serum (Samant *et al.*, 2008; Andersen-Civil *et al.*, 2018; Poulsen *et al.*, 2019; Zhu *et al.*, 2021). Similarly to biotin synthesis, in our model, mutants in the purine biosynthetic pathway clearly displayed better survival in plasma (Fig. 2A). Transposon-insertion mutants in three main operons encoding enzymes of purine biogenesis were found overrepresented in the screen. Notably, mutants in *purF*, encoding an amido-phospho-ribosyltransferase were significantly enriched with a Log₂(FC) of 17. To further study the link between purine biosynthesis and increased plasma resistance, we designed a *purD* deletion mutant ($\Delta purD$) and investigated its killing kinetics over 6-hours in plasma. In accordance with Hill *et al.*, 2021 in an antibiotic context, a $\Delta purD$ mutant displayed a tolerant phenotype in plasma (Fig. 2B). To note, no genes were found to be enriched to the same level in the pyrimidine pathway, suggesting that the specific product of the purine pathway and not the global nucleotide metabolism, contributed to increased survival in human plasma.

ATP levels influence *P. aeruginosa* tolerance to plasma

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

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

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

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 $\Delta bioB$ suggesting a distinct molecular
293 mechanism for evader formation in this mutant.

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

One mutant isolated during the screen was significantly enriched in plasma with a $\text{Log}_2(\text{FC})$ of 13.2 (Fig. 4A). The Sanger sequencing determined the exact transposon insertion within the intergenic region between an uncharacterized predicted three-gene operon *IHMA87_01573-IHMA87_01571* (predicted in PA14 by Wurtzel *et al.*, 2012, corresponding to *PA3369-PA3371* in PAO1) and *panM* (*IHMA87_01574/PA3368*), encoding a shorter version of a probable acetyltransferase (Fig. 4B). The isolated mutant displayed a tolerant phenotype over a 6-hour period (Fig. S1) and a 100 fold increase in survival compared to the parental strain after 3h of incubation (Fig. 4C). The position of the transposon and the RT-qPCR analysis did not allow us to assess the effect of the insertion. In addition, strains carrying deletions either in *panM* or in *IHMA87_01573-IHMA87_01571* in the IHMA87 background showed no difference in survival in plasma compared to the wild-type strain. Therefore, to globally apprehend the transposon-induced changes in the mutant, we determined the transcriptomic profile of the mutant and compared it to the parental strain in LB. The RNA-seq data showed the overexpression of the predicted operon *IHMA87_01573-IHMA87_01571*, named *srgABC* for serum resistance genes, with about a 20-fold change compared to the parental strain. Thus, the transposon insertion mutant will be referred to as $\text{Tn}::P_{srg}$. All together 90 genes were differentially expressed ($|\text{FC}| > 2$, $p_{\text{adj}} < 1 \times 10^{-10}$) between $\text{Tn}::P_{srg}$ and the wild-type strain (Table 2S), including *algU* encoding the alternative sigma factor AlgU and five other alginate genes (*algR* and *mucABCD* operon, Fig. 4D). Consistent with the observed upregulation of *algU*, colonies displayed characteristic mucoid phenotype on solid medium. The *srg* operon encodes three small putative proteins of 9.9 (SrgA), 5.3 (SrgB) and 6.5 (SrgC) kDa. Based on structural predictions, SrgA is a periplasmic or secreted protein

