Bacterial behavior in human blood reveals complement evaders with persister-like features

Short title: Complement evaders

Stéphane Pont[1], Nathan Fraikin[2], Yvan Caspar [3, 4], Laurence Van Melderen[2], Ina Attrée*[1] and François Cretin*[1]

[1]Université Grenoble Alpes, Bacterial Pathogenesis and Cellular Responses team, CNRS ERL5261, CEA IRIG-BCI, INSERM UMR1036, Grenoble, France
[2]Université Libre de Bruxelles, Department of Molecular Biology, Cellular & Molecular Microbiology, B-6041 Gosselies, Belgium
[3]Centre Hospitalier Universitaire Grenoble Alpes, Laboratoire de bactériologie-Hygiène hospitalière, Grenoble, France
[4]Université Grenoble Alpes, CNRS, Grenoble INP, TIMC-IMAG, F-38000 Grenoble, France

*Correspondance to francois.cretin@cea.fr; ina.attree-delic@cea.fr

Abstract

Bacterial bloodstream infections (BSI) are a major health concern and can cause up to 40% mortality. *Pseudomonas aeruginosa* BSI is often of nosocomial origin and is associated with a particularly poor prognosis. The mechanism of bacterial persistence in blood is still largely unknown. Here, we analyzed the behavior of a cohort of clinical and laboratory *Pseudomonas aeruginosa* strains in human blood. In this specific environment, complement was the main defensive mechanism, acting either by direct bacterial lysis or by opsonophagocytosis, which required recognition by immune cells. We found highly variable survival rates for different strains in blood, whatever their origin, serotype, or the nature of their secreted toxins and despite their detection by immune cells. We identified and characterized a complement-tolerant subpopulation of bacterial cells that we named “evaders”. Evaders represented 0.1-0.001% of the initial bacterial load and displayed transient tolerance. Although evaders shared some features with bacterial persisters, which tolerate antibiotic treatment, they appear to have evolved distinct strategies to escape complement. We detected the evaders for five other major human pathogens: *Acinetobacter baumannii, Burkholderia multivorans,* enteroaggregative *Escherichia coli*, *Klebsiella pneumoniae*, and *Yersinia enterocolitica*. Thus, the evaders could allow the pathogen to persist within the bloodstream, and may be the cause of fatal bacteremia or dissemination, notably in the absence of effective antibiotic treatments.

Author summary for "Complement evaders"

Blood infections by antibiotic resistant bacteria, notably *Pseudomonas aeruginosa*, are major concerns in hospital settings. The complex interplay between *P. aeruginosa* and the innate immune system in the context of human blood is still poorly understood. By studying the behavior of various *P. aeruginosa* strains in human whole blood and plasma, we showed that bacterial strains display different rate of tolerance to the complement system. Despite the complement microbicide activity, most bacteria withstand elimination through phenotypic heterogeneity creating a tiny (<0.1%) subpopulation of transiently tolerant evaders. While genetically identical to the rest of the complement-sensitive population, evaders allow the bacteria to persist in plasma. This phenotypic heterogeneity thus prevents total elimination of the pathogen from the circulation, and represent a new strategy to disseminate within the organism.

Introduction

The incidence of bacterial bloodstream infections (BSI) in high-income countries is as extensive as that of strokes, ranging from 113 to 204 cases per 100,000 inhabitants [1]. BSI, whether nosocomial or community-acquired, have poor prognosis, with mortality rates up to 40% [1,2]. They are also a leading cause of healthcare-associated infections in intensive care units (ICUs) [3] and neonatal wards [4], and are particularly prevalent in elderly patients (6,000 cases per 100,000 population). In this demographic, mortality rates can be up to 70% [5]. Their extensive impact on overall hospital costs (>$40,000 per patient in the US) [6] make BSI a major public health concern.

Many different bacterial species can cause BSI, among which *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* species, *Pseudomonas aeruginosa*, *enterococci*, *streptococci* and coagulase-negative *staphylococci* are the most prominent [1]. *P. aeruginosa* is mainly associated with nosocomial infections, and bacteremia caused by this pathogen has a poor prognosis [2,7,8] with very high mortality rates [9]. *P. aeruginosa* can survive in many different environments and colonizes plants, animals, and humans [10]. In addition to BSI, it is responsible for a number of life-threatening complications including acute pneumonia and skin infection in immunocompromised and elderly patients, as well as degradation of lung function in chronically-infected cystic fibrosis patients [11]. The major health concerns related to *P. aeruginosa* are linked to intrinsic and acquired resistance to currently available antibiotics [12].

The capacity of *P. aeruginosa* to survive in the human body hinges on a balance between its numerous virulence factors and the presence of multiple host-defense mechanisms. Regardless of the primary site of infection, *P. aeruginosa* can cross the

epithelial and endothelial barriers to reach the bloodstream [13,14]. In the blood, the bacteria encounter the innate immune system, composed essentially of neutrophils, monocytes, and the complement system. Interactions between *P. aeruginosa* and this innate immune system have mainly been studied using selected strains and purified components, such as isolated complement proteins or phagocytes, or serum [15–20], in conditions differing from those found in the human blood [21,22]. Recent data indicated that systemic *P. aeruginosa* infection could lead to pathogen transmission as the bacteria were found to disseminate and propagate through the gallbladder and intestinal tract in a murine model of infection [23]. However, the mechanisms allowing bacteria to persist in the blood remained unclear. Within this study, we examined the behavior of a number of laboratory and recently isolated clinical *P. aeruginosa* strains in a standardized assay using fresh whole blood from healthy donors. Our results showed that, although complement exerts an essential antibacterial activity in the blood, individual bacterial strains display variable levels of tolerance. We evidenced, even for the most sensitive strains, the characteristic biphasic killing curves reminiscent of antibiotic persisters, and characterized a small subpopulation of phenotypic variants that we named complement evaders. These rare cells withstand complement-mediated lysis through phenotypic heterogeneity. Moreover, we discovered that several other major Gram-negative human pathogens shared the same capacity to escape human complement by forming intrinsically plasma-resistant evaders. The complement evaders may have a very significant impact on bacterial dissemination.

Results

Laboratory and clinical strains display diverse survival rates in human blood

Using a highly standardized method in human whole blood (HWB), we examined the survival of six *P. aeruginosa* strains with distinct toxin repertoires and serotypes (Fig 1). These assays included the commonly used laboratory strains PAO1 [24], PA14 [25] and PA7 [26], which belong to distinct phylogenetic lineages/groups . PAO1 and PA14 both possess the type III secretion system (T3SS) which they use to translocate ExoS or ExoU, respectively, into target host cells. PA7 lacks T3SS genes, but encodes the pore-forming toxin Exolysin A [26,27]. In addition to these laboratory strains, we included in the survey three *P. aeruginosa* strains recently isolated from infected patients [27–29]. *E. coli* CF7968, a derivative of the K12 laboratory strain [30] was added as a control (Table S1). Bacteria were incubated for 3 h in HWB from healthy donors, and bacterial survival was assessed in ten independent experiments by counting colony-forming units (CFU) (Fig 1A). Strains showed clearly distinguishable and reproducible survival rates. Approximately 10% of the laboratory and reference strains, PAO1 (ExoS$^+$) and PA7 (ExlA$^+$), survived, whereas only around 1% of the most sensitive strain, PA14 (ExoU$^+$), was still present following the 3-h incubation in HWB. Very different survival rates were measured for the three recent clinical strains, YIK (ExoU$^+$), CLJ1 and IHMA87 (ExlA$^+$), ranging from 0.05% for CLJ1 to complete tolerance for YIK. Only the non-pathogenic laboratory strain *E. coli* CF7968 was completely eliminated, with no detectable CFU after 3 h exposure to HWB. The nature of the strain's virulence factors (T3SS versus ExlA) did not appear to confer any significant benefit for survival, as similar sensitivities were measured for ExlA$^+$ strains

and T3SS$^+$ strains (e.g. IHMA87 versus PA14, or PA7 versus PAO1). In addition, survival in HWB did not correlate with a given serotype, as highly variable survival rates were measured for the three O12 strains (Fig 1B). Lack of the O antigen was detrimental for the bacteria, as illustrated by the hypersensitivity of *E. coli* CF7968.

Intrinsic *P. aeruginosa* tolerance in blood is linked to complement activity, through either MAC insertion or opsonophagocytosis

To explore the origin of the extensive differences in survival measured in HWB, we first determined how well each strain was recognized by immune cells. While YIK induced marginal TNF**α** and IL6 production, all the other strains tested triggered similar high levels of cytokines (Fig S1). The toxins ExoS, ExoU, and Exolysin A are known to induce apoptosis or necrosis in a variety of eukaryotic cells, including white blood cells which may play a role in bacterial clearance from the blood [16,31–34]. We therefore examined the cytotoxic potential of each strain toward circulating leukocytes. YIK induced the highest cell death in both neutrophils and mononuclear cells, whereas most other strains showed similar limited levels of cytotoxic potential with no significant differences (Fig S2). Thus, except for YIK, the extent to which the bacterial strains tested were recognized by and destroyed circulating leukocytes could not explain the different survival rates measured in HWB.

We next assessed the capacity of the strains to cope with the complement system in plasma (Fig 2A). As with bacterial survival in HWB, in plasma the survival rates for the six selected *P. aeruginosa* strains revealed a range of sensitivities from resistance to almost complete eradication. In agreement with previous reports [17,35,36], the two laboratory strains PAO1 and PA14 had contrasting survival patterns: PAO1 was

tolerant, whereas PA14 was sensitive. The strains that were most tolerant to HWB (PA7, PAO1 and YIK) were fully resistant to complement-mediated killing. The similar survival profiles between HWB and plasma, and the fact that survival of the complement-sensitive strains CLJ1, IHMA87, and PA14 was 10-fold lower in plasma than in whole blood suggest an important role for complement in bacterial clearance within HWB. Due to its complete resistance to killing, the YIK strain was excluded from subsequent experiments.

