Antibiotic persister cells in Acinetobacter baumannii : overview of molecular mechanisms and removal strategies

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February 14, 2025

Abstract

Acinetobacter baumannii is a bacterium classified as priority 1 by the World Health Organization due to the rapid development of antibiotic resistance leading to many therapeutic failures. However, the existence of dormant bacterial subpopulations, like persister cells, may also partly explain these therapeutic failures. Persister cells are a bacterial subpopulation that may survive to high concentrations of antibiotics compared to isogenic individuals. This temporary and reversible phenotype may eventually lead to the development of new antibiotic resistance or to the recurrence of infection. Persister cells are often studied, but the mechanisms involved in their selection / formation are complex and often poorly described (toxin/antitoxin; second messengers; SOS response; phenyl acetic catabolic pathway; membranes). The mechanisms involved in the selection of persister cells are complex, and some appear to be species- or strain-specific. For example, some systems, such as antitoxin toxin, are described by others as directly involved in the formation of these cells. In fact, *A. baumannii* presents a great diversity of systems of antitoxin toxin. This review focuses on current advances in knowledge concerning the molecular mechanisms involved in the physiology of persister cells in *A. baumannii*, and the eradication strategies developed to combat this dormant subpopulation.

1. Introduction

Acinetobacter baumannii is a pathogen responsible for infections that are extremely problematic in hospitals. It has no typical virulence factors such as toxins to explain its pathogenicity [1]. Therefore, this pathogen has been referred to have a "persist and resist" virulence strategy [2]. This means that it is capable of surviving in very hostile environments [3]. More particularly, this bacterium is well known for its resistance to desiccation, its ability to form a biofilm to protect itself from external stresses and also its capacity to develop easily antibiotic resistances, notably to the carbapenem class [4], [5], [6]. The difficulty of eradicating this bacterium makes it particularly dangerous when found in a hospital environment[7]. Consequently, it has been classified in the "Priority 1" pathogen group by the World a Health Organization in 2017 - this status was confirmed in 2024 - to prioritize the development of new antibacterial agents [W1]. This phenomenon of multi-resistance, becoming increasingly widespread, is the root of many therapeutic failures [8], [9]. Persister cells in bacteria were first described by Hobby in 1942 and Joseph Bigger in 1944 [10], [11]. Persister cells are a bacterial subpopulation able of surviving to high concentrations of antibiotics compared to isogenic individuals [12], [13]. Recently, it was shown that phages also produce a subpopulation of persister cells similar to those generated/selected by antibiotics [14].

This recalcitrant phenotype is reversible and thus temporary since persister cells are able to regrow after the removal of selection pressure, to reform a population similar to the original, *i.e.* susceptible to antibiotics [13]. Persister cells are generally characterized by a growth arrest. However, it seems that this feature is neither necessary nor sufficient to enter into persistence [15]. Two types of persister cells have been described (Figure 1) [12]. One is the result of a spontaneous formation and are cells, very poorly represented in a population, which do not present a response to any particular stress but which can enter into a very

slow multiplication phase even under favorable conditions (Figure 1A) [16]. The other type, the triggered persister, formerly called type I [16] is generated by an environmental stressor like a late stationary phase starvation [17], a high cell density [18] , or an antibiotic exposure[12] (Figure 1B). It should be noticed that the viable but non-culturable cells (VBNC) could sometimes be considered as persister exhibiting a longer latency [19]. The VBNC remain intact and metabolically active after exposure to an antibiotic, but unlike persister cells, they can not resume growth after antibiotic removal [20]. However, it has been shown that under certain conditions, some VBNC could regrow with a transcriptomic profile very similar to the one of persister [19]. Two main methodologies are available for studying persister cells: the study at individual cell level using microfluidics, growth reporters and ScanLag as examples or the study of the subpopulation level *via* genetic screens, evolution of whole genome sequencing, -omics techniques or mathematical modeling ..etc [21], [22], [23], [24]. These have been used to highlight some mechanisms involved in the formation or in the selection or that may play a role in the physiology of persister cells, like the toxin-antitoxin systems, some second messengers activating specific transcription factors, the activation of the SOS response, the oxidative stress response and the membrane modifications. It is still difficult to determine whether these mechanisms are directly or indirectly involved, whether they are a cause or a consequence, but they play a role in the physiology of persister cell.

It is admitted that persister cells can represent up to 10 % of the cells living in biofilm mode and <1 % of the cells in planktonic growth mode [25]. As they might be partly responsible for the recurrence of chronic infections [26], [27], [28] and could also promote resistance mechanisms development [29], [30], the "persister" field of research is increasingly being explored. In this review, we bring together the main findings about the molecular mechanisms by which persister cells of *A. baumannii* could appear or that are involved in their physiology, and which molecules may be used to prevent their appearance or destroy them.