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

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

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

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

To establish the relationship between the *srg* operon, alginate production and bacterial tolerance to complement, we inactivated *algD*, encoding GDP-mannose 6-dehydrogenase, a checkpoint in alginate biosynthesis (Deretic, Gill and Chakrabarty, 1987; Berry, DeVault and Chakrabarty, 1989; Wood and Ohman, 2012) both in the parental strain and in Tn::*P_{srg}*, and investigated relevant phenotypes. In the assay, we included alginate-overproducing mutant Tn::*mucB* isolated during the screen. We also designed a deletion mutant in the gene *IHMA87_01134* (*PA3819*), an uncharacterized gene that was overexpressed ($\text{Log}_2(\text{FC})=2.35$, $p_{\text{adj}}=2 \times 10^{-153}$) in the Tn::*P_{srg}* strain. For each strain, we established the survival rate by CFU counting, the quantities of alginates using carbazole assay and assessed the colony morphology on plates (Fig. 4E-F). The mutant Tn::*P_{srg}* displayed a 100-fold higher survival in human plasma compared to the parental strain, along with an increased alginate production. Although alginate overproduction can lead to higher resistance to human plasma, as seen for the mutant Tn::*mucB*, the alginates in Tn::*P_{srg}* did not account significantly for its tolerance to plasma. Finally, the deletion of *IHMA87_01134* had no impact neither on alginate production nor on plasma resistance. Overall, these results demonstrate that increased plasma tolerance in a *srg* overexpressing mutant is alginate-independent, and that SrgA alone modulates plasma tolerance.

The specificity of mutants toward MAC-induced killing

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

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

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 OmCI complement inhibitor.

Discussion

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

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

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

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

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

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

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

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

Materials and methods

Bacterial strains and genetic manipulations

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

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

Generation of the IHMA87 transposon library

The IHMA87 transposon mutant library was constructed essentially as previously described (Skurnik *et al.*, 2013; Trouillon *et al.*, 2020). Briefly, IHMA87, grown overnight at 42°C with shaking, was mixed with two *E. coli* strains carrying pRK2013 (Figurski and Helinski, 1979) and pBTK24. The conjugation mixtures containing 100 µl of each bacterial culture adjusted to optical densities measured at 600 nm (OD₆₀₀) of 1, were centrifuged, washed with LB, deposited onto pre-warmed LB plates and let at 37°C for 5 h. Approximately 100 conjugation mixtures yielded a library of > 300,000 mutants. Bacteria were scraped off into liquid LB and aliquots were plated on LB plates containing irgasan (25 µg/ml) and gentamicin (75 µg/ml). Following growth at 37°C, the colonies were collected directly into LB containing 20% glycerol, aliquoted and stored at -80 °C.

Plasma pools preparation

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

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

Screen in plasma

A vial containing the IHMA87 mutant library was thawed on ice, transferred into a flask containing 30 mL of LB and grown 16h under agitation at 27°C to limit bacterial growth. The next day, bacterial suspension was diluted to OD_{600nm} ~0.1 and grown at 37°C under agitation. Meanwhile, plasma and HIP were prepared. At an OD_{600nm} ~1, bacteria were harvested and resuspended in PBS supplemented with calcium and magnesium ions (ThermoFisher Scientific) before infecting plasma, HIP or LB samples to a concentration of 2.25x10⁷ CFU/mL (90% final plasma concentration). The precise initial CFU count of each experiment was determined by serial dilution. Samples were incubated at 37°C for 3h on a rotating wheel. At the end of the challenge, part of the sample was transferred to LB and grown overnight, part was spread on LB agar plates to isolate individual mutants. Finally, bacterial survival was calculated by CFU counts of each sample. In parallel, the same protocol was performed with the wild-type strain for survival comparison. The next day, vials were taken from the culture and stored for further mutant isolation. About 1x10⁹ bacteria were harvested for each sample and subjected to DNA extraction.

Illumina library construction, sequencing and data analysis

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

Library construction. Fragmented gDNA (1 µg) were end-repaired using an End-repair module (NEB#E6050) followed by a dA-tailing with Klenow (NEB#6053). Short and Long adaptors (Table 1S) were annealed in 10 mM MgCl₂ in thermocycler with a program allowing decrease of 1°C/cycle, from 95°C to 20°C. Ligation of gDNA fragments with annealed adaptors was achieved overnight at 16°C with T4 DNA ligase (NEB#M0202). A size selection was performed on a 2% agarose gel by extracting the bands from 200 to 400 bp (Monarch DNA Gel Extraction Kit, NEB#T1020L). Amplification of purified adaptor-DNA fragments was carried out with Phusion polymerase (NEB#M0530) and specific primers (PCR1 Tn-spe direct and PCR1 Adaptor comp.). A second amplification step was performed with a primer carrying a P5 illumina sequence and Indexed-P7 illumina primers (NEB#E7335S). Primer sequences are available on request. After each step, quantification was performed using the Qubit dsDNA HS Assay kit (Q33230). The quality of the libraries was assessed on the Agilent Bioanalyzer 2100 using high-sensitivity DNA chips (Ref