To assess how complement contributes to bacterial elimination in HWB, we examined the survival of the different strains by counting CFUs following incubation in HWB reconstituted after heat-inactivation of the plasma (see Material and Methods). Heat-inactivation, which eradicates complement activity, resulted in full survival of all strains, including the hypersensitive *E. coli* CF7968 (Fig 2B). Indeed, most strains multiplied when incubated in blood lacking functional complement, as observed by the increased CFUs compared to the starting population.

The antimicrobial activity of complement relies on three main mechanisms: i/ bacterial lysis due to the insertion of the membrane attack complex (MAC) into the bacterial envelope [37], ii/ opsonophagocytosis, which combines C3b binding at the pathogen's surface and its recognition by complement receptors [37], and iii/ formation of neutrophil extracellular traps (NETs) to trap and kill invading bacteria [38]. To elucidate which of these mechanisms was involved in bacterial clearance, we treated the blood with Cytochalasin D to inactivate phagocytosis, or with DNase I to prevent NETs formation [22,39] and monitored bacterial survival. Following treatment with DNase I, the same level of bacterial elimination was observed as with untreated HWB (Fig 2C), indicating that NETs play a negligible role in the process observed here. In contrast,

PA7 and PAO1 elimination appear to involve some internalization by phagocytes, as their survival was consistently increased in Cytochalasin D-treated blood (Fig 2C). Because the clearance of these two strains was also complement-dependent, we conclude that they are eliminated through opsonophagocytosis. Blocking phagocytosis had no impact on the other complement-sensitive strains CLJ1, IHMA87, PA14, and E*. coli* CF7968, suggesting that they are killed through direct MAC-induced lysis.

Importantly, even though most of the strains were highly sensitive to complement, we recurrently detected a subpopulation of survivors corresponding to <1% or even 0.002% of the initial inoculum, in HWB and plasma, respectively (Fig 1A and 2A). These results suggest that a small bacterial subpopulation, that we termed “evaders”, differ in phenotype from the majority of the population, and display increased tolerance to complement-mediated killing.

Complement evaders display persister-like features

As indicated above, only the laboratory strain *E. coli* CF7968 was entirely eliminated upon exposure to complement. The subpopulation of *P. aeruginosa* evaders in plasma ranged from 0.1% down to 0.002% of the initial bacterial load, depending on the strain (Fig 2A). We further investigated this intriguing difference in sensitivities using the three complement-sensitive strains PA14, CLJ1, and IHMA87, by carefully examining the kinetics of bactericidal activity in plasma over a 6-h incubation (Fig 3A). A biphasic curve of bacterial killing was observed, with the majority of the sensitive population (> 99.9%) eliminated within 2 h. Following this first phase, killing slowed down, reached a plateau and left a minor subpopulation of surviving cells. However, this subpopulation failed to grow, even after 6 h. We verified that the drop off in killing rate was not due to

204 depletion of complement activity after 2 h by retesting the used plasma. The used
205 plasma was still sufficiently active to kill > 10^7 *P. aeruginosa* PA14 cells during a 1-h
206 incubation (Fig S3A). Thus, the plasma had a residual bactericidal capacity, sufficient
207 to eliminate a population at least 4-log more numerous than the number of evaders.
208 As further evidence that evaders are not simply a result of bacterial overload of the
209 complement system, inoculating HWB with 10-fold fewer bacterial cells resulted in the
210 same proportion of evaders (Fig S3B). Based on these results, evaders correspond to
211 phenotypic variants displaying complement tolerance, and appear to be present in
212 similar proportions to antibiotic-tolerant persisters [40,41].

213 To further phenotypically characterize evaders, we re-cultured the survivors recovered
214 from a first incubation in plasma and challenged their progeny. As shown in Fig 3B,
215 following re-culture, the bacterial population had a similar sensitivity profile to
216 previously unchallenged cells. In some cases, the number of evaders in these repeat
217 challenges was below the limit of detection in our experimental settings (e.g. CLJ1
218 after 2 h). This apparent paradox is a hallmark of antibiotic persisters [42]. Thus,
219 neither evaders nor persisters reflect the emergence of resistant mutants, rather the
220 evader phenotype is transient and reversible.

221 As complement evaders were detected for all sensitive *P. aeruginosa* strains, we
222 tested whether this behavior could be extended to other Gram-negative bacteria. To
223 that aim, we selected strains of seven Gram-negative species (Table S1) and
224 assessed their survival in pooled plasma. Among the strains tested,
225 *Stenotrophomonas maltophilia* was undetectable after 1 h of incubation, and *Serratia*
226 *marcescens* presented what we called a tolerant phenotype in plasma, with a slow but
227 constant elimination rate. In contrast, the five other strains tested – *Acinetobacter*

baumannii, *Burkholderia multivorans*, enteroaggregative (EA) *E. coli* 17-2, *Klebsiella pneumoniae, and Yersinia enterocolitica* – presented a biphasic survival curve similar to the one recorded for *P. aeruginosa* (Fig 3C). For these strains, the proportion of evaders withstanding complement-mediated lysis after 3 h of incubation ranged from 1% to 0.0002% of the initial population. When individual evader colonies were re-cultured, a population as sensitive as the parental one was recovered, as seen for *P. aeruginosa*. Unexpectedly, for *K. pneumoniae*, one evader colony out of the three that were randomly selected gave rise to a resistant population (Fig 3D). Thus, complement-resistant mutants can be selected and we do not currently know whether selection occurs over the course of the pre-challenge culture steps, or during contact with plasma.

Evaders and persisters are distinct subpopulations

To further describe the features of complement evaders, we compared them with antibiotic-tolerant persisters. Antibiotic persisters can correspond to up to 100% of cells in the stationary phase of growth, presumably due to growth arrest [43–45]. Thus, after verifying bacterial growth-rates and states (log versus stationary) (Fig S4), we tested whether the evaders observed in exponentially growing cultures corresponded to residual non-growing cells from the previous overnight culture, or to rare cells that had already entered the stationary state after a few hours of culture. To eliminate possible artefacts due to the growth phases [42,46], we challenged stationary-phase cells or exponentially growing cells with plasma. For the three strains *P. aeruginosa* IHMA87, *A. baumannii* and *Y. enterocolitica*, we observed that the level of evaders was higher in actively growing cultures, while for the other strains their proportions were similar

between the two states (Fig 4A). Therefore, exponentially growing bacteria had a similar or higher capacity to produce evaders, suggesting that the emergence of evaders is unrelated to dormancy before complement challenge. To assess whether metabolic shut-down could increase the proportion of evaders, prior to exposure to plasma, *P. aeruginosa* IHMA87 was treated with the protonophore cyanide m-chlorophenylhydrazone (CCCP), which uncouples oxidative phosphorylation. Exposure to CCCP has been reported to increase the proportion of antibiotic persisters in *P. aeruginosa* by almost 2-log [47]. The addition of CCCP had no effect on bacterial survival in LB (Fig S5), but its use before the plasma challenge completely abolished the detection of evaders from 2 h of incubation (Fig 4B), suggesting that proton-motive force is necessary for survival. To assess more growth parameters of evaders and the possibility that they display a ''slow growth phenotype'', we performed time-lapse microscopy of IHMA87-GFP bacteria on agarose pads after incubation in normal or heat-inactivated plasma (Fig 4C to 4F). Our results show that evaders immediately and actively grow following plasma removal and spotting on agarose pads, forming exponentially-growing microcolonies (Fig 4C). In both normal and heat-inactivated plasma-treated conditions, growth rate was low upon spotting (0.6±0.3 h^{-1}) but stabilized at levels similar to batch cultures (1.2±0.1 h^{-1}) after 2.5 h (Fig 4D). Division was not impaired in evaders as the mean time to reach a first division did not vary between normal (1.08±0.35 h) and heat-inactivated plasma-treated cells (0.98±0.34 h) (Fig 4E and 4F). Altogether, these results suggest that survival to plasma treatment is not dependent on growth rate but requires energy and active metabolism.

BSI isolates show full range of complement resistance and presence of evaders

As we had demonstrated that the central driver of bacterial clearance from blood was the complement system, mainly through its direct lytic activity, we next investigated survival in plasma of a cohort of twelve clinical strains isolated from patients with BSI (Table S1 and S2) to determine their capacity to form evaders. Like the data obtained with the initial six selected strains, BSI isolates displayed differences in survival rates in plasma, of up to five orders of magnitude. Four strains (PaG2, PaG5, PaG6, and PaG10) were tolerant to complement killing, displaying > 50% survival, with some even able to multiply in these conditions (PaG2, PaG6 and PaG10) (Fig 5A). In contrast, for other strains (PaG8, PaG9, PaG14) just < 0.02% of the initial population survived (Fig 5A). The limited number of strains and the high diversity of serotypes identified (Table S1) would make any attempt to correlate bacterial survival in plasma with strain serotype too speculative. Even though some strains were highly sensitive to complement, none were fully eliminated. To verify that these surviving cells corresponded to complement evaders, we also assessed their survival kinetics in plasma (Fig. 5B). The three isolates PaG3, 9, and 17 presented a tolerant phenotype, with a constant rate of elimination, never reaching a plateau even after 4 h incubation. In contrast, a biphasic killing curve was recorded for the five other isolates, the kinetics of the curve varied from strain to strain, sometimes reaching a plateau after just 1 h, whereas for others the death rate started to slow from the 3-h time point. As indicated above, in some cases, surviving cells were scarce and bellow the limit of detection (e.g. PaG8 at 2 and 3 h). Thus, most clinical isolates form evaders which can withstand complement-mediated lysis, suggesting that this phenomenon could be exploited by *P. aeruginosa* to persist within the bloodstream in clinical settings.