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Figure 1 : **Persister cells selection by antibiotics** . Triggered persister cells, on the other hand, are induced by an environmental change such as an antibiotic shock. (Adapted from [12], [31]).

2. Molecular mechanisms of *A. baumannii* involved in the physiology of persister cells

Factors influencing the level of persister cells

Several studies have investigated the formation of persister cells by *A. baumannii.* The study by Bart et al., (2013) was the first investigation on this type of cells in *A. baumannii* . Thirty-seven clinical strains from the hospital of Porto Alegre (Brazil), isolated from January to September 2012, were tested for persister cell formation with two antibiotics, polymyxin B and tobramycin (Table 1) [32]. This study showed the proportion of persister cells strongly depended on the strain and varied between 0.0007% to 10.1% and 0.0003 % to 11.84 % of the total population, for polymyxin B and tobramycin respectively [32]. However, for the majority of isolates, the most likely proportion was about 1.2% for polymyxin B and 0.5% for tobramycin.

In another study about the same hospital [33] twenty other clinical isolates were classified according to their ability to form biofilms. As known, the proportion of persister cells in biofilm mode was much higher than in planktonic mode but surprisingly, strong biofilm producers had some of the lowest persister levels (Table 1). Persister levels show a higher heterogeneity (0.2 to 7.2%) for isolates producing low biofilm compared with those producing more biofilm. Among the isolates, 5 were tested in planktonic mode at different concentrations (15 to 200 times the minimum inhibitory concentration (MIC) of meropenem). The proportion of persisters did not vary as a function of antibiotic concentration. The authors suggested that this persister level heterogeneity could have four distinct and non-exclusive origins: the individual ability to produce persistence stochastically, specific regulatory mechanisms, the metabolic structure of each isolate and the influence of other previous stresses.

Persister cells were also observed after ciprofloxacin treatment of non multidrug resistance (MDR) strong biofilm former clinical isolates [34]. This treatment also rose the level of ciprofloxacin resistance in regrown planktonic cells suggesting a link between biofilm, persistence and resistance (Table 1).

Treatments by aminoglycosides and tobramycin in particular were also shown to promote persister formation (Table 1) [35], the level of which was modulated by oxygenation level and composition of the culture medium [35], [36]. This modulation *via* nutriment availability was also observed on persister levels obtained after ciprofloxacin treatment (Table 1) [36]. The growth phase is another factor that was shown to strongly influence the proportion of persister cells [37], but this has not yet been demonstrated for *A. baumannii* . Thus, if the level of persister formation depend weakly on the culture parameters or on the antibiotic used, it was suggested to be essentially related to the strain in *A. baumannii* . Indeed, the diversity of the *A. baumannii* genomes reflects a high degree of diversity in the strains themselves (Table 1) [38]. In fact, out of the 2 500 *A. baumannii* genomes, only 2 221 genes are shared between each strain (core genome), out of the 19 272 that correspond to the pangenome. Strain diversity may be a main explanation for the difference in persister formation levels.

Toxin/antitoxin systems related to the formation of persister cells

The toxin/antitoxin (TA) systems are composed of a stable toxin and an unstable antitoxin, which neutralizes the activity of the toxin. TA systems are currently grouped into five types (types I to V) according to the nature of the antitoxin and the mode of interaction between toxin and antitoxin. In all cases, toxins are proteins, while an antitoxin may be either RNA or protein [39].

Initially presented as a plasmid maintenance system [40], new advances have shown that these systems act also on the physiology of the bacteria. Indeed, they allow slowing down bacterial growth in response to a stress factor, *promoting thus* an entry into bacterial persister state [41]. The involvement of "high persistence" genes (*hipA/hipB* TA operon) in the persister phenomenon was discovered in 1983 in *E. coli* K12 [42]. This system is the most documented for its involvement in persister cell formation. Under unfavorable conditions, a cascade of reactions activates the TA system. Indeed, the presence of stress induces activation of the stringent response, leading to 5'-diphosphate 3'-diphosphate (ppGpp) accumulation. Although not essential, ppGpp accumulation leads to the formation of polyphosphate (polyP) by the polyphosphate kinase (PPK) [43]. This polyP then interacts with the protease complex Lon, activating a complex polyP - Lon that promotes protein degradation of the HipB antitoxin from the TA. HipA toxin is thus released and inhibits protein synthesis*via* an inhibition of the glutamyl-tRNA synthetase [44]. This leads to an accumulation of uncharged tRNAGlu in the cell and results in failure of protein biosynthesis. The TA systems remain partially described in *A. baumannii* and present, as already described, a significant variability depending on the strain [45], [46], [47].