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

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

Plasma killing assay

Killing assay was performed as described previously (Pont *et al.*, 2020). Unless stated otherwise, bacterial pre-cultures were diluted in LB to $OD_{600nm} = 0.1$ and grown at 37°C under agitation until $OD_{600nm} \sim 1$. Bacteria were resuspended into PBS +/- (Thermofisher Scientific) and incubated in plasma to a concentration of 2.25×10^7 CFU/mL (90 % final plasma concentration). The precise initial CFU count of each experiment was determined by serial dilution. Bacterial survival was calculated by CFU counts at the indicated time(s) and compared to the initial count. ATP, biotin and purine were added to the plasma prior to bacterial addition at the indicated concentrations.

Transcriptomics

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

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

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

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

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

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

Alginate dosage

Equivalent amounts of OD_{600nm} adjusted bacterial cultures were spread onto *Pseudomonas* isolation agar (PIA) plates and incubated for 24h at 37°C. Bacterial lawns were flushed with saline solution (0.9% NaCl) and scraped off the plates. Bacteria were pelleted and supernatant was stored in a separated tube. Bacterial pellet was resuspended in LB and incubated on a rotating wheel for 1-2 hours to avoid bacterial aggregates. Bacterial titer was calculated by serial dilutions and subsequent CFU counting. The alginate dosage was done as described in Knutson and Jeanes, 1968 and May and Chakrabarty, 1994. Briefly, 600µL of ice cold borate working solution (borate stock solution (4 M BO₃³⁻) diluted to a final concentration of 100 mM in H₂SO₄) was added to 70 µL of the bacteria supernatant, saline solution for a blank, and to commercial sodium alginate solution to obtain a standard curve. Keeping on ice, 20 µL of carbazole solution (0.1% carbazole (Sigma #442506) in 100% ethanol) was added, and samples were incubated at 55°C for 30 min after mixing. The absorbance was measured at 530 nm.

Quantitative measurement of intracellular bacterial ATP

For ATP measurement in LB, about 1 x 10⁸ bacteria were pelleted and resuspended in PBS. HIP was inoculated with a final concentration of 2.025 x 10⁷ bacterial/mL in PBS (90% final HIP concentration). Samples were incubated for 2h at 37°C on a rotating wheel. At the end of the incubation, samples were centrifuged and resuspended in PBS prior to a gentle sonication step using Qsonica sonicator (Q700) for 45 sec with 10% intensity and 5 sec ON-5 sec OFF intervals in order to dissociate

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

Residual complement activity

Sample preparation. Bacteria grown to $OD_{600nm} \sim 1$ were resuspended into PBS +/- (ThermoFisher Scientific) and incubated in plasma to a concentration of 2.25×10^9 CFU/mL (90% final plasma concentration) or PBS only for the reference. Samples were incubated for 3 hours at 37°C on a rotating wheel to allow complement activation by bacteria. The initial CFU count was determined by serial dilution. At the end of the incubation, samples were centrifuged and supernatant was filtered through 0.22 μ m membrane to remove any remaining bacteria. Samples were aliquoted and frozen at -80°C until use. The remaining complement activity in the bacterial/plasma supernatant was measured using a total hemolytic assay for classical and alternative pathways, as described by (Dumestre-Pérard *et al.*, 2008).

Classical complement hemolytic activity. Briefly, sheep erythrocytes (Orgentec) were sensitized using anti-erythrocytes antibodies (1/40000 - Hemolysin, Orgentec) for 15min at 37°C. Bacterial/plasma supernatant (25 μ L) was mixed with 3 mL of sensitized erythrocytes (4×10^6 cells/mL) diluted in DGVB²⁺ (2.5% glucose, 0.05%

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

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

C3b and C5b-9 binding assays

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

C9 binding

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

Nisin assay

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

Transmission electron microscopy

Bacterial pre-cultures were diluted to an OD_{600nm} of 0.1 in flasks containing 30 mL of LB and incubated at 37°C under agitation. Plasma was then inoculated with bacterial suspension to reach a bacterial concentration of 1.125 x10⁹ bacteria/mL and incubated on a rotating wheel for 1 hour at 37°C. At the end of the challenge, bacteria were pelleted by centrifugation and resuspended in LB prior to fixation. At the same time, bacteria grown in LB to an OD_{600nm} of 1 were pelleted by centrifugation and resuspended in LB prior to fixation.