Discussion

Bacteremia caused by the multi-drug resistant opportunistic pathogen *P. aeruginosa* presents a particular threat to hospitalized patients. To gain more knowledge on host and pathogen strategies associated with BSI, we undertook an extensive analysis of a cohort of *P. aeruginosa* strains in an *ex vivo* HWB model of infection. Although many previous studies have addressed bacterial transmigration across epithelial and endothelial barriers, the interplay between the immune system and bacterial survival in HWB has been less extensively documented. We found that the nature of *P. aeruginosa* toxins (ExoS, ExoU or ExlA) was unrelated to a strain's capacity to survive in HWB, even though these toxins play important roles in breaching epithelial and endothelial barriers [13,27,31,48,49]. The levels of bacterial survival measured in HWB were highly variable and directly related to the action of the complement system, as previously reported for carbapenem-resistant epidemic clones of *Klebsiella pneumoniae* [50]. NETs did not contribute significantly to bacterial killing. In some cases, PMNs contributed to bacterial elimination through complement-dependent opsonophagocytosis, but this process was less efficient than direct MAC-induced lysis. In line with this observation, Thanabalasuriar and colleagues [51] observed that neutrophils had a limited capacity to phagocytose *P. aeruginosa* in the mouse lung vasculature, in contrast to PMNs recruited to the organ at the site of infection [52]. Complement resistance is the main driver of survival in the bactericidal environment that human blood represents. Nevertheless, we observed that to fully resist the immune system, strains had to display both complement resistance and cytotoxicity toward immune cells, as documented for the highly virulent YIK strain recently isolated from a 49-year-old individual with no known immunodeficiency [29].

A plethora of previously identified bacterial factors could interfere with the complement system in the HWB model. Thus, bacteria could alter C3b binding [19,36,51,53–56], recruit negative complement regulators [18,20,57,58], break down complement proteins [15,59,60], or stabilize outer membrane integrity [61,62]. Consequently, bacterial resistance to complement activity appears to be multifactorial and strain-dependent, and should thus be investigated in a more systematic and uniform way to obtain a better overall picture of the complex interactions involved. Indeed, a very recent Tn-Seq approach performed in parallel on four *Klebsiella pneumoniae* strains revealed a few general, but mainly strain-dependent, factors contributing to complement-resistance [63].

The most prominent result from our study is the evidence that strains highly sensitive to plasma can nevertheless escape complement's bactericidal activity by forming phenotypic variants, or evaders. In some instances, complement evaders represent < 0.01% of the initial population, with survival kinetics reaching a plateau reminiscent of bacterial persisters following antibiotic challenges [42]. As with antibiotic persisters [42], we found that complement evaders do not harbor genetic mutations and that they lose their complement-tolerant phenotype upon elimination of the stress. However, complement evaders differ from antibiotic persisters through several features. First, persisters are more numerous in stationary-phase cultures [45], whereas the fraction of evaders is similar or even higher in exponentially growing cells. Second, we observed no growth defect/delay of these rare cells following removal of the stress. CCCP treatment, which decreases ATP production by membrane-bound ATPases as a result of inhibition of proton motive force was previously shown to increase the number of persister cells by 2-log upon challenge with ciprofloxacin [47]. Here, metabolic shut-down with CCCP prior to the plasma challenge abolished the

emergence of evaders. This result further evidences that complement evaders are not in a dormant state, and that they emerge through an energy-dependent process. Based on these results, we propose that complement evaders share few characteristics with antibiotic persisters to withstand a stress [40,64–67], however the mechanisms leading to the development of these characteristics remain a subject of debate [68].

Interestingly, following serum challenge of uropathogenic *E. coli*, Putrinš and colleagues [69] reported the emergence of complement-resistant stationary-phase persisters. This result suggests that, in addition to complement evaders, bacteria may have developed a number of ways to transiently hide from the immune system when present in the blood. Putrinš and colleagues also identified a subpopulation of non-quiescent cells undergoing rapid division in serum, which could withstand complement-mediated lysis but was sensitive to antibiotics. This population may be the same as the evaders described here. However, Putrinš and colleagues only observed these cells from stationary-phase cultures, as exponentially growing bacteria appeared to be serum-resistant in their experiments.

Host immunity can amplify a pathogen's phenotypic heterogeneity, promoting the formation of antibiotic persisters both *in vivo* and *in vitro*. For example, upon lung infection with *M. tuberculosis*, cell-to-cell variations in ribosomal RNA transcription patterns increased markedly compared to growth-permissive *in vitro* conditions [70]. Bacterial uptake by macrophages was also recently shown to induce persistence in both *Salmonella enterica* and *S. aureus*, in response to the stress conditions encountered during vacuolar internalization [71–73]. Exposure to human serum has also been linked to an increased frequency of antibiotic persisters in *Vibrio vulnificus*

374 [74]. The process involved in the emergence of this population is at least partly
375 mediated by complement activity, as a lower proportion of surviving cells was detected
376 following exposure to heat-inactivated serum. Our results show that human plasma
377 can trigger phenotypic diversity in addition to antibiotic persistence, although we still
378 lack information on whether the evader phenotype emerges spontaneously (are these
379 cells already present in the population prior to the stress?) or in response to a trigger
380 (do they only appear upon contact with complement?) [46]. This minor population of
381 evaders has been ignored so far as it can represent less than 0.01% of the initial
382 population, and because bacterial survival in serum is often monitored at a single time
383 point (usually after a 1-h challenge), rather than examining the kinetics of survival over
384 a long enough period to reach a plateau.

385 Although BSIs were historically considered “dead-ends” for infectious agents, Bachta
386 and colleagues [23] recently reported that once in the blood, a subpopulation of *P.*
387 *aeruginosa* migrates to the gallbladder in mice, where it replicates and can exit the
388 organism through the intestinal tract, causing contamination of cage-mates. In our
389 experiments, not all BSI isolates were complement-tolerant, but most of the sensitive
390 strains could form evaders, making the bacteria potentially transmittable.

391 Numerous bacterial pathogens affecting humans may form an evader population,
392 which could represent a reservoir of complement-resistant cells capable of
393 disseminating and spreading throughout the organism. By elucidating the molecular
394 mechanisms through which complement evaders emerge, notably by performing
395 transcriptomic/proteomic profiling of these populations, we hope to identify ways to
396 diminish the risks of bacteremia caused by various bacterial pathogens.

397

Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. Bacteria were grown in liquid Lysogeny Broth (LB) prepared according to Miller's formulation (0.5% yeast extract, 1% tryptone, 1% NaCl) for >16 h with agitation (300 rpm) at 37 °C, except for *Y. enterocolitica*, which was cultured at 28 °C. If not otherwise specified, the culture was diluted and placed at 300 rpm until the OD_{600nm} reached ~ 1.

Whole blood and plasma killing assays

Heparinized HWB from healthy donors was provided by the French National blood service (EFS, Grenoble, France) and was used within 3 h of collection. Bacteria resuspended in RPMI (Thermo Fisher Scientific, Illkirch, France) were incubated in HWB (90% final blood concentration) at a theoretical multiplicity of infection (MOI) of 5 per phagocyte (monocytes and granulocytes), which corresponded to a final bacterial concentration of $2.25x10^7$ mL^{-1}. The precise value was verified for each experiment by plating the bacteria on LB agar plates at t_0 and counting colony-forming units (CFU) after ~ 15 h incubation at 37 °C. Tubes were incubated for 3 h on a rotating wheel at 37 °C in a 5% CO_2 atmosphere. Following incubation, bacterial survival was determined following serial dilutions in H_2O by colony counting on LB or selective PIA (Pseudomonas Isolation Agar) medium. Bacterial survival was expressed as a percentage (%) of survivors calculated from the CFU number after 3 h (t_{3h}) incubation relative to the CFU measured in the initial inoculum (t_0). To inhibit the potential bactericidal effect of phagocytosis and Neutrophil Extracellular Traps (NETs), Cytochalasin D (10 µM) and DNase I (200 U/mL) (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were applied to the HWB for 30 min at 37 °C prior to the addition of

422 bacteria. In the non-treated condition, RPMI medium without inhibitors was added to
423 the blood.

424 To inactivate complement, HWB was centrifuged for 5 min at 400 g to isolate plasma,
425 which was subsequently heat-inactivated for 30 min at 56 °C. Meanwhile, cells were
426 washed twice with RPMI and pelleted by centrifugation for 5 min at 400 g.
427 Complement-inactivated whole blood was reconstituted by mixing the heat-inactivated
428 plasma with the washed cells. A control condition was also prepared, combining
429 washed cells with untreated plasma.

430 For plasma killing assays, heparinized HWB was centrifuged for 10 min at 1000 g. The
431 supernatant was recovered and filtered through a 0.2-μm membrane prior to storage
432 at -80 °C until needed. Pools of plasma used in experiments were from ten individual
433 donors. After thawing, plasma was systematically filtered once again through a 0.2-μm
434 membrane. Bacteria in PBS supplemented with calcium and magnesium (Thermo
435 Fisher Scientific, Illkirch, France) were incubated in plasma (90% final plasma
436 concentration) at the same concentration as that used for whole blood assays
437 ($2.25x10^7$ CFU/mL). At various time points (see figures), survival was determined by
438 counting colonies on LB or PIA, following serial dilutions in PBS supplemented with
439 calcium and magnesium. As for the HWB assays, the CFU count for the starting
440 inoculum was taken as reference (100% survival) to quantify bacterial killing.