The study of 476 genomes of *A. baumannii* isolates predicted 15 different TA systems. They were detected either on the strain genome or on their plasmids (ACICU, AYE, SDF, MDR-ZJ06, p3ABAYE...) [45]. The authors subcloned the toxins and expressed them in *E. coli* under an inducible promoter. They then investigated the toxic effect of the protein and whether it was functional. In this group, five of them appear to be functional (Table 1). These five TA systems are all type II. Three of these TA are orthologs of the bacterial and archaeal systems RelB/RelE, HicA/HicB and HigB/HigA, and two are unique TA modules, AbkA/AbkB (also called SplT/SplA) and CheT/CheA [45], [48], [49].

AbkA/AbkB : Among these five potential TA systems, the AbkA/AbkB system was the only system studied for its involvement in persister formation in *A. baumannii* . It is mainly carried by plasmid but does not appear to be directly involved in plasmid maintenance [50], [51], [52]. The toxin AbkB is an endoribonuclease that inhibits translation, and AbkA as its cognate antitoxin [50]. The genes *abkA/abkB* were over-expressed (2.7 fold change) in persister cells under antibiotic treatment (10 × MIC of imipenem) [53]. The strains overexpressed AbkA and presented a high proportion of persister while a drastic decrease was observed in the other strains without this system. It is also interesting to note that a significant correlation was observed between the presence of *abkA* and *blaOXA-24* genes in carbapenem-resistant isolates obtained from patients

in Iran [48]. In this case, it was suggested a potential role for these systems as plasmid stabilization systems, contributing to the evolution of antibiotic resistance in *A. baumannii [45], [49].*

Concerning the other TA systems, their function was hypothesized regarding their identity with already described TA systems in different bacteria.

HigA/B : Indeed, for HigB/HigA, no link has been yet established for its involvement in *A. baumannii* persisters. However, in *Mycobacterium tuberculosis* it is involved in stress response [54]. In *Pseudomonas aeruginosa,* the toxin HigB regulates virulence factor production (*e.g* pyocyanin production, pyochelin, swarming mobility) and promote biofilm formation where a high proportion of cells are known to persist [55], [56]. HigA antitoxin represses the expression of a key transcriptional regulator of the virulence systems by directly binding to their promoter regions, and it controls pyocyanin synthesis, type III secretion system (T3SS), and type VI secretion system (T6SS) expression [56]. In *A. baumannii,* the HigB2/HigA2 system was found on the 11kb plasmid pAB120 also carrying genes for resistance to carbapenems [57]. The hetero-oligomer has a feedback control on its expression. It was also modulate by environmental stresses and was shown to be overexpressed in stationary phase or under iron deficiency [57]. Both HigB/HigA and AbkA/AbkB are the most abundant systems, and only detected to be carried by plasmids up to now. They are predominantly present in isolates from the international clones one and two (IC-I and IC-II) [45], [48], [57], [58], [59].

CheT/CheA : Regarding *A. baumannii* CheT/CheA system, CheA presented 25 % identity with the *E. coli* DinJ antitoxin, and has 37 % identity with an N-acetyltransferase superfamily [48]. . The predicted structure of the protein seems to show that CheA possesses helix-turn-helix (HTH) domains characteristic of DinJ. In *E. coli* , DinJ/YafQ system is involved in the formation of persister cells [60]. Kill-rescue assays on *A. baumannii* isolates showed that CheT/CheA served as a TA module: the HTH domain of the protein CheT acted as a toxin and the GNAT domain of CheA protein neutralized the HTH effect acting thus as an antitoxin [48].

RelB/RelE : The RelB/RelE system is detected at genomic level in some *A. baumannii* clinical strains (AC1633, ACICU, MDR-ZJ06, TCDC-AB0715, TYTH1 or AB 5075) [38], [45], [46], [61]. In *E. coli* , the target of the RelB toxin is the A ribosomal site. The blockage of this site promotes the cleavage of the messenger ribonucleic acid (mRNA) during translation [62]. This allows a translation stop and thus a stop of protein production[62].

HicA/B: The last TA system *hicA/hicB* was shown to be overexpressed in *A. baumannii* ATCC 17978 upon ciprofloxacin treatment (50 × MIC), suggesting an involvement of this system in the response of bacteria to antibiotic shocks [63]. In *E. coli* K12, the toxin HicA cleaves mRNA and also the tRNA in a ribosome-independent manner and HicA is reported to be inactivated by HicB [64]. In *Burkholderia pseudomallei* , this system was suggested to be involved in the formation of persister cells [65].