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

Bioinformatics analysis and protein conservation

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

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

Statistical analysis

Sigmaplot software was used to perform statistical analysis. Multiple groups comparison was performed using one-way ANOVA or Kruskal-Wallis tests depending on data normality followed by a Student-Newman-Keuls pairwise comparison. Non-normally distributed data were converted to normally-distributed dataset by log₁₀-transformation where indicated. Figures were designed using Graphpad Prism9.

Data availability

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

Acknowledgments

The work was supported by grants from Agence Nationale de la Recherche (ANR-15-CE11-0018-01), the Laboratory of Excellence GRAL, financed within the University Grenoble Alpes graduate school (Écoles Universitaires de Recherche) CBH-EUR-GS (ANR-17-EURE-0003), the Fondation pour la Recherche Médicale (Team FRM 2017, DEQ20170336705) to I.A., the European Union's Horizon 2020 research programs H2020-EU-ITN-EJD (CORVOS #860044 to F.M. and SHMR). The sequencing part

has benefited from the facilities and expertise of the high throughput sequencing core facility of I2BC (Centre de Recherche de Gif – <http://www.i2bc.paris-saclay.fr/>). *P. aeruginosa* IHMA87 was obtained from the International Health Management Association, USA. This work used the platforms of the Grenoble Instruct-ERIC center (ISBG; UAR 3518 CNRS-CEA-UGA-EMBL) within the Grenoble Partnership for Structural Biology (PSB), supported by FRISBI (ANR-10-INBS-0005-02) and GRAL, financed within the University Grenoble Alpes graduate school (Ecoles Universitaires de Recherche) CBH-EUR-GS (ANR-17-EURE-0003). M. JM received a Ph.D. fellowship from the French Ministry of Education and Research. The IBS Electron Microscope facility is supported by the Auvergne Rhône-Alpes Region, the Fonds Feder, the Fondation pour la Recherche Médicale and GIS-IBiSA. The authors thank Benoît Gallet and Emily Rey for their help for TEM and mutant analyses, respectively. M.JM thanks Tweeter followers for indicating literature on PolyP granules.

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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 (same 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
<i>Pseudomonas aeruginosa</i>		
IHMA879472/AZPAE1 5042	Urinary isolate, plasma sensitive	IHMA ¹ collection (Kos <i>et al.</i> , 2015; Pont <i>et al.</i> , 2020; Trouillon <i>et al.</i> , 2020) (Kos, Trouillon, Pont)
IHMA87Δ <i>bioB</i>	IHMA87 with <i>bioB</i> deletion	This work
IHMA87Δ <i>purD</i>	IHMA87 with <i>purD</i> deletion	This work
IHMA87Δ <i>algD</i>	IHMA87 with <i>algD</i> deletion	This work
IHMA87Δ <i>O1134</i>	IHMA87 with <i>O1134</i> deletion	This work
IHMA87 Tn::P _{srg}	IHMA87 with mariner transposon inserted in the promoter of <i>srg</i> operon (Gm ^R)	This work
IHMA87 Tn::P _{srg} Δ <i>srgABC</i>	IHMA87 Tn::P _{srg} with <i>srgABC</i> deletion (Gm ^R)	This work