441 **Cyanide m-chlorophenylhydrazone treatment before plasma challenge**

442 CCCP (Sigma-Aldrich, Saint-Quentin-Fallavier, France) solubilized in dimethyl
443 sulfoxide (DMSO) was added to the LB culture once an OD_{600nm} ~ 1 was reached. The
444 final CCCP and DMSO concentrations were 200 μg/mL and 0.5%, respectively.
445 Following 1 h incubation at 37 °C with agitation (300 rpm), bacteria were recovered by

centrifugation for 5 min at 5,000 g. The bacterial pellet was resuspended in PBS supplemented with calcium and magnesium.

Cytokine quantification in whole blood

Cytokine concentrations in whole blood were assessed by flow cytometry. Briefly, bacteria suspended in RPMI were added to HWB ($2.25x10^7$ CFU/mL) and incubated for 3 or 6 h. Plasma was recovered following a 5-min centrifugation step at 400 g and stored at -80 °C until required. Analytes were quantified using the LEGENDplex Human Inflammation Panel 1 (Biolegend, San Diego, USA) according to the supplier's instructions, and samples were analyzed on a FACSCalibur (Becton Dickinson, Pont de Claix, France). For the non-infected control (NI), RPMI was added to the blood.

Bacterial cytotoxicity in whole blood

Heparinized whole blood from healthy donors (100 µL) was infected with bacteria in the same conditions described for the killing assays (MOI 5). After incubation for 3 h at 37 °C on a rotating wheel, CD45VioBright 667 antibody from Miltenyi Biotec (Bergisch Gladbach, Germany) was added. Samples were incubated for 20 min at room temperature, and then diluted 10x with RPMI medium without phenol red but containing penicillin and streptomycin. Flow cytometry was performed on a FACSCalibur, and data were analyzed using FCS Express software (DeNovo software). Leucocytes were first defined based on $CD45^+$ expression to exclude red blood cells. Lymphocyte/Monocyte and Neutrophil gates were then defined according to size and granulometry of events, and quantified. The percentage of lymphocytes/monocytes and neutrophils was determined for each infection condition, and compared to uninfected conditions.

Time-lapse microscopy to assess the regrowth of complement-resilient bacteria

After incubation in normal plasma (or in heat-inactivated plasma for the control condition), bacteria were collected by centrifugation for 5 min at 5,000 g, washed and resuspended in PBS and then spotted on a 2% agarose pad containing LB. After complete absorption of the liquid into the pad, the preparation was sealed under a 0.17-mm glass coverslip using an adhesive plastic frame (Gene Frame 125 µL, Thermo Fisher Scientific, Illkirch, France). Time-lapse microscopy was performed at 37 °C using a 100x oil immersion objective on an inverted microscope (Axio Observer Z1, Zeiss, Germany). Phase-contrast and fluorescent images were recorded every 15 min with a Hamamatsu ORCA-Flash 4.0 digital camera and a Zeiss 38HE filterset. In each experiment performed (two for normal and two for heat-inactivated plasma), eight isolated microcolonies were segmented using MicrobeJ (27572972) to determine their surface, growth rate and time of first division.

Serotyping of BSI isolates

The twelve clinical isolates were serotyped using 16 monovalent antisera directed against *P. aeruginosa* LPS (Bio-Rad, Marnes-la-Coquettes, France), according to the manufacturer's instructions.

Statistical analysis

Statistical tests were performed using SigmaPlot software. To analyze multiple comparisons, a one-way ANOVA or Kruskall-Wallis test were applied, depending on the normality of the data. A Student-Newman-Keuls pairwise comparison was then performed. Mann-Whitney U test or Student's t-test were used to compare two groups, depending of the normality of the data. Where indicated, values were log-transformed to convert initially non-normally-distributed data into a normally-distributed dataset. GraphPad Prism was used to create graphs.

Ethics statement

Medical data from patients infected with *P. aeruginosa* strains were extracted from medical records. No nominative or sensitive personal data were recorded, and the study only involved the reuse of already available data. This study falls within the scope of the French Reference Methodology MR-004 for studies not involving human subjects.

Data Availability Statement

All relevant information are provided in the article and the supporting information files.

Funding

This work was supported by grants from the French National Agency for Research (ANR-15-CE11-0018-01), the Laboratory of Excellence GRAL, funded through the University Grenoble Alpes graduate school (Ecoles Universitaires de Recherche) CBH-EUR-GS (ANR-17-EURE-0003), and the Fondation pour la Recherche Médicale (Team FRM 2017, DEQ20170336705) to I.A. S.P. was awarded a Ph.D. fellowship from the French Ministry of Education and Research, and acknowledges the European Molecular Biology Organization for the short-term fellowship enabling 2-month stay in L. van Melderen's laboratory allowing further characterization of the evader population. Institutional funding support from CNRS, INSERM, CEA, and Grenoble Alpes University is also acknowledged. The funders had no role in study design, data collection or analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Thomas Hindré (University Grenoble Alpes, France), Pierre Marcoux (LETI, CEA Grenoble, France) and Eric Cascales (CNRS, Aix-Marseille Université, France) for gift of *E. coli* CF7968, *K. pneumoniae* NCTC96 and enteroaggregative *E. coli* 17-2, respectively. *P. aeruginosa* strain IHMA879472 was kindly provided by International Health Management Association (IHMA; USA). We are grateful to Peter Panchev for English editing of the initial manuscript, and to Maighread Gallagher-Gambarelli (TWS Editing) for suggestions on language usage for the final manuscript.

Conflict of interest

All authors declare no competing interests.

References

[1] Kern WV, Rieg S. Burden of bacterial bloodstream infection—a brief update on epidemiology and significance of multidrug-resistant pathogens. Clin Microbiol Infect 2020;26:151–7. https://doi.org/10.1016/j.cmi.2019.10.031.

[2] Kang C, Kim S, Kim H, Park S, Choe Y, Oh M, et al. Pseudomonas aeruginosa Bacteremia: Risk Factors for Mortality and Influence of Delayed Receipt of Effective Antimicrobial Therapy on Clinical Outcome. Clin Infect Dis 2003;37:745–51. https://doi.org/10.1086/377200.

[3] Vincent J-L, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, et al. International study of the prevalence and outcomes of infection in intensive care units. JAMA 2009;302:2323–9. https://doi.org/10.1001/jama.2009.1754.

[4] Wójkowska-Mach, Chmielarczyk, Strus, Lauterbach, Heczko. Neonate Bloodstream Infections in Organization for Economic Cooperation and Development Countries: An Update on Epidemiology and Prevention. J Clin Med 2019;8:1750. https://doi.org/10.3390/jcm8101750.

[5] Leibovici-Weissman Y, Tau N, Yahav D. Bloodstream infections in the elderly: what is the real goal? Aging Clin Exp Res 2019;In press. https://doi.org/10.1007/s40520-019-01337-w.

[6] Kaye KS, Marchaim D, Chen T-Y, Baures T, Anderson DJ, Choi Y, et al. Effect of Nosocomial Bloodstream Infections on Mortality, Length of Stay, and Hospital Costs in Older Adults. J Am Geriatr Soc 2014;62:306–11. https://doi.org/10.1111/jgs.12634.

[7] Hattemer A, Hauser A, Diaz M, Scheetz M, Shah N, Allen JP, et al. Bacterial and Clinical Characteristics of Health Care- and Community-Acquired Bloodstream Infections Due to Pseudomonas aeruginosa. Antimicrob Agents Chemother 2013;57:3969–75. https://doi.org/10.1128/AAC.02467-12.

[8] Guillamet CV, Vazquez R, Noe J, Micek ST, Kollef MH. A cohort study of bacteremic pneumonia: The importance of antibiotic resistance and appropriate initial therapy? Medicine (Baltimore) 2016;95:e4708. https://doi.org/10.1097/MD.0000000000004708.

[9] Thaden JT, Park LP, Maskarinec SA, Ruffin F, Fowler VG, van Duin D. Results from a 13-Year Prospective Cohort Study Show Increased Mortality Associated with Bloodstream Infections Caused by Pseudomonas aeruginosa Compared to Other Bacteria. Antimicrob Agents Chemother 2017;61. https://doi.org/10.1128/AAC.02671-16.

[10] Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, et al. The environmental occurrence of Pseudomonas aeruginosa. APMIS Acta Pathol Microbiol Immunol Scand 2020;128:220–31. https://doi.org/10.1111/apm.13010.

[11] Williams BJ, Dehnbostel J, Blackwell TS. Pseudomonas aeruginosa: Host defence in lung diseases: P. aeruginosa: host defence in the lung. Respirology 2010;15:1037–56. https://doi.org/10.1111/j.1440-1843.2010.01819.x.

[12] Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018;18:318–27. https://doi.org/10.1016/S1473-3099(17)30753-3.

[13] Rangel SM, Diaz MH, Knoten CA, Zhang A, Hauser AR. The Role of ExoS in Dissemination of Pseudomonas aeruginosa during Pneumonia. PLoS Pathog 2015;11:e1004945. https://doi.org/10.1371/journal.ppat.1004945.

[14] Golovkine G, Faudry E, Bouillot S, Elsen S, Attrée I, Huber P. Pseudomonas aeruginosa Transmigrates at Epithelial Cell-Cell Junctions, Exploiting Sites of Cell Division and Senescent Cell Extrusion. PLoS Pathog 2016;12:e1005377. https://doi.org/10.1371/journal.ppat.1005377.

[15] Hong Y, Ghebrehiwet B. Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol 1992;62:133–8. https://doi.org/10.1016/0090-1229(92)90065-V.

[16] Dacheux D, Attree I, Schneider C, Toussaint B. Cell death of human polymorphonuclear neutrophils induced by a Pseudomonas aeruginosa cystic fibrosis isolate requires a functional type III secretion system. Infect Immun 1999;67:6164–7.