Finally, a Zeta/Epsilon system has been predicted and detected in the plasmids p2ABTCDC, pAbSK-OXA-82, pABTJ1 and pACICU2 [45]. Zeta toxins are notably larger (360 amino acids) than their currently known functional orthologs (270 amino acids). The Zeta toxin in *A. baumannii* could block cell wall biosynthesis by phosphorylation of the peptidoglycan precursor uridine diphosphate-N-acetylglucosamine (UNAG) [66]. Indeed, Zeta would form an ATP-UNAG-Zeta complex. This complex will block the action of MurA, responsible for peptidoglycan biosynthesis [67], which is the initial step in membrane biosynthesis [66]. Zeta then appears to have a strong implication in cell multiplication and would be an interesting target, as it stops bacteria to grow rapidly. Although not an antitoxin-toxin system, PPK kinase is also involved in protein degradation, particularly in antitoxin degradation. A study in *A. baumannii* investigated the effect of polyP *via* a mutant of PPK [68]. In this study, ATCC 17978 strains deficient in PPK1 (Dppk1::Apr) and supplemented with PPK1 (Dppk1::Apr/PJL02-ppk1) were studied. As expected, the authors showed that PPK1 is essential for polyP formation *in vivo* . The PPK1 mutant had a reduced motility by a destabilisation of the pili structure, inhibited biofilm formation and decreased persister under antibiotic (40 × MIC of Ampicillin) but also under other stresses such as H_2O_2, heat shock and starvation stress. These phenotypes were restored by complementation. Interestingly, metabolomics analysis revealed that PPK1 was associated

with glycerophospholipid metabolism and fatty acid biosynthesis. The authors suggested thus alteration of glycerophospholipid metabolism could alter the membrane charge, resulting in a resistance of bacteria to the tested stresses [68].

2.3 Second Messengers

The second messengers, (p)ppGpp and cyclic dimeric diguanosine monophosphate (*c* -di-GMP), are main signal molecules, the level of which determines the entry in persister state [69], [70], [71].

(p)ppGpp : Indeed, in *E. coli* , (p)ppGpp is clearly a major player in induction of persister state [72]. Increased levels of (p)ppGpp induce transcription of RpoS and RpoE [73]. ppGpp inhibits deoxyribonucleic acid (DNA) primase[74], and ribosomal ribonucleic acid (rRNA) transcription [75], deregulates an early step in lipopolysaccharide (LPS) formation [76], and binds to ribonucleic acid (RNA) polymerase [77]. ppGpp could directly inhibit the negative DNA supercoiling [78], and indirectly induces transcription of ribosomal modulation factor (Rmf) and hibernation promotion factor (Hpf), which plays a role in ribosome dimerization (90S and 100S) [79]. In *A. baumannii* it was shown that the formation of (p)ppGpp, is mediated by RelA (ABUW_3302) (Table1) [80]. RelA is a monofunctional protein only contributing to pppGpp synthesis. Moreover, the reduction of pppGpp into ppGpp is mediated by SpoT, a monofunctional (p)ppGpp hydrolase [81]. The*relA* deletion resulted in the formation of 4-fold less persister cells than in WT AB5075 strain under 50 × MIC colistin or 80 × MIC rifampin treatments. The mutant *relA* underwent also variations of its motility, morphology or colony morphology [80]. In other strains like ATCC 17978, it was shown that a mutant deficient in (p)ppGpp production (Δ*A1S _0579*) is 2- to 4-fold more susceptible to antibiotics, *i.e.* MICs are reduced by a factor of 1 to 4 compared to wide type (WT) or complemented strain [82]. The authors suggest this susceptibility could be due to the under-expression of efflux transporters (*AdeI, AdeJ* and *AdeK*) and, less obviously, to the over-expression of certain proteins belonging to the transporter systems, such as AetA, AdeB and AdeM [82], [83].

c-di-GMP : It was already shown that this second messenger molecule increases the proportion of persister cells in*E. coli [84].* It also influences virulence factors in many bacteria [85], [86], [87]. In *P. aeruginosa* , HigB decreases the intracellular level of *c* -di-GMP, which is responsible for the increased expression of the T3SS genes and the repression of biofilm formation [88]. It is worth noting that the enzymes, diguanylate cyclases, involved in *c* -di-GMP synthesis contain a catalytic site with a GGDEF/EAL motif [89]. In *A. baumannii* ATCC 17978 strain, several diguanylate cyclases were identified and characterized but their involvement in persister cells has never been studied [90].

2.4 SOS response in persister cells

The SOS pathway is an inducible DNA damage repair system that is pivotal for bacterial adaptation, pathogenesis, and diversification of population. The SOS response activates systems to carry out adaptation processes to face hostile or even toxic environments. These systems can contribute to growth arrest, DNA repair, repair of replication errors or mobilization of mobile genetic elements (transposons and insertion sequences) [91], [92], [93]. Activation of the SOS response in persister cells could explain the appearance of adaptative mutations, especially in the presence of antibiotics inducing reactive oxygen species (ROS) production leading therefore to DNA damages [94]. Persister could thus be a key stage in the course of genetic resistance development [95]. The SOS response seems to be a key player in persister physiology, for both maintaining DNA integrity and remodeling it [96]. However, DNA damage response is a multifactorial response that appear to be species-specific and may have a major influence on the development of new capabilities of the species [97].

