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MINI REVIEW

Proton motive force and antibiotic tolerance in bacteria

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Abstract

Bacterial antibiotic tolerance is a decades-old phenomenon in which a bacterial sub-population, commonly known as persisters, does not respond to antibiotics and remains viable upon prolonged antimicrobial treatment. Persisters are detectable in populations of bacterial strains that are not antibiotic-resistant and are known to be responsible for treatment failure and the occurrence of chronic and recurrent infection. The clinical significance of antibiotic tolerance is increasingly being recognized and comparable to antibiotic resistance. To eradicate persisters, it is necessary to understand the cellular mechanisms underlying tolerance development. Previous works showed that bacterial antibiotic tolerance was attributed to the reduction in metabolic activities and activation of the stringent response, SOS response and the toxin–antitoxin system which down-regulates transcription functions. The latest research findings, however, showed that decreased metabolic activities alone do not confer a long-lasting tolerance phenotype in persisters, and that active defence mechanisms such as efflux and DNA repair are required for the long-term maintenance of phenotypic tolerance. As such active tolerance-maintenance mechanisms are energy-demanding, persisters need to generate and maintain the transmembrane proton motive force (PMF) for oxidative phosphorylation. This minireview summarizes the current understanding of cellular mechanisms essential for prolonged expression of phenotypic antibiotic tolerance in bacteria, with an emphasis on the importance of generation and maintenance of PMF in enabling proper functioning of the active tolerance mechanisms in persisters. How such mechanisms can be utilized as targets for the development of anti-persister strategies will be discussed.

INTRODUCTION

This year marks the 80th anniversary of the pioneering study by Joseph Bigger which demonstrated that bacterial cultures could not be completely sterilized by antibiotics due to the existence of a sub-population that did not respond to antibiotics. The study, commonly regarded as the first report of the phenomenon of antibiotic tolerance in bacteria, was published in the journal *Lancet* in 1944, 16 years after the discovery of the first antibiotic penicillin (Bigger, 1944). It should be noted, however, that Gladys Hobby should be recognized as the first person who observed the antibiotic tolerance phenomenon. In 1942, she found that penicillin could only kill 99% of a streptococcal culture, and that the other 1% remained viable (Hobby et al., 1942). The term 'antibiotic tolerance' has since been used to describe the phenotype of a bacterial population that exhibits a slower rate of killing by antibiotics, most commonly due to the reduced growth rate in an unfavourable environment (Boeck, 2023). An antibiotic-tolerant sub-population may also be described as 'persisters' if they exhibit a significantly higher level of tolerance than the rest of the population during antimicrobial treatment and

cannot be eradicated by antibiotics over a long period of time (Peyrusson et al., 2020; Verstraete et al., 2022). A bacterial population that contains persisters therefore exhibits a tolerance phenotype characterized by biphasic killing (Huemer et al., 2020). Based on this observation, antibiotic tolerance and persistence are viewed as two different concepts; such terms remain rather confusing to most members of the medical and scientific community who are not familiar with this topic, especially after the emergence of penicillin-resistant strains, also in the 1940s (Bigger, 1944). Antibiotic-resistant strains can be differentiated from the antibiotic-tolerant or persistent sub-population by the observation that only the resistant strains can grow in an environment where the antibiotic is present and exhibit an elevated MIC, whereas an antibiotic-tolerant bacterial population or persisters do not replicate in the presence of the antibiotic but can re-grow when the antibiotic is no longer present in the environment (Balaban et al., 2019; Brauner et al., 2016). It should be noted that cellular mechanisms underlying the development of antibiotic tolerance and phenotypic features of antibiotic-tolerant bacterial cells and persisters had not been investigated for decades until the early 2000s, when researchers began to show, on the basis of phenotypic studies, that bacterial antibiotic tolerant population may contain two types of persisters: those which form in response to environmental stress (Type I) and those which are stochastic in nature and form spontaneously even within a population that grows under favourable conditions (Type II) (Balaban et al., 2004; Shultis et al., 2022). In 2019, a group of researchers reached a consensus on the definition of persisters and suggested that the phenotype of Type I and Type II persisters should be defined as bacterial cells that exhibited triggered and spontaneous persistence, respectively (Balaban et al., 2004, 2013, 2019; Niu et al., 2024; Zhou et al., 2023). Based on this definition, it is conceivable that almost all bacterial populations, including those of antibiotic-resistant strains, contain both types of persisters at any given time. First, different types of environmental stresses always exist, inducing the formation of Type I or stress-triggered persisters constantly; second, if Type II or spontaneous persisters intrinsically exist in non-tolerant bacterial populations, they may also be found in the tolerant populations of Type I or stress-triggered persisters. Subsequent studies showed that almost all bacterial species were able to exhibit antibiotic tolerance and that the degree and phenotypic features of antibiotic tolerance varied according to changes in environmental conditions, such as the types of stresses that bacteria encounter (Zheng et al., 2022). In reality, it may be difficult to distinguish between the two types of persisters; in addition, adverse environmental conditions may simultaneously induce the formation of persisters and render existing persisters to stay in the stationary or lag phase (Bollen et al., 2023; Eisenreich et al., 2021). In this review, the term tolerant population has the same meaning as persisters, and the two terms are used interchangeably to describe all persisters.