[17] Priebe GP, Dean CR, Zaidi T, Meluleni GJ, Coleman FT, Coutinho YS, et al. The galU Gene of Pseudomonas aeruginosa Is Required for Corneal Infection and Efficient Systemic Spread following Pneumonia but Not for Infection Confined to the

590 Lung. Infect Immun 2004;72:4224–32. https://doi.org/10.1128/IAI.72.7.4224-
591 4232.2004.

592 [18] Kunert A, Losse J, Gruszin C, Hühn M, Kaendler K, Mikkat S, et al. Immune
593 Evasion of the Human Pathogen *Pseudomonas aeruginosa* : Elongation Factor Tuf Is
594 a Factor H and Plasminogen Binding Protein. J Immunol 2007;179:2979–88.
595 https://doi.org/10.4049/jimmunol.179.5.2979.

596 [19] Mishra M, Byrd MS, Sergeant S, Azad AK, Parsek MR, McPhail L, et al.
597 Pseudomonas aeruginosa Psl polysaccharide reduces neutrophil phagocytosis and
598 the oxidative response by limiting complement-mediated opsonization: Psl and the
599 innate immune response towards P. aeruginosa. Cell Microbiol 2012;14:95–106.
600 https://doi.org/10.1111/j.1462-5822.2011.01704.x.

601 [20] Hallström T, Uhde M, Singh B, Skerka C, Riesbeck K, Zipfel PF. Pseudomonas
602 aeruginosa Uses Dihydrolipoamide Dehydrogenase (Lpd) to Bind to the Human
603 Terminal Pathway Regulators Vitronectin and Clusterin to Inhibit Terminal Pathway
604 Complement Attack. PLOS ONE 2015;10:e0137630.
605 https://doi.org/10.1371/journal.pone.0137630.

606 [21] Brodin P, Davis MM. Human immune system variation. Nat Rev Immunol
607 2017;17:21–9. https://doi.org/10.1038/nri.2016.125.

608 [22] van der Maten E, de Jonge MI, de Groot R, van der Flier M, Langereis JD. A
609 versatile assay to determine bacterial and host factors contributing to
610 opsonophagocytotic killing in hirudin-anticoagulated whole blood. Sci Rep
611 2017;7:42137. https://doi.org/10.1038/srep42137.

612 [23] Bachta KER, Allen JP, Cheung BH, Chiu C-H, Hauser AR. Systemic infection
613 facilitates transmission of Pseudomonas aeruginosa in mice. Nat Commun
614 2020;11:543. https://doi.org/10.1038/s41467-020-14363-4.

615 [24] Holloway BW. Genetic Recombination in Pseudomonas aeruginosa.
616 Microbiology 1955;13:572–81. https://doi.org/10.1099/00221287-13-3-572.

617 [25] Rahme L, Stevens E, Wolfort S, Shao J, Tompkins R, Ausubel F. Common
618 virulence factors for bacterial pathogenicity in plants and animals. Science
619 1995;268:1899–902. https://doi.org/10.1126/science.7604262.

620 [26] Roy PH, Tetu SG, Larouche A, Elbourne L, Tremblay S, Ren Q, et al. Complete
621 genome sequence of the multiresistant taxonomic outlier Pseudomonas aeruginosa
622 PA7. PloS One 2010;5:e8842. https://doi.org/10.1371/journal.pone.0008842.

623 [27] Elsen S, Huber P, Bouillot S, Couté Y, Fournier P, Dubois Y, et al. A Type III
624 Secretion Negative Clinical Strain of Pseudomonas aeruginosa Employs a Two-
625 Partner Secreted Exolysin to Induce Hemorrhagic Pneumonia. Cell Host Microbe
626 2014;15:164–76. https://doi.org/10.1016/j.chom.2014.01.003.

627 [28] Kos VN, Déraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, et al. The
628 Resistome of Pseudomonas aeruginosa in Relationship to Phenotypic Susceptibility.
629 Antimicrob Agents Chemother 2015;59:427–36. https://doi.org/10.1128/AAC.03954-
630 14.

631 [29] Elabbadi A, Pont S, Verdet C, Plésiat P, Cretin F, Voiriot G, et al. An unusual
632 community-acquired invasive and multi systemic infection due to ExoU-harboring

633 Pseudomonas aeruginosa strain: Clinical disease and microbiological characteristics.
634 J Microbiol Immunol Infect 2019;In press. https://doi.org/10.1016/j.jmii.2019.06.008.

635 [30] Hirsch M, Elliott T. Role of ppGpp in rpoS Stationary-Phase Regulation in
636 Escherichia coli. J Bacteriol 2002;184:5077–87.
637 https://doi.org/10.1128/JB.184.18.5077-5087.2002.

638 [31] Hauser AR, Engel JN. Pseudomonas aeruginosa induces type-III-secretion-
639 mediated apoptosis of macrophages and epithelial cells. Infect Immun 1999;67:5530–
640 7.

641 [32] Diaz MH, Hauser AR. Pseudomonas aeruginosa cytotoxin ExoU is injected into
642 phagocytic cells during acute pneumonia. Infect Immun 2010;78:1447–56.
643 https://doi.org/10.1128/IAI.01134-09.

644 [33] Rangel SM, Logan LK, Hauser AR. The ADP-ribosyltransferase domain of the
645 effector protein ExoS inhibits phagocytosis of Pseudomonas aeruginosa during
646 pneumonia. MBio 2014;5:e01080-01014. https://doi.org/10.1128/mBio.01080-14.

647 [34] Reboud E, Elsen S, Bouillot S, Golovkine G, Basso P, Jeannot K, et al.
648 Phenotype and toxicity of the recently discovered exlA-positive Pseudomonas
649 aeruginosa strains collected worldwide: Virulence of exlA+ strains. Environ Microbiol
650 2016;18:3425–39. https://doi.org/10.1111/1462-2920.13262.

651 [35] Skurnik D, Roux D, Cattoir V, Danilchanka O, Lu X, Yoder-Himes DR, et al.
652 Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas
653 aeruginosa revealed through high-throughput sequencing. Proc Natl Acad Sci
654 2013;110:20747–52. https://doi.org/10.1073/pnas.1221552110.

655 [36] Martínez-Ramos I, Mulet X, Moyá B, Barbier M, Oliver A, Albertí S.
656 Overexpression of MexCD-OprJ Reduces Pseudomonas aeruginosa Virulence by
657 Increasing Its Susceptibility to Complement-Mediated Killing. Antimicrob Agents
658 Chemother 2014;58:2426–9. https://doi.org/10.1128/AAC.02012-13.

659 [37] Heesterbeek DAC, Angelier ML, Harrison RA, Rooijakkers SHM. Complement
660 and Bacterial Infections: From Molecular Mechanisms to Therapeutic Applications. J
661 Innate Immun 2018;10:455–64. https://doi.org/10.1159/000491439.

662 [38] de Bont CM, Boelens WC, Pruijn GJM. NETosis, complement, and coagulation:
663 a triangular relationship. Cell Mol Immunol 2019;16:19–27.
664 https://doi.org/10.1038/s41423-018-0024-0.

665 [39] Brinkmann V, Reichard U, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, et
666 al. Neutrophil Extracellular Traps Kill Bacteria. Science 2004;303:1532–5.
667 https://doi.org/10.1126/science.1092385.

668 [40] Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a
669 phenotypic switch. Science 2004;305:1622–5.
670 https://doi.org/10.1126/science.1099390.

671 [41] Fisher RA, Gollan B, Helaine S. Persistent bacterial infections and persister
672 cells. Nat Rev Microbiol 2017;15:453–64. https://doi.org/10.1038/nrmicro.2017.42.

673 [42] Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, et
674 al. Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol

2019;17:441–8. https://doi.org/10.1038/s41579-019-0196-3.

[43] Spoering AL, Lewis K. Biofilms and Planktonic Cells of Pseudomonas aeruginosa Have Similar Resistance to Killing by Antimicrobials. J Bacteriol 2001;183:6746–51. https://doi.org/10.1128/JB.183.23.6746-6751.2001.

[44] Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 2004;230:13–8. https://doi.org/10.1016/S0378-1097(03)00856-5.

[45] Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, et al. Persister formation in Staphylococcus aureus is associated with ATP depletion. Nat Microbiol 2016;1:16051. https://doi.org/10.1038/nmicrobiol.2016.51.

[46] Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 2016;14:320–30. https://doi.org/10.1038/nrmicro.2016.34.

[47] Grassi L, Di Luca M, Maisetta G, Rinaldi AC, Esin S, Trampuz A, et al. Generation of Persister Cells of Pseudomonas aeruginosa and Staphylococcus aureus by Chemical Treatment and Evaluation of Their Susceptibility to Membrane-Targeting Agents. Front Microbiol 2017;8:1917. https://doi.org/10.3389/fmicb.2017.01917.

[48] Lee VT, Smith RS, Tummler B, Lory S. Activities of Pseudomonas aeruginosa Effectors Secreted by the Type III Secretion System In Vitro and during Infection. Infect Immun 2005;73:1695–705. https://doi.org/10.1128/IAI.73.3.1695-1705.2005.

[49] Bouillot S, Munro P, Gallet B, Reboud E, Cretin F, Golovkine G, et al. Pseudomonas aeruginosa Exolysin promotes bacterial growth in lungs, alveolar damage and bacterial dissemination. Sci Rep 2017;7:2120. https://doi.org/10.1038/s41598-017-02349-0.

[50] DeLeo FR, Kobayashi SD, Porter AR, Freedman B, Dorward DW, Chen L, et al. Survival of Carbapenem-Resistant Klebsiella pneumoniae Sequence Type 258 in Human Blood. Antimicrob Agents Chemother 2017;61. https://doi.org/10.1128/AAC.02533-16.