The DNA damages induced by ROS can be produced by antibacterial molecules and leads to an SOS response [98]. This response is mediated by RecA, which binds to single-stranded DNA and protects it from degradation. In many bacteria, LexA is the transcriptional repressor of RecA and of all SOS response genes. It is cleaved by RecA if the latter is bound to a single strand of DNA activating the transcription of SOS genes. In *Acinetobacter* , the mechanism seems different, as the genome lacks the *lexA* gene [99], [100]. Indeed, the error-prone reserve polymerase UmuD (polymerase V) is recruited during SOS response. *UmuD*

is repressed by UmuDAb, itself repressed by DdrR. *DdrR* overexpression represses the DNA repair genes [101].

It was shown that, after 1h tobramycin treatment at 10 × MIC,*umuD* and *ddrR* of ATCC 17978 strain were over-expressed [102]. The transcriptomics of ATCC 17978 persisters selected by treatment of 50 × MIC of ciprofloxacin, showed overexpression of different systems, like the DNA repair system, the phenylacetic acid degradation pathway, the leucine catabolism, HicAB toxin-antitoxin system and ROS response (Table1) [63]. Several genes displayed significant changes like *pare* (A1S_3359); *recA* (A1S_1962); *umuC* (A1S_2008); *umuD* (A1S_1174, A1S_1389); *ddrR* (A1S_1388); *ruvB* (A1S_2588);*recB* (A1S_0356) and *repA* (A1S_0663). The construction of deletion mutants of *recA* (A1S_1962) and *umuD* homolog (A1S_1389) was performed. The *umuD* mutant formed fewer persister cells than the WT strain, when the *recA* mutant did not form any persister cells after 3 hour (h) of antibiotic treatment [63]. These results suggested that these proteins (*umuD* and *recA*) are involved in persister formation. It should be also noticed that RecA is involved in resistance to some β-lactam antibiotics and contributes to survival after ultraviolet (UV) treatment, and to virulence in macrophages *in vitro* and in mice *in vivo* [103], [104].

Other transcriptomic analyses on ATCC 19606 and two clinical strains treated by 10 mg/L of ciprofloxacin and 10 mg/L of imipenem for 6 h were also performed [105]. They showed similarly that the three strains overexpressed *dam, recC, umuD, phoU* , and *glpD* . The methyltransferase Dam, when subcloned under the control of an inducible promoter, positively regulated *recC* , *umuD* , *phoU* , and *glpD* genes. Consequently, the percentage of persister cells was more abundant in the Dam-overexpressing mutant. The RecC overexpressing-mutant presented also an increased rate of persister cell formation (Table 1) [105].

Antibiotic shock treatment frequently leads to the generation of ROS [106], [107], [108], [109]. As seen previously, ROS may damage DNA leading to the SOS response [96]. However, the bacteria may also activate some other mechanisms to face the ROS stress and maintain the cell integrity. These mechanisms are essential for a rapid adaptation or the bacterial survival. This response is indirectly involved in the state of persister cells [110]. As an example, pyocyanin generates ROS in *A. baumannii* . It was shown ROS production leads to catalase and Sod increased expression in order to eliminate ROS [111]. Clearance of *A. baumannii* polymyxin B persister by Rifampicin was shown to be related to a significant down regulation of *sodB* and therefore to an enhanced generation of ROS (Table 1) [112]. Additionally, it was shown that a SodB *A. baumannii* mutant was more sensitive to antibiotics and generated less persister cells [112].

2.5 The phenyl acetic catabolic pathway

In ATCC 19606 strain under a ceftazidime (50 × MIC) antibiotic shock, the aromatic compound degradation (PAA) genes were overexpressed in persister cells (Table 1) [113]. The authors suggest that the degradation of the aromatic ring of antibiotics was used by *A. baumannii* under conditions of nutrient limitation. In ATCC 17978, under antibiotic subinhibitory concentrations, the PAA operon was shown to be overexpressed while Csu pilus expression and biofilm formation were repressed [114]. This suggests that this pathway may participate to the regulatory network used in response to antibiotic treatment.

2.6 Changes at membrane level in persister cells

The next section of this review which deals with therapeutics, will show that the membrane is considered to be a main target for anti-persister cell agent development [115].