Difficulty in identifying persisters in clinical samples means that the clinical significance of bacterial antibiotic tolerance or persistence has not been fully recognized until recent years when new studies showed that antibiotic-tolerant persisters were mostly responsible for causing chronic and recurrent infections, especially amongst immunocompromised patients who cannot completely eradicate bacterial persisters from the human body through various immune mechanisms (Defraine et al., 2018; Huemer et al., 2020). This is because only organisms in the tolerance mode can live within the human body for a prolonged period by exhibiting the ability to survive against multiple stresses, including those inflicted by the host immune system and lack of nutrients. It has also been postulated that switching to the tolerance mode provides bacteria sufficient time to evolve various resistance mechanisms if antibiotic selection pressure persists (Bakkeren et al., 2020; Levin-Reisman et al., 2017; Stower, 2020; Windels et al., 2019).

To combat tolerance, it is necessary to determine how tolerance is induced physiologically in bacteria, yet conditions that give rise to tolerance formation remain poorly defined. The size of any given antibiotic-tolerant sub-population apparently varies in a presumably highly complex manner according to changes in environmental conditions (Berkvens et al., 2022). When environmental conditions become unfavourable, bacteria cease to grow and replicate, and become insensitive to antibiotics whose activity depends on bacterial metabolism (Berkvens et al., 2022); hence the size of the antibiotic-tolerant population will rapidly expand under adverse environmental conditions (Brauner et al., 2016). In this regard, all bacterial strains, including those that are susceptible to antibiotics, may switch to a tolerance status from time to time and according to the nature of changes in environmental conditions, resulting in constant alteration in the degree of susceptibility to antibiotics (Meylan et al., 2018). It should be noted, however, that bacterial persisters also exhibit tolerance to membrane-targeting antibiotics which can kill bacteria that are not metabolically active, indicating that the onset of bacterial antibiotic tolerance or persistence does not merely rely on shutting down metabolic processes (Fauvart et al., 2018). It is also not clear how long this initial phase of tolerance onset due to growth arrest-induced inactivation of the antibiotic target can be maintained in the stress-triggered persisters without further activation of other tolerance or stress protection mechanisms. On the other hand, when environmental conditions improve, the tolerant sub-population can resume growth and only then the non-resistant strains would become susceptible to antibiotics, but a small proportion of the spontaneous persisters may still exist (Bollen et al., 2023). How the tolerance mechanisms of the two types of persisters