[51] Thanabalasuriar A, Surewaard BGJ, Willson ME, Neupane AS, Stover CK, Warrener P, et al. Bispecific antibody targets multiple Pseudomonas aeruginosa evasion mechanisms in the lung vasculature. J Clin Invest 2017;127:2249–61. https://doi.org/10.1172/JCI89652.

[52] Koh AY, Priebe GP, Ray C, Van Rooijen N, Pier GB. Inescapable Need for Neutrophils as Mediators of Cellular Innate Immunity to Acute Pseudomonas aeruginosa Pneumonia. Infect Immun 2009;77:5300–10. https://doi.org/10.1128/IAI.00501-09.

[53] Qadi M, Izquierdo-Rabassa S, Mateu Borrás M, Doménech-Sánchez A, Juan C, Goldberg JB, et al. Sensing Mg2+ contributes to the resistance of Pseudomonas aeruginosa to complement-mediated opsonophagocytosis: Mg 2+ -dependent opsonophagocytosis. Environ Microbiol 2017;19:4278–86. https://doi.org/10.1111/1462-2920.13889.

[54] Pier G. Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. Int J Med Microbiol

718 2007;297:277–95. https://doi.org/10.1016/j.ijmm.2007.03.012.

719 [55] Mishra M, Ressler A, Schlesinger LS, Wozniak DJ. Identification of OprF as a
720 complement component C3 binding acceptor molecule on the surface of
721 Pseudomonas aeruginosa. Infect Immun 2015;83:3006–14.
722 https://doi.org/10.1128/IAI.00081-15.

723 [56] Jones CJ, Wozniak DJ. Psl Produced by Mucoid Pseudomonas aeruginosa
724 Contributes to the Establishment of Biofilms and Immune Evasion. MBio 2017;8.
725 https://doi.org/10.1128/mBio.00864-17.

726 [57] Hallström T, Mörgelin M, Barthel D, Raguse M, Kunert A, Hoffmann R, et al.
727 Dihydrolipoamide Dehydrogenase of *Pseudomonas aeruginosa* Is a Surface-Exposed
728 Immune Evasion Protein That Binds Three Members of the Factor H Family and
729 Plasminogen. J Immunol 2012;189:4939–50.
730 https://doi.org/10.4049/jimmunol.1200386.

731 [58] Paulsson M, Singh B, Al-Jubair T, Su Y-C, Høiby N, Riesbeck K. Identification
732 of outer membrane Porin D as a vitronectin-binding factor in cystic fibrosis clinical
733 isolates of Pseudomonas aeruginosa. J Cyst Fibros 2015;14:600–7.
734 https://doi.org/10.1016/j.jcf.2015.05.005.

735 [59] Laarman AJ, Bardoel BW, Ruyken M, Fernie J, Milder FJ, van Strijp JAG, et al.
736 Pseudomonas aeruginosa Alkaline Protease Blocks Complement Activation via the
737 Classical and Lectin Pathways. J Immunol 2012;188:386–93.
738 https://doi.org/10.4049/jimmunol.1102162.

739 [60] Bastaert F, Kheir S, Saint-Criq V, Villeret B, Dang PM-C, El-Benna J, et al.
740 Pseudomonas aeruginosa LasB Subverts Alveolar Macrophage Activity by Interfering
741 With Bacterial Killing Through Downregulation of Innate Immune Defense, Reactive
742 Oxygen Species Generation, and Complement Activation. Front Immunol
743 2018;9:1675. https://doi.org/10.3389/fimmu.2018.01675.

744 [61] Munguia J, LaRock DL, Tsunemoto H, Olson J, Cornax I, Pogliano J, et al. The
745 Mla pathway is critical for Pseudomonas aeruginosa resistance to outer membrane
746 permeabilization and host innate immune clearance. J Mol Med Berl Ger
747 2017;95:1127–36. https://doi.org/10.1007/s00109-017-1579-4.

748 [62] Sanchez-Larrayoz AF, Elhosseiny NM, Chevrette MG, Fu Y, Giunta P,
749 Spallanzani RG, et al. Complexity of Complement Resistance Factors Expressed by
750 Acinetobacter baumannii Needed for Survival in Human Serum. J Immunol Baltim Md
751 1950 2017;199:2803–14. https://doi.org/10.4049/jimmunol.1700877.

752 [63] Short FL, Di Sario G, Reichmann NT, Kleanthous C, Parkhill J, Taylor PW.
753 Genomic profiling reveals distinct routes to complement resistance in Klebsiella
754 pneumoniae. Infect Immun 2020. https://doi.org/10.1128/IAI.00043-20.

755 [64] Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. The rate of killing of
756 Escherichia coli by beta-lactam antibiotics is strictly proportional to the rate of bacterial
757 growth. J Gen Microbiol 1986;132:1297–304. https://doi.org/10.1099/00221287-132-
758 5-1297.

759 [65] Lewis K. Persister cells and the riddle of biofilm survival. Biochem Biokhimiia
760 2005;70:267–74. https://doi.org/10.1007/s10541-005-0111-6.

[66] Dhar N, McKinney JD. Microbial phenotypic heterogeneity and antibiotic tolerance. Curr Opin Microbiol 2007;10:30–8. https://doi.org/10.1016/j.mib.2006.12.007.

[67] Pontes MH, Groisman EA. Slow growth determines nonheritable antibiotic resistance in Salmonella enterica. Sci Signal 2019;12. https://doi.org/10.1126/scisignal.aax3938.

[68] Goormaghtigh F, Van Melderen L. Single-cell imaging and characterization of Escherichia coli persister cells to ofloxacin in exponential cultures. Sci Adv 2019;5:eaav9462. https://doi.org/10.1126/sciadv.aav9462.

[69] Putrinš M, Kogermann K, Lukk E, Lippus M, Varik V, Tenson T. Phenotypic heterogeneity enables uropathogenic Escherichia coli to evade killing by antibiotics and serum complement. Infect Immun 2015;83:1056–67. https://doi.org/10.1128/IAI.02725-14.

[70] Manina G, Dhar N, McKinney JD. Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell Host Microbe 2015;17:32–46. https://doi.org/10.1016/j.chom.2014.11.016.

[71] Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science 2014;343:204–8. https://doi.org/10.1126/science.1244705.

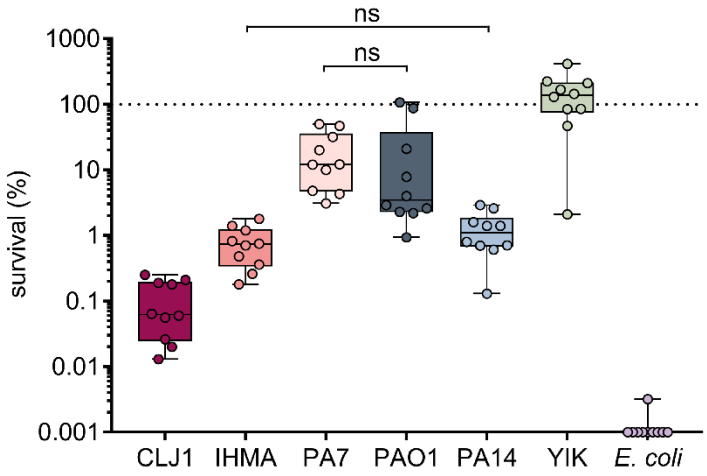
[72] Stapels DAC, Hill PWS, Westermann AJ, Fisher RA, Thurston TL, Saliba A-E, et al. Salmonella persisters undermine host immune defenses during antibiotic treatment. Science 2018;362:1156–60. https://doi.org/10.1126/science.aat7148.

[73] Peyrusson F, Varet H, Nguyen TK, Legendre R, Sismeiro O, Coppée J-Y, et al. Intracellular Staphylococcus aureus persisters upon antibiotic exposure. Nat Commun 2020;11:2200. https://doi.org/10.1038/s41467-020-15966-7.

[74] Ayrapetyan M, Williams TC, Baxter R, Oliver JD. Viable but Nonculturable and Persister Cells Coexist Stochastically and Are Induced by Human Serum. Infect Immun 2015;83:4194–203. https://doi.org/10.1128/IAI.00404-15.

Figures

A



B

Strain	T3SS		ExlA	LPS
	ExoS	ExoU		
PAO1	+	-	-	O5
PA14	-	+	-	O10
YIK	-	+	-	O8
CLJ1	-		+	O12
IHMA	-		+	O11+O12
PA7	-		+	O12
E. coli	-		-	-

Fig1. *P. aeruginosa* strains display various rates of resilience in the HWB model. **(A)** *P. aeruginosa* survival in blood is independent of strain origin or the secreted toxin profile (ExoU, ExoS, or ExlA). Exponentially-growing bacteria were incubated for 3 h in blood obtained from 10 different healthy donors (n=10), and bacterial survival was determined by serial dilutions and CFU counting. ns: non-significant. When not stated, differences in survival were significant. Kruskal-Wallis test, $p < 0.001$; Student-Newman-Keuls post-hoc test: $p < 0.05$**. (B)** Nature of the toxins secreted by each strain, and serotypes of these bacteria. Note that the IHMA87 strain cross-reacts with the two antisera O11 and O12, while *E. coli* CF7968 lacks O antigens.