IHMA87 Tn:: <i>P_{srg} ΔsrgA</i>	IHMA87 Tn:: <i>P_{srg}</i> with <i>srgA</i> deletion (Gm ^R)	This work
IHMA87 Tn:: <i>P_{srg} ΔsrgB</i>	IHMA87 Tn:: <i>P_{srg}</i> with <i>srgB</i> deletion (Gm ^R)	This work
IHMA87 Tn:: <i>P_{srg} ΔsrgC</i>	IHMA87 Tn:: <i>P_{srg}</i> with <i>srgC</i> deletion (Gm ^R)	This work
IHMA87 Tn:: <i>P_{srg} ΔsrgBC</i>	IHMA87 Tn:: <i>P_{srg}</i> with <i>srgBC</i> deletion (Gm ^R)	This work
IHMA87 Tn:: <i>P_{srg} ΔalgD</i>	IHMA87 Tn:: <i>P_{srg}</i> with <i>algD</i> deletion (Gm ^R)	This work
IHMA87 Tn:: <i>P_{srg} Δ01134</i>	IHMA87 Tn:: <i>P_{srg}</i> with <i>01134</i> deletion (Gm ^R)	This work
IHMA87 <i>ladS</i> ::Tn	IHMA87 with mariner transposon inserted in <i>ladS</i> gene (Gm ^R)	This work
IHMA87 <i>bioA</i> ::Tn	IHMA87 with mariner transposon inserted in <i>bioA</i> gene (Gm ^R)	This work
IHMA87 <i>bioB</i> ::Tn	IHMA87 with mariner transposon inserted in <i>bioB</i> gene (Gm ^R)	This work
IHMA87 <i>mucE</i> ::Tn	IHMA87 with mariner transposon inserted in <i>mucE</i> gene (Gm ^R)	This work
IHMA87 <i>mucA</i> ::Tn	IHMA87 with mariner transposon inserted in <i>mucA</i> gene (Gm ^R)	This work
IHMA87 <i>mucD</i> ::Tn	IHMA87 with mariner transposon inserted in <i>mucD</i> gene (Gm ^R)	This work
IHMA87 <i>pprB</i> ::Tn	IHMA87 with mariner transposon inserted in <i>pprB</i> gene (Gm ^R)	This work
IHMA87 <i>mucB</i> ::Tn	IHMA87 with mariner transposon inserted in <i>mucB</i> gene (Gm ^R)	This work
<i>Escherichia coli</i>		
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 <i>et al.</i> , 2005)
pRK600	Helper plasmid with conjugative properties (Cm ^R)	(Kessler, de Lorenzo and Timmis, 1992)
pEXG2	Allelic exchange vector (Gm ^R), <i>sacB</i>	(Rietsch <i>et al.</i> , 2005)
pEX18Tc	Allelic exchange vector (Tc ^R), <i>sacB</i>	(Li <i>et al.</i> , 2011)
pEX100T	Allelic exchange vector (Cb ^R), <i>sacB</i>	(Schweizer and Hoang, 1995)
pminiCTX- <i>pX2</i> -GFP	pminiCTX harboring the <i>gfp</i> gene under the control of a constitutive promoter (<i>attP</i> site, Tc ^R)	(Thibault <i>et al.</i> , 2009)
pminiCTX- <i>Psrg-lacZ</i>	pminiCTX- <i>lacZ</i> harboring the <i>srg</i> promoter fused to <i>lacZ</i> reporter gene (<i>attP</i> site, Tc ^R)	This work
pEXG2-mut- <i>bioB</i>	pEXG2 carrying DNA fragment for <i>bioB</i> deletion obtained by SLIC (Gm ^R)	This work
pEXG2-mut- <i>purD</i>	pEXG2 carrying DNA fragment for <i>purD</i> deletion obtained by SLIC (Gm ^R)	This work
pUC57-mut- <i>srgABC</i>	pUC57 carrying the synthetic DNA fragment for <i>srgABC</i> deletion in Tn:: <i>P_{srg}</i> (Genewiz)	This work
pEX18Tc-mut- <i>srgABC</i>	pEX18Tc carrying DNA fragment for <i>srgABC</i> deletion in Tn:: <i>P_{srg}</i> mutant obtained by SLIC (Tc ^R)	This work
<i>pEX18Tc-mut-01134</i>	pEX18Tc carrying DNA fragment for IHMA87_01134 deletion obtained by SLIC (Tc ^R)	This work
pEX100T-mut- <i>srgA</i>	pEX100T carrying DNA fragment for <i>srgA</i> deletion obtained by SLIC (Cb ^R)	This work
pEX100T-mut- <i>srgB</i>	pEX100T carrying DNA fragment for <i>srgB</i> deletion obtained by SLIC (Cb ^R)	This work