However, few studies have investigated the lipidome of persister cells (Table 1). In *Mycobacterium tuberculosis* dormant cells, an accumulation of triacylglycerol (TG) linked to the membrane or as inclusion bodies [116], [117] was highlighted [118]. TG could be involved in energy storage used during hibernation [119], [120] and used to reduce the toxic burden of free fatty acids causing a reductive stress [121]. It could also contribute to antibiotic tolerance by redirecting cellular carbon fluxes away from the tricarboxylic acid cycle [122]. In addition, genes encoding TG biosynthetic enzymes were found predominantly in virulent *M. tuberculosis* strains, suggesting a potential role of these genes in the infection [118]. In *A. baumannii* , the lipidome of persister cells under ciprofloxacin treatment (100 × MIC) was analyzed using LC-MS (Table 1) [123]. It was demons-

trated that membranes of persister cells displayed specific lipids like lyso-1-phosphatidyl-2-acyl-glycerol-3-phosphoethanolamines (LPAGPE) and 1-phosphatidyl-2-acyl-glycerol-3-phosphoethanolamines (PAGPE). These specific lipids may compensate a repulsive effect of the phosphate negative charges due to an increase in cardiolipin abundance. Moreover, the acyl chains of their lipids were modified by hydroxylation or di-unsaturations. The presence of di-unsaturation and hydroxylation could contribute to a change in membrane fluidity. In persister cells, a rigidification of the membrane could allow bacteria to modify the permeability of their membranes in order to survive, and may accompany to the bacterial growth arrest. Finally, the persister cells were shown to accumulate particular wax esters (WE) composed of two fatty acids and a fatty diol. These uncommon WE could be a specific metabolic key for *A. baumannii* to store energy during growth arrest and used for rapid regrowth? [123].

Concerning membrane proteins, genes coding for OmpA and OmpW were shown overexpressed by a factor of 5.5 and 10.5 respectively, in 15 × MIC meropenem persisters of the Acb-1 strain (Table 1) [124]. In agreement with this, virulence assays on *Galleria mellonella* indicated that these persisters were more virulent than control cells [124]. On the contrary, *ompW* was under-expressed in tobramycin persisters. [63] Its expression in persisters may thus be drug-dependent. The authors suggest that over-expression can help *A. baumannii* survive exposure to meropenem by increasing drug efflux.

Finally, it was already shown that *A. baumannii* persister cells underwent morphological changes. Indeed, a study on persisters generated by 100 × MIC meropenem showed that cells had a spherical shape with a reduced permeability (Table 1) [125]. These morphological changes were also observed for the ATCC 17978 strain under a 6 µg/mL meropenem treatment [126]. Furthermore, images of persister cells under tobramycin (10 mg/L) or meropenem (15 mg/L) treatment by transmission and scanning electron microscopies showed the presence of a division septa at the cell midpoint [35]. The presence of this septum was intriguing. It was suggested that the persister cells could still be in a multiplying state to maintain a balance between multiplication and cell death, as also suggested in *Mycobacterium smegmatis* [127].

Table 1 : **Molecular mechanisms involved in *A. baumannii* persister cells physiology** .

Mechanisms	Antibiotic of selection	Str
TA systems		
abkA/abkB	-Imipenem (10 × MIC) -Imipenem combined with chlorhexidine (0.25 × MIC)	Ref
RelB/RelE		
$HigB_{Ab}/HigA_{Ab}$	-Ceftazidime (50 × MIC)	Ref
hicA/hicB	-Ciprofloxacin (50 × MIC)	Ref
PPK	-Ampicillin (40 × MIC)	Ref
Secondary messenger		
(p)ppGpp relA	-Colistin (50 × MIC) -Rifampin (80 × MIC)	Cli
SOS response	-Ciprofloxacin (50 × MIC) -Ciprofloxacin (10 mg/L) -Imipenem (10 mg/L)	Ref
ROS	-Amikacin (2 × MIC) -Carbenicillin (5 × MIC)	clin
Membrane changes	-Ciprofloxacin (100 × MIC) -Meropenem (15 × MIC) -Meropenem (100 × MIC + 10 × MIC)	Ref
PAA operon	-Ceftazidime (50 × MIC)	Ref

3. Strategies against *A. baumannii* persister cells

Two main approaches can be used to fight against persister cells: i) the prevention approach that uses antibacterial compounds that do not form persister cells. In this approach, the use of antibacterial agents' combination is very useful. ii) the development of direct killing agents that do not generate persister cells itself or that are able to kill persister specifically. In this approach, the most effective strategy for eliminating persister cells is to take growth arrest into account and find a target not linked to multiplication activity [128]. Most effective anti-persister agents take often the bacterial membrane as a primary target. In *A. baumannii* , combination of treatments strategies and direct killing have been explored and will be presented

in this section [128], [129].

3.1 Preventing approach: combination of treatments

Combining antibiotics to prevent the formation of persister cells is a strategy that generally uses antibiotics with radically different targets, the membrane being one of the preferred targets. Thus, most of the studies combine the use of the polymyxin B or colistin with β-lactams.