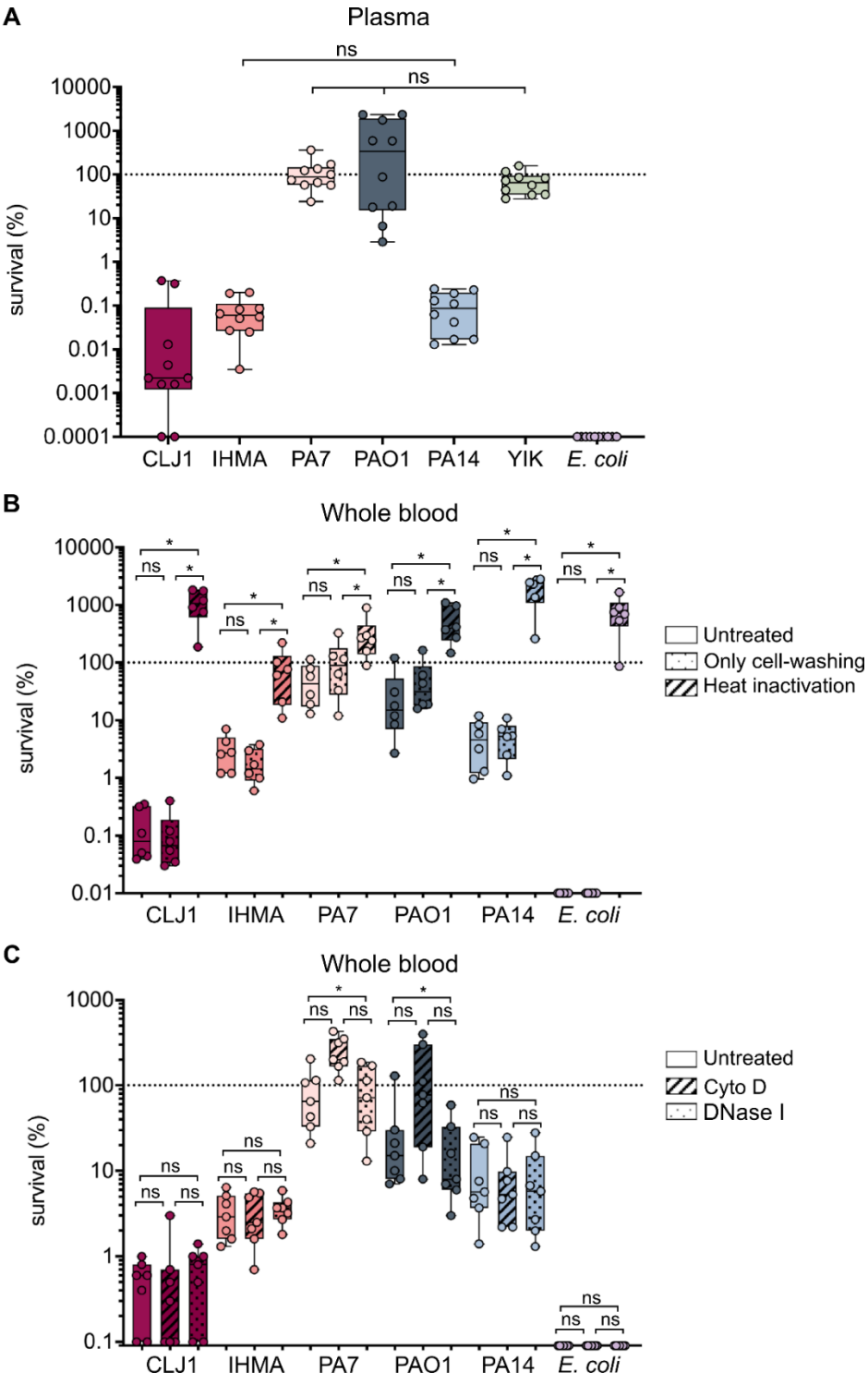


Fig2. Complement is the main immune factor involved in bacterial clearance in HWB. (A) Survival of *P. aeruginosa* strains in human plasma. Strains were incubated in plasma from different healthy donors (n=10), and survival was measured based on CFU counts. ns: non-significant. When not stated, differences in survival were

819 significant. Kruskal-Wallis test, p <0.001; Student-Newman-Keuls post-hoc test: p
820 <0.05. Note similarities in survival profiles to those shown in Figure 1. **(B)** Heat-treating
821 plasma prevents elimination of bacteria from HWB. Bacteria were incubated for 3 h in
822 HWB from different donors (n=6) with (hashed) and without (solid) heat-treatment to
823 inactivate complement, or only with washed blood cells without plasma heating
824 (dotted). The effect of this treatment on bacterial survival was assessed based on CFU
825 counts. **(C)** Phagocytes are involved in the elimination of a limited number of strains.
826 Bacteria were incubated for 3 h in HWB from different donors (n=7) in the absence
827 (solid) or presence of Cytochalasin D (hashed) or DNase I (dotted), to monitor the
828 impact of these treatments on strain survival. **(B)** and **(C)**: Kruskal-Wallis test, p <0.05;
829 Student-Newman-Keuls post-hoc test: * p <0.05.