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differ from each other is also not clear. In view of the fact that the majority of persisters are stress-triggered and arise as a result of deteriorating growth conditions, we envisage a need to look beyond cellular changes associated with the initial stress-induced growth arrest or failure to switch back to the growth mode, and identify other tolerance mechanisms that confer long term survival of persisters, if we wish to develop effective strategy to combat tolerance. For the drug-resistant strains, data on the related stress-induced tolerance phenotype are not available, but currently available evidence shows that cellular mechanisms that help maintain a tolerance phenotype differ from major resistance mechanisms, as organisms that do not express known resistance mechanisms or carry resistance-conferring genetic mutations still exhibit phenotypic antibiotic tolerance (Xie et al., 2022). Therefore, strains that are known to be resistant to one antibiotic may also become tolerant to other drugs under tolerance-inducing conditions. If resistant strains cannot switch to or maintain a tolerance phenotype, theoretically they would eventually die under environmental stresses even if they exhibit multiple resistance mechanisms. Taken together, these independent lines of evidence converge upon the conclusion that the old phenomenon of bacterial antibiotic tolerance or persistence is highly clinically relevant and that unveiling the cellular mechanisms involved in tolerance expression should be a priority research topic in the future. Importantly, research breakthroughs in recent years began to suggest that the expression of the bacterial antibiotic tolerance phenotype needs to be actively managed by various regulatory, defence and repair mechanisms (Wan et al., 2021). One important tolerance mechanism is the maintenance and continuous generation of the transmembrane proton motive force (PMF), which is required to support a number of membrane protein functions that are specifically turned on in persisters (Wan et al., 2023). These new findings have important implications in the development of new antimicrobial strategies because they mean that inhibiting bacterial tolerance maintenance mechanisms offers a new way to eradicate bacteria regardless of their drug susceptibility status, as targeting tolerance mechanisms would undermine the ability of even drug-resistant bacterial strains to survive for a prolonged period under adverse environmental conditions. This review summarizes the latest evidence gathered in our laboratories and those of others in the past few years, which demonstrate the importance of PMF maintenance in the expression of bacterial antibiotic tolerance (Wang et al., 2021). We shall explain why inhibiting the ability of bacteria to actively express and maintain stress tolerance phenotypes can promisingly lead to the invention of a novel antimicrobial strategy that can effectively eradicate bacterial antibiotic persisters and target antibiotic-resistant strains of all nature by inhibiting their ability to maintain a tolerance phenotype.

CURRENT THEORIES OF BACTERIAL ANTIBIOTIC TOLERANCE

Tuning down the activities of the major metabolic pathways when growth conditions turn unfavourable has for decades been regarded as the main underlying factor that results in antibiotic tolerance in bacteria (Wood et al., 2013). This convenient explanation of the cause of bacterial antibiotic tolerance, however, cannot explain why two types of persisters exist. A more complex picture emerged when genetic analysis became possible in the 1990s, when various signalling and physiological regulatory systems, including the stringent response, the SOS response and the toxin–antitoxin system, were postulated to play a role in regulating the expression of stress tolerance phenotypes (Debbia et al., 2001; Dörr et al., 2009; Masuda et al., 2020). A wide range of tolerance mechanisms and a number of putative 'tolerance' genes have also been identified and summarized in a comprehensive review presented by Wilmaerts et al. (2019). However, none of the tolerance mechanisms reported to date has been regarded as a major mechanism, because the deletion of a single tolerance gene had a limited impact on the tolerance phenotype (Pietrzykowski & Treistman, 2008). It should be noted that complete eradication of the entire persister population is important in the development of effective anti-tolerance strategies to prevent chronic and recurrent infections, as the re-growth of a single persister cell would still pose an infection risk in immunocompromised patients. Key stress responses identified at the time, such as the stringent response, at most suggest that bacteria actively shut down major physiological and metabolic processes upon encountering adverse environmental conditions, but provide no hint of whether bacteria can actively defend themselves against environmental stresses, including the bactericidal effects of antibiotics (Salzer & Wolz, 2023). Tolerance that develops simply by growth arrest or by active defence represents a significant difference in the degree of technical challenge in the development of strategies to target bacterial persisters, as persisters would be killed much more effectively by inhibiting actively expressed protective mechanisms if such mechanisms play a key role in tolerance development and maintenance.