Recently, it has been shown that meropenem or carbenicillin treatments resulted in the appearance of a particular cell phenotype that survive: round, metabolically active cells that have lost their cell wall [126]. A modification of their envelope leads to a susceptibility to treatment with β-lactam (ampicillin and carbenicillin) with an increase in the killing power from 100 to 1000 fold [126]. These results suggest that antibiotic treatment can cause a modification of the envelope, making the bacteria more sensitive to another antibiotic targeting the membrane. Another study focused on three clinical strains (Acb-1, Acb-8 and Acb-2) and determined the level of persister cells generated by polymyxin B and meropenem, tested at different concentrations alone or in combination [130]. Mono-treatment with these antibiotics at 5, 10 or 15 mg/L, induced between 0.0031 to 0.9743 % of persisters depending on the choice of antibiotics. However, these antibiotics in combination did not produce persister cells. This study highlighted and confirmed the useful effects of combined therapy.

Synergistic effects were also observed with other antibiotic combinations: 30 µg/mL carbenicillin with 2 µg/mL colistin or 200 µg/mL ampicillin with 2 µg/mL colistin. They increased the killing efficiency on two clinical isolates [126]. These combinations provide a two-log increase in bacterial eradication.

Colistin has also been tested in combination with other antibiotics, *e.g* . colistin plus tobramycin, rifampicin or colistin [131]. These authors show that these combinations eradicate all cells. Sequential antibiotic combinations were also examined [132]. It was demonstrated that treatment by colistin followed by amikacin eradicated the entire population, when the reverse sequence did not. Even though no high antibiotic concentration was used to select persisters, this study clearly shows that sensitization by a membranolytic agent is highly effective. Colistin in combination with the econazole, an imidazole derivative, was also tested [133]. This combination showed a synergic effect with a reduction of MIC colistin by 128 fold with 10 µg/mL econazole.

The combination of an antimicrobial agent with a phage cocktail to control or limit biofilm formation has also been investigated [134]. Combinations of four temperate bacteriophages (SA1, Eve, Ftm and Gln) and different antibiotics (ampicillin/sulbactam, meropenem and colistin) enhanced biofilm inhibition and degradation on thirty extensively drug-resistant (XDR) strains of *A. baumannii* . Moreover, it has been shown that a combination of Paride and meropenem can sterilize deep-dormant cultures *in vitro* and greatly reduce a resilient bacterial infection of a tissue cage implant in mice [135]. In this context, it would be interesting to test these phage/antibiotic combinations on persister cells.

3.2 Persister eradication approach

In *A. baumannii* , the first bactericidal agent used to eradicate persister cells was Art-175 from the Artilysine family [136]. Art-175 is a phagic endolysin which degrades peptidoglycan, fused with specific peptides that destabilize the outer membrane [136]. The specificity between peptidoglycan and endolysin results from the long and intense coevolution between phages and bacteria. Art-175 (60 µg/ml) was highly effective at 1 h and eradicated all strains after 24 h of treatment. Persister selected with tobramycin and treated with 30 × MIC Art-157 on the RUH134 strain led to a drastic population reduction in just 20 min. It took only 120 min to reach the limit of bacteria detection.

The effect of molecules with an aromatic core (1-BC or 2-NC), an L-lysine moiety (K), and a variable lipophilic chain (8, 10, 12 carbon chain) were also tested on *E. coli* , *Klebsiella pneumoniae, P. aeruginosa* and *A. baumannii* persister cells [137]. NCK-10 and BCK-12 activity on *A. baumannii* clinical isolates (MTCC 1425, R676 and R674) showed lytic activity between 1.5 and 9 µM. NCK-10 (8 × MIC) did not induce resistance even after 20 cycles in *E. coli* . NCK-10 appears to depolarized and permeabilize the membranes of *E. coli*

persister cells (induced by 300 µg/mL of ampicillin for 3 h). NCK-10 was burn-tested on female Balb/c mice infected with *A. baumannii* at concentrations of 17.5 mg/kg and did not induce any toxic effects. Moreover, this compound significantly reduced the bacterial load after a 7-day application.

Squalamine, a polycationic aminosterol isolated from the shark*Squalus acanthias* with antibacterial properties, was tested on persister cells generated by ciprofloxacin [36]. This molecule contains a polyamine tail, which is known to be able to lead to a fast membrane depolarization and disruption [138]. In this study, unlike those presented above, the methodology is slightly different. The authors generated persister cells using an antibiotic (ciprofloxacin 1000 × MIC). They first characterized the selected population to determine the proportion of persister cells and VNBCs. Then the authors tested squalamine, the molecule of interest, with colistin as the control for cell lysis. Squalamine and colistin were able to eradicate persister cells at 100 mg/L (50 × MIC) and 500 mg/L (100 × MIC) within 9 and 12 h respectively. Colistin concentrations (100 × MIC) are higher than those of squalamine (50 × MIC), and require more time to eradicate the persistent population, which underlines the efficacy of squalamine. It was mentioned that squalamine was used at concentrations below the minimum hemolytic concentration (IC50 > 66 mg/L [139]) demonstrating a poor toxicity.