830

831

832

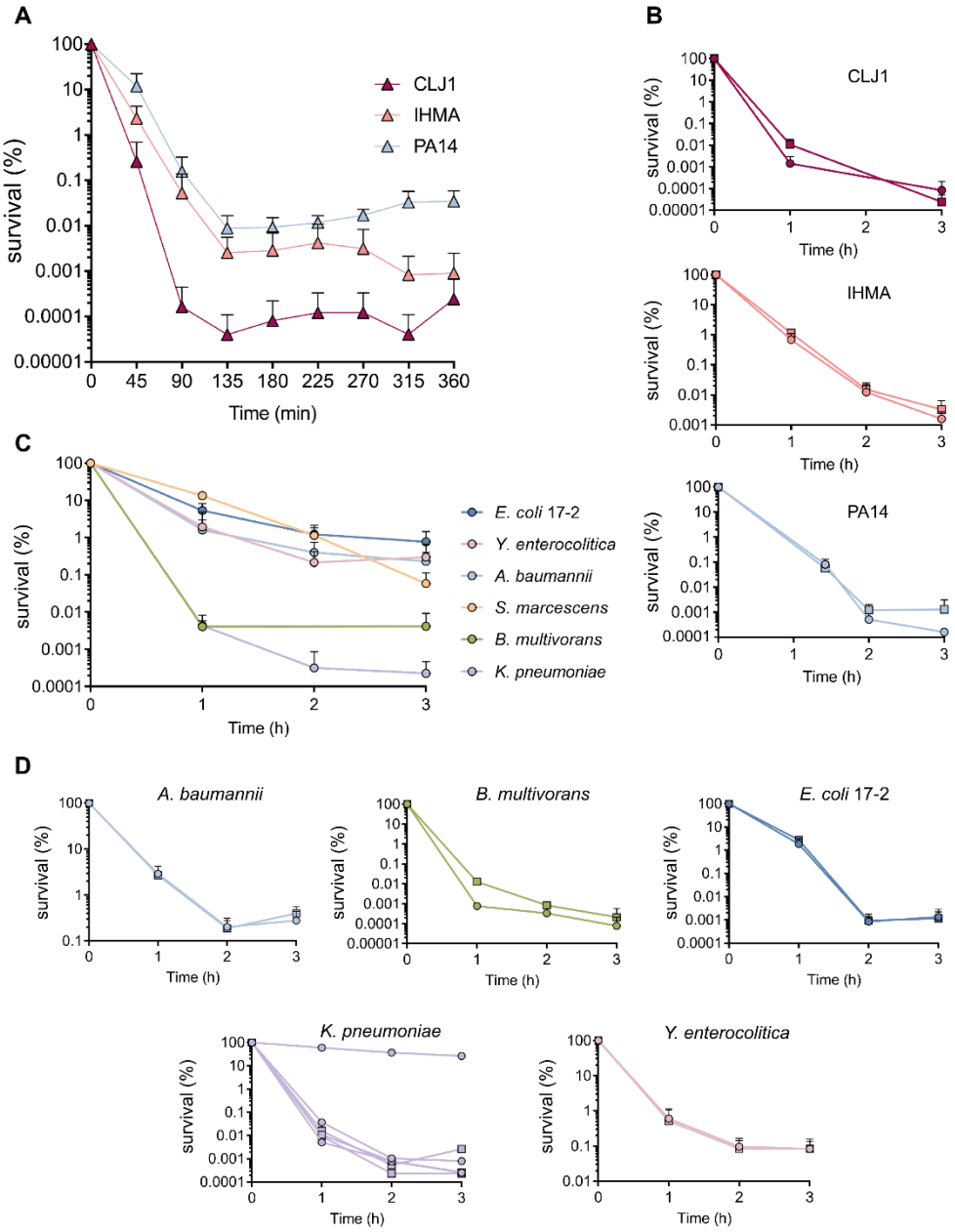
833

834

835

836

837



838

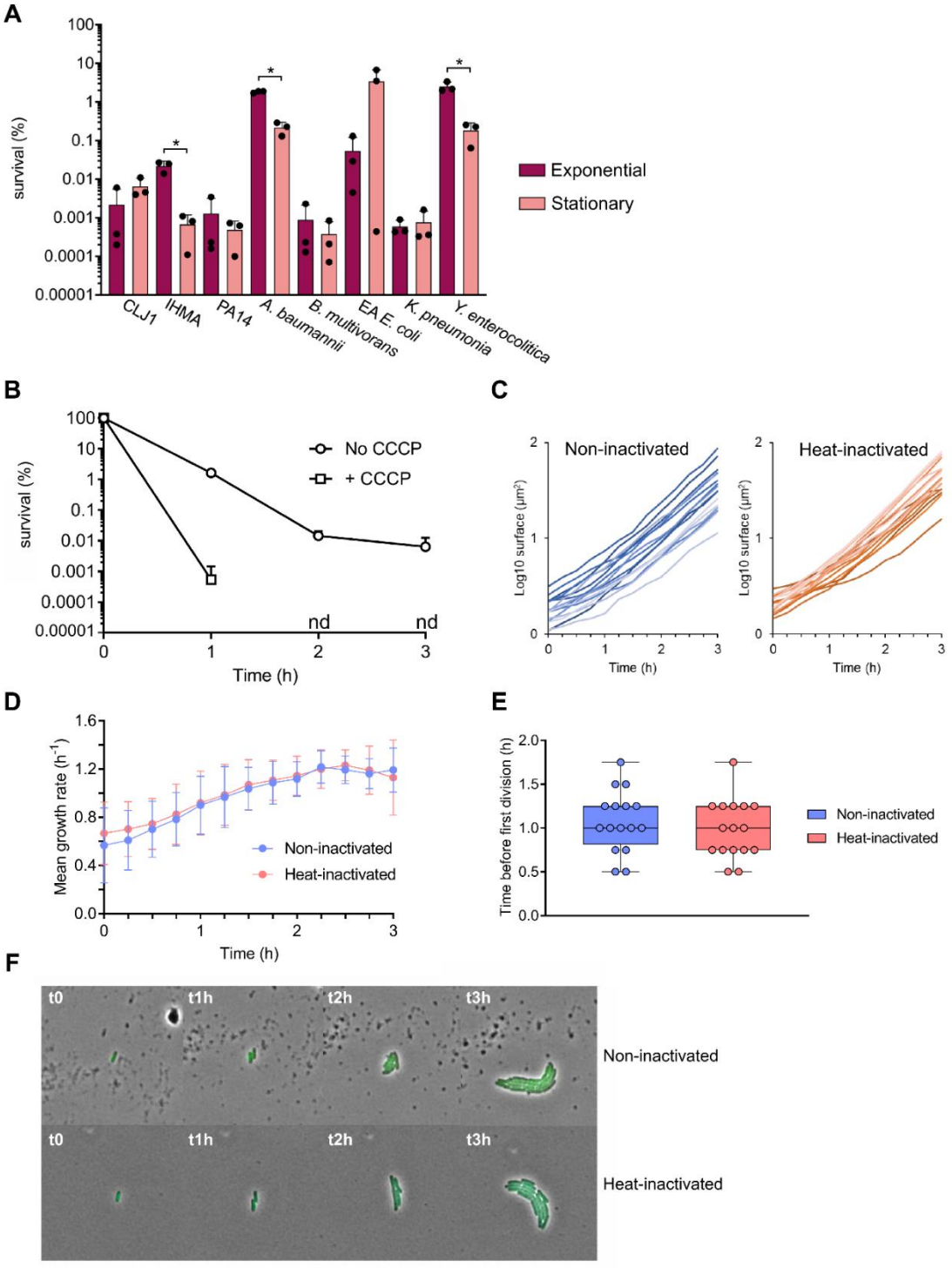
839 **Fig3. Evaders are rare, complement-resistant, phenotypic variants. (A)** Prolonged
840 exposure of *P. aeruginosa* to plasma results in killing curves reminiscent of those that
841 characterize persister formation. The three complement-sensitive strains PA14, CLJ1
842 and IHMA87 were incubated for 6 h in a pool of human plasma, and their survival was
843 measured every 45 min to determine the kinetics of their survival. **(C)** Evaders are a
844 common feature among Gram-negative bacteria. Various Gram-negative species
845 including *A. baumannii*, *B. multivorans*, enteroaggregative *E. coli*, *K. pneumoniae*, and

846 *Y. enterocolitica* were incubated in pooled human plasma for 3 h, and their survival
847 was assessed hourly. **(B)** and **(D)** The evader phenotype is reversible and not the
848 result of fixed mutations. Following a first challenge, a single “evader” colony was
849 recultured to assess the survival of its progeny (circle) in pooled plasma during 3 h.
850 Survival was compared to that of a population that was never exposed to complement
851 (square). Data represent mean ± SD of three independent experiments **(A** to **D)**. Note
852 that for *K. pneumoniae* in **(D)**, the three experimental points were not pooled as one
853 resistant mutant was isolated, presenting a survival profile different from the two other
854 evader clones.

855

856

857



858

859 **Fig4. Complement evaders differ from antibiotic persisters. (A)** Cells in the
860 stationary phase do not form more evaders than exponentially growing bacteria.
861 Bacteria from stationary phase and exponentially growing cultures from every evader-
862 forming strain were challenged in a pool of plasma for 3 h to compare their ability to

form evaders. Student's t-test: * p <0.01, performed on log-transformed values. **(B)** Formation of evaders requires active metabolism. Survival kinetics of IHMA87 in a pool of plasma, following 1-h treatment with the protonophore CCCP in LB, or not. nd: non-detected. **(C) to (E)** Following stress removal, evaders show no growth defect. After 3 h incubation of IHMA87-GFP in pooled plasma following heat-inactivation or not, surviving cells were recovered and spotted on a 2% agarose pad containing LB, allowing the bacteria to regrow. Using time-lapse microscopy, individual microcolony surface **(C)**, mean growth rate **(D)** and elapsed time before the first division **(E)** were determined. From two independent experiments, 16 evader cells were analyzed and compared with 16 non-evader cells from the control condition (heat-inactivated plasma). **(F)** Time-lapse microscopy of two representative microcolonies from each conditions. Data represent mean ± SD of three independent experiments **(A** and **B)**.

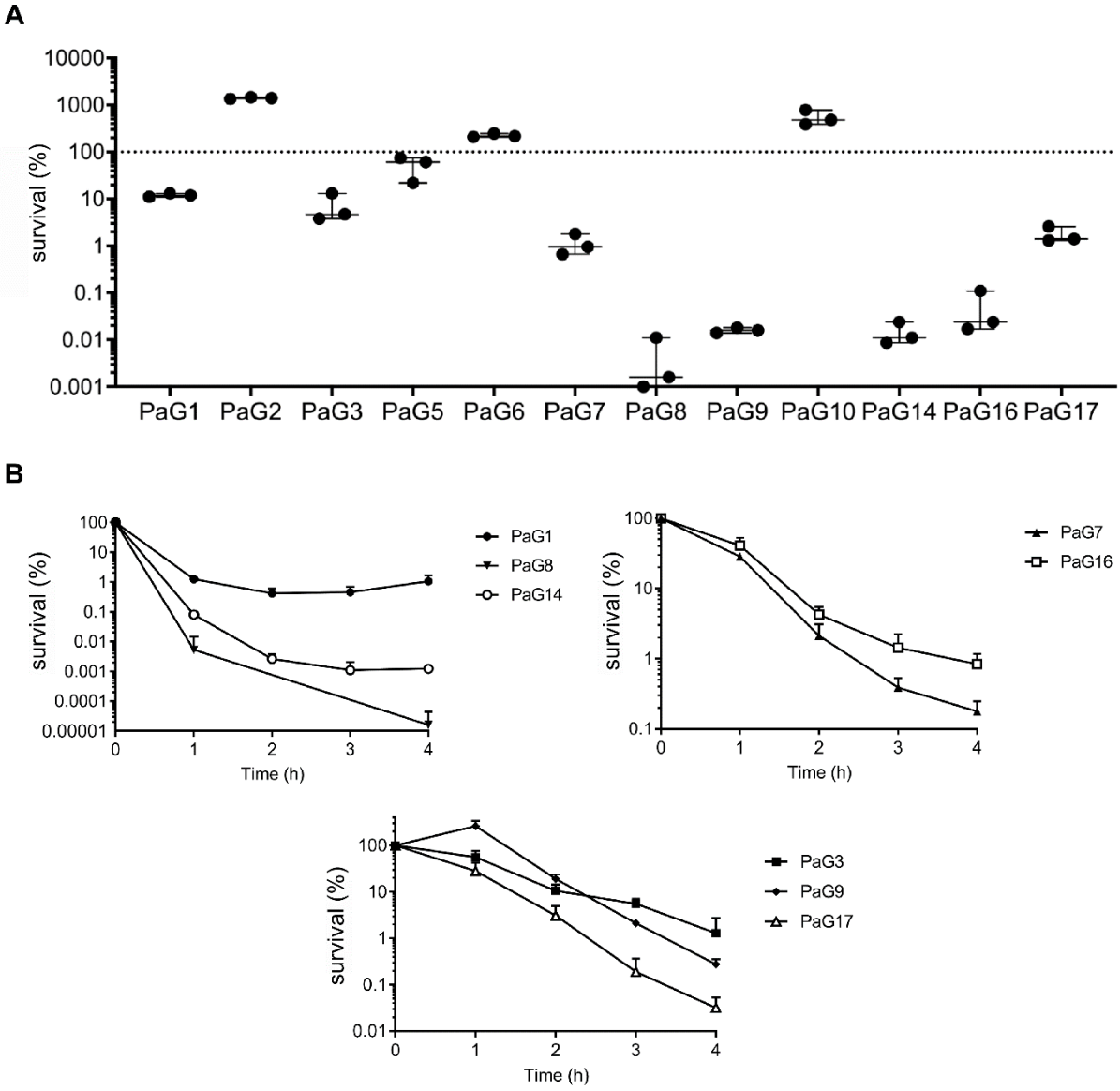


Fig5. BSI isolates avoid elimination mainly through complement evaders. (A) Complement-resistance is not common to all BSI isolates. Twelve *P. aeruginosa* isolates from bloodstream infections were incubated in pooled plasma for 3 h, and their survival was assessed at the end of the incubation. **(B)** Most complement-sensitive isolates form evaders. Kinetics of survival in pooled plasma for 3 h of eight complement-sensitive clinical isolates. Three different panels are used for clarity and to allow better visualization of the inflection points. Data represent mean ± SD of three independent experiments (**A** and **B**).