ACTIVE TOLERANCE OR STRESS DEFENCE MECHANISMS

Several studies conducted after the year 2010 began to suggest that physiological dormancy alone is insufficient for long-term maintenance of the antibiotic tolerance phenotype in bacteria. Nguyen et al. (2011) showed that antibiotic tolerance inducible by nutrient depletion involved curtailing

the production of pro-oxidant metabolites such as 4-hydroxy-2-alkylquinolines (HAQ), thereby actively enhancing the antioxidant defence capability. However, this tolerance mechanism has only been observable in *Pseudomonas aeruginosa*. It is not clear whether other bacterial species also produce HAQ. Evidence that showed that persisters could be derived from rapidly growing bacterial populations, and that dormancy is not necessarily required for the formation of persisters, were also reported (Goormaghtigh & Van Melderen, 2019; Orman & Brynildsen, 2013). Researchers also began to utilize more sophisticated genetic approaches to characterize the underlying mechanisms of tolerance development. Through systematic analysis of gene deletion mutants, Hansen et al. (2008) identified a number of genetic loci involved in the upstream control of a vast tolerance response network (Hansen et al., 2008), suggesting that antibiotic tolerance was not simply due to passive shutdown of drug target activities as a result of retarded bacterial growth in an unfavourable environment. Nevertheless, most of the genes for which deletion had a detectable effect on antibiotic tolerance in Hansen's study were global regulators of nutrient metabolism, stress sensing, protection mechanisms, as well as essential cellular processes such as transcription, but the key tolerance mechanism could not be elucidated in this study. On the other hand, evidence of expression of actively regulated tolerance mechanisms was also provided by Fung et al. (2010). Their study involved the utilization of a nutrient starvation model to test the tolerance induction effect of depletion of specific nutrients and establish a functional link between bacterial starvation responses and the development of antibiotic tolerance. Their data suggested the existence of complex cellular mechanisms that regulated bacterial physiology in accordance with the nutritional status in the environment and effectively exerted sensitive control over the strength and specificity of antibiotic tolerance induction, with depletion of amino acids exerting the strongest tolerance induction effect. Since cellular replication of organisms subjected to all test conditions had halted but they still exhibited significantly different levels of tolerance, this experiment provided some of the strongest evidence that bacteria actively express a variety of cellular mechanisms to defend themselves against different environmental stresses. Consistently, this study showed that the use of bacteriostatic antibiotics to inhibit bacterial growth in an exponentially growing population did not induce the same strength of antibiotic tolerance in such a population as that observed in organisms that encountered nutrient starvation. These findings depict a need to delineate the underlying mechanisms by which nutrient depletion induces the onset of antibiotic tolerance.

Another important piece of work that shows that bacteria actively defend themselves against the deleterious effect of antibiotics upon encountering conditions when they need to exhibit phenotypic tolerance was performed by Pu et al. (2016). Their study showed that the efflux activity of bacteria was enhanced upon the development of antibiotic tolerance so that a lower amount of antibiotics accumulated inside the bacterial cell. Through measurement of the amount of the efflux pump component TolC, Pu et al. found that the elevated efflux activity in persisters was mediated by an increased number of efflux pumps expressed in those cells. In 2017, a study by Bergmiller et al. (2017) also showed that biased partitioning of membrane-bound efflux pumps during cell division could lead to the formation of cells in clonal populations that had more efflux pumps and were more tolerant to antibiotics that are pump substrates than other bacterial cells in the population. Consistently, Dunlop and colleagues also demonstrated that heterogenous efflux pump expression was associated with both antibiotic tolerance formation and resistance development (El Meouche & Dunlop, 2018). These findings are intriguing as they imply that persisters need to undergo active efflux and synthesize a range of proteins that support such activities, even in adverse environments.

Around the same time as Pu et al. reported the discovery of active efflux in persisters, another group found that the DNA repair mechanism was active in persisters that were tolerant to ofloxacin, especially in the recovery stage when the drug is removed (Völzing & Brynildsen, 2015). Ofloxacin is a fluoroquinolone antibiotic that may cause DNA breakage and trigger the RecA-mediated SOS response, which also involves the expression of a number of proteins (Goormaghtigh & Van Melderen, 2019; Leshchiner et al., 2022). Like efflux, DNA repair is metabolically demanding and energy intensive even in bacteria growing in favourable conditions. To date, there is no evidence that shows that persisters undergo active efflux and DNA repair simultaneously, if this is the case, they would need to undergo active energy metabolism to support such activities. To meet such demand, persisters would also need to maintain a substantial level of transmembrane PMF for energy production. How persisters achieve this goal under nutrient starvation or other stress conditions is intriguing.