Carvacrol, Eugenol, and Thymol are compounds approved by the US Food and Drug Administration (FDA), having a GRAS (generally recognized as safe) status [125]. They can be used as food additives. These compounds were tested on persister cells selected with meropenem (100 × MIC). Thymol used alone at its MIC concentration lead to a complete eradication of meropenem induced persisters. In combination with meropenem, it completely eradicated persisters at lower concentration (0.5 × MIC). It allowed also the total eradication of 48 h mature biofilm at concentration 1 × MIC. Concerning eugenol and carvacrol, they eradicated biofilm persisters but at much higher concentrations than thymol. Thymol treatment significantly inhibited meropenem persisters of MDR *A. baumannii* isolates, both as monotherapy and co-therapy. Thymol was also effective on rifampicin, tigecycline and polymyxin B persisters at concentrations equal to or less than 1 × MIC. Thymol was demonstrated to induce a membrane depolarization and permeability, as well as an increased ROS production. It inhibited proton motive force (PMF) and multiple drug efflux pumps.

Recently, it has been shown that phages can have an effect on dormant cells [140]. The action of the phage Paride on dormant cells of*Pseudomonas aeruginosa* has been studied. This study showed that this phage has an active lytic cycle on *P. aeruginosa* cells in deep dormancy [135]. To our knowledge, phages with an effective lytic cycle on persistent *A. baumannii* cells have not yet been studied.

Table 2 : Therapeutic investigation for anti- persister cells in *A. baumannii*

Molecule	**Concentration**
Membranolytic agent associated with antibacterial agents	
Meropenem, ampicillin + carbenicillin	Combination of 30 µg/mL carbenicillin with 2 µg/mL
Polymyxin B + Meropenem	5, 10 and 15 µg/mL
Colistin + tobramycin or rifampicin	10, 20 or 40 × MIC
Colistin then amikacin or *vice versa*	Colistin 5 × MIC
Colistin + econazole	Econazole 10 µg/mL
Art-175	Tobramycin 30 × MIC
Persister eradication strategy	
Squalamine Colistine	Squalamine 50 × MIC Colistine 100 × MIC
Carvacrol, Eugenol, Thymol	Thymol 1 × MIC Meropenem 100 × MIC.

4. Conclusion

Persister formation in *A. baumannii* is highly dependant on bacterial strain and culture conditions. This physiological complexity potentially involves various molecular determinants, currently under investigation. Among them, toxin-antitoxin systems could modulate the formation of these persistent cells (Abka...).

Other systems involved in persister cell formation for other bacteria are still poorly explored in *A. baumannii* : second messengers, the SOS response and PAA. Membrane modifications appear to be an important factor in the physiology of *A. baumannii* . Further research into the physiology of this particular bacterial population is required. This could lead to the identification of specific biomarkers and, consequently, to new therapeutic targets.

Current approaches to eradicating persisters focus on different strategies: the use of molecules that inhibit persister formation, such as naturally-occurring compounds like Art-175, phages and squalamine, or the combination of antibiotics, one of which acts mechanistically on the bacterial membrane.

Many questions relating to this physiology remain unanswered, particularly with regard to its fundamental understanding and the identification of effective therapeutic targets. The development of strategies targeting persister cells could make a significant contribution to limiting the development of new bacterial resistances.

Acknowledgements

This work has been partially supported by University of Rouen Normandy, INSA-Rouen Normandy, the Centre National de la Recherche Scientifique (CNRS), European Regional Development Fund (ERDF), SéSAD research network, France.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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List of used abbreviations

c-di-GMP : cyclic dimeric diguanosine monophosphate
DNA : deoxyribonucleic acid
FDA : Food and Drug Administration
h : hour

c-di-GMP : cyclic dimeric diguanosine monophosphate

IC-I : international clones one
IC-II : international clones two
LPAGPE : lyso-1-phosphatidyl-2-acyl-glycerol-3-phosphoethanolamines
LPS : lipopolysaccharide
MDR : multidrug resistance
MIC : minimum inhibitory concentration
mRNA : messenger ribonucleic acid
PAA : aromatic compound degradation
PAGPE : 1-phosphatidyl-2-acyl-glycerol-3-phosphoethanolamines
PMF : proton motive force
polyP : polyphosphate
ppGpp : guanosine 5'-diphosphate 3'-diphosphate
PPK : polyphosphate kinase
RNA : ribonucleic acid
ROS : reactive oxygen species
rRNA : ribosomal ribonucleic acid
T3SS : type III secretion system
T6SS : type VI secretion system
TA : toxin/antitoxin
TG : triacylglycerol
UNAG : uridine diphosphate-N-acetylglucosamine
UV : ultraviolet
VBNC : viable but non-culturable cells
WE : wax esters
XDR : extensively drug-resistant