pEX100T-mut- <i>srgC</i>	pEX100T carrying DNA fragment for <i>srgC</i> deletion obtained by SLIC (Cb ^R)	This work
pEX100T-mut- <i>srgAB</i>	pEX100T carrying DNA fragment for <i>srgAB</i> deletion obtained by SLIC (Cb ^R)	This work
pEX100T-mut- <i>srgBC</i>	pEX100T carrying DNA fragment for <i>srgBC</i> deletion obtained by SLIC (Cb ^R)	This work
pEX100T-mut- <i>algD</i>	pEX100T carrying DNA fragment for <i>algD</i> 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- <i>bioB</i> -sF1	GGTCGACTCTAGAGGATCCCC GTTCGG ACCCCTCGAACATCG	<i>bioB</i> deletion
pEXG2-mut- <i>bioB</i> -sR1	GGTGGCGACGGCTGCGGAT	<i>bioB</i> deletion
pEXG2-mut- <i>bioB</i> -sF2	GCGCATCCGCAGCCGTGCGCACCC AGC TGTTCTATAACGCCGCCT	<i>bioB</i> deletion
pEXG2-mut- <i>bioB</i> -sR2	ACCGAATTCGAGCTCGAGCCC CTTGCC GACCAGCGCGGTGA	<i>bioB</i> deletion
F0-mut- <i>bioB</i>	GCGAGCAGCCGTTGCGCG	PCR verification for <i>bioB</i> deletion
R0-mut- <i>bioB</i>	TTGAGGCGGTCTCCAGCA	PCR verification for <i>bioB</i> deletion
pEXG2-mut- <i>purD</i> -sF1	GGTCGACTCTAGAGGATCCCC CAGGAG ATCCACGACCTGATCT	<i>purD</i> deletion
pEXG2-mut- <i>purD</i> -sR1	ACCGCCGCTGCCGATGATGA	<i>purD</i> deletion
pEXG2-mut- <i>purD</i> -sF2	TACTCATCATCGGCAGCGGCGGT GAGC GCGGCGAGTCCTGAC	<i>purD</i> deletion
pEXG2-mut- <i>purD</i> -sR2	ACCGAATTCGAGCTCGAGCCC TCGCGG ACCAGCTGGCCGT	<i>purD</i> deletion
F0-mut- <i>purD</i>	CTGAAGATCGTCACTCGCCG	PCR verification for <i>purD</i> deletion
R0-mut- <i>purD</i>	GACTCGCCGGTGTGCTGGT	PCR verification for <i>purD</i> deletion
pEX18Tc-mut- <i>srgABC</i> -sF1	GTCGACTCTAGAGGATCCCC GGATCCC CGACATTCGGCTA	Transfer DNA fragment from pUC57 to pEX18Tc for <i>srgABC</i> deletion in Tn::P _{srg}
pEX18Tc-mut- <i>srgABC</i> -sR1	CGAATTCGAGCTCGGTACCC AAGCTTT GATCTACGTGCAAGC	
F0-mut- <i>srgABC</i>	GCCTCGCCGACCTCTACA	PCR verification for <i>srgA</i> , <i>srgB</i> , <i>srgC</i> and <i>srgABC</i> deletion
RO-Tn::P _{srg} -mut- <i>srgABC</i>	GCTTGCTGCCTTCGACCAAG	PCR verification for <i>srgA</i> , <i>srgB</i> , <i>srgC</i> and <i>srgABC</i> deletion
pEX18Tc-mut-01134-sF1	GTCGACTCTAGAGGATCCCC CACCTCG GTGTCCACGCTGC	IHMA87_01134 deletion
pEX18Tc-mut-01134-sR1	CGACGAGCATCGACTTGTTAC	IHMA87_01134 deletion
pEX18Tc-mut-01134-sF2	TGAACAAGTCGATGCTCGTC GACAAGA ACGGTCGGCTGGT	IHMA87_01134 deletion
pEX18Tc-mut-01134-sR2	CGAATTCGAGCTCGGTACCC ATGTTCC GCAGCCTGGTCGG	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- <i>srgA</i> -sF1	ACCCTGTTATCCCTACCC GTGATCTACG	<i>srgA</i> and <i>srgAB</i> deletion in

	TGCAAGCAGA	Tn::P _{srg}
pEX100T-mut-srgA-sR1	CAGGTTGCGTGAGCTGCTCA	srgA and srgAB deletion in Tn::P _{srg}
pEX100T-mut-srgA-sF2	TGAGCAGCTCACGCAACCTGTGACCTG GACATTCTGACGAGGTA	srgA deletion in Tn::P _{srg}
pEX100T-mut-srgA-sR2	GGATAACAGGGTAATCCCC ACCAGCGC AAGCAGCAGC	srgA deletion in Tn::P _{srg}
pEX100T-mut-srgB-sF1	ACCCTGTTATCCCTACCC ATGGAGGTGA ACATGAGCAGC	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	GAAACATGTGGATTCTGGCTTGAACGG AAGCTCGGCCCACT	srgB deletion in Tn::P _{srg}
pEX100T-mut-srgB-sR2	GGATAACAGGGTAATCCCC CGTGTTGAC TCACGTCGGAC	srgB deletion in Tn::P _{srg}
pEX100T-mut-srgC-sF1	ACCCTGTTATCCCTACCC CGACGAACA GGCCTGGACAT	srgC deletion in Tn::P _{srg}
pEX100T-mut-srgC-sR1	AAGAATGACGGCGGATCGTGA	srgC deletion in Tn::P _{srg}
pEX100T-mut-srgC-sF2	TCACGATCCGCCGTCATTCTTTG AGATC CCAGCCTGGACTGATC	srgC deletion in Tn::P _{srg}
pEX100T-mut-srgC-sR2	GGATAACAGGGTAATCCCC CACTCGAA GCCGACATTCTG	srgC deletion in Tn::P _{srg}
pEX100T-mut-srgAB-sF2	TGAGCAGCTCACGCAACCTGTGAACGG AAGCTCGGCCCACT	srgAB deletion in Tn::P _{srg}
pEX100T-mut-srgAB-sR2	GGATAACAGGGTAATCCCC CGTGTTGAC TCACGTCGGAC	srgAB deletion in Tn::P _{srg}
pEX100T-mut-srgBC-sF2	GAAACATGTGGATTCTGGCTTGA GATC CCAGCCTGGACTGATC	srgBC deletion in Tn::P _{srg}
pEX100T-mut-srgBC-sR2	GGATAACAGGGTAATCCCC CACTCGAA GCCGACATTCTG	srgBC deletion in Tn::P _{srg}
pEX100T-mut-algD-sF1	ACCCTGTTATCCCTACCC CGAAACGC CATCAAGTTGGTA	algD deletion
pEX100T-mut-algD-sR1	GCCAGCACATACCGCACCGA	algD deletion
pEX100T-mut-algD-sF2	TCGGTGCGGTATGTGCTGGCC AGGCCG AGGGCATCTGCT	algD deletion
pEX100T-mut-algD-sR2	GGATAACAGGGTAATCCCC CGTGTCGAT GGCTTCGCGGA	algD deletion
F0-mut-algD	CTCGTGCGCAATAGGCCTAC	PCR verification for algD deletion
R0-mut-algD	GCGAAGCGCAGCTTGTC	PCR verification for algD deletion
Primers for Tn-seq		
Short adaptor	TACCACGACCA-NH2	
Long adaptor	GTGACTGGAGTTCAGACGTGTGCTCTTC CGATCTGGTCTGTTAT	
PCR1 Tn-specific	CACAGGAAACAGGACTCTAGAGG	
PCR2 adaptor complementary	GTGACTGGAGTTCAGACGTGTG	
P5+ Illumina	AATGATACGGCGACCACCGAGATCTACA CTCTTTCCCTACACGACGCTCTTCCGAT CTCTAGAGACCGGGGACTTATCAGC	
P7-index	CAAGCAGAAGACGGCATACGAGAT CGT GAT	

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