PMF AND ANTIBIOTIC TOLERANCE

The idea that the status of bacterial transmembrane PMF is tightly linked to antibiotic tolerance was first introduced by Verstraeten et al. (2015), who reported that the Obg protein mediates tolerance formation by causing dissipation of PMF; in a follow-up study, they

further showed that awakening persister cells and activation of their re-growth relied on PMF repolarization (Michiels et al., 2016). Likewise, expression of the TisB protein, which is a membrane-targeting toxin molecule, was also shown to play a role in inducing persister formation by disrupting PMF and causing membrane depolarization (Edelmann & Berghoff, 2022). Recently, a study by Lee et al. (2023) showed that members of a bacterial population exhibited variable PMF levels in a manner suggestive of a hedging strategy to survive against antibiotic stresses, as organisms with lower PMF were expected to be physiologically less active and hence more tolerant to antibiotics (Lee et al., 2023). In other words, these studies suggested that the dissipation of PMF would slow down bacterial growth and therefore trigger the onset of tolerance. It should be noted, however, that this theory is contradictory to other reports which showed that PMF is required for tolerance development. In a study by Rao et al. (2008), for example, it was shown that PMF is required for maintaining the survival of hypoxic non-growing *Mycobacterium tuberculosis* (Rao et al., 2008). Although this study did not investigate bacterial antibiotic tolerance, latent *M. tuberculosis* organisms that can reside in the human body for decades are physiologically almost identical to persisters of other bacterial species. Hence the observation that maintaining PMF is important for survival of latent *M. tuberculosis* infers that this function is also required for maintaining phenotypic antibiotic tolerance in bacteria. Interestingly, high redox activity was found to yield a larger number of non-growing tolerant cells, presumably due to the fact that such activity is required to generate PMF (Spoering & Lewis, 2001). This finding is somewhat surprising as it is physiologically expensive to maintain a considerable level of oxidative phosphorylation in tolerant cells or physiologically dormant organisms such as latent *M. tuberculosis*. On the other hand, Feng et al. (2015) showed that most of the new tuberculosis drug candidates discovered by phenotypic screens or genome sequencing are highly lipophilic in nature, and that such compounds act by targeting the hydrophobic cell membrane of *Mycobacterium* species, inhibiting membrane proteins, and causing the collapse of PMF (Feng et al., 2015). Consistently, inhibition of respiration by deletion of genes that encode TCA cycle enzymes or by suppressing electron transport chain (ETC) activities that generate PMF was found to negatively affect tolerance formation in another study (Orman & Brynildsen, 2015). Expression of the caa_3-encoded oxidase, which is highly efficient in creating a proton gradient, was found to play a role in enhancing the survival fitness of *Pseudomonas aeruginosa* in nutrient starvation conditions (Osamura et al., 2017). Likewise, bacteria became less tolerant to stress upon deletion of the *rgpF* gene, which resulted in PMF disruption (Kovacs et al., 2017). The idea that maintaining PMF is necessary for the survival of antibiotic persisters is also supported by the work of Yamamoto et al. (2018), who showed that the expression level of the *ldhA* gene, which encodes for the enzyme lactose dehydrogenase, was elevated in a bacterial population which contained a larger number of persisters (Yamamoto et al., 2018). The group also showed that artificially induced expression of the *ldhA* gene exhibited a tolerance induction effect through enhancement of generation of PMF and ATP synthesis. More direct evidence of the functional importance of PMF in tolerance formation is the observation in our recent study that compounds that cause the dissipation of PMF, such as the ionophores, were found to exhibit the ability to kill antibiotic-tolerant cells (Wang et al., 2021). Taken together, these findings suggest that, although PMF dissipation may lead to tolerance formation in exponentially growing bacterial cells, PMF is actually required for maintaining the viability of tolerant cells in the long term. It is therefore necessary to delineate the underlying mechanisms by which bacteria persisters generate or maintain PMF and investigate whether PMF maintenance in persisters is required to support other important physiological functions essential for the survival of the persisters.

PMF MAINTENANCE IS AN ESSENTIAL ANTIBIOTIC TOLERANCE MECHANISM

A recent study performed in our laboratory confirmed that bacteria need to actively maintain a tolerance phenotype in the long term and that inhibiting the ability to actively maintain the tolerance phenotype can eradicate antibiotic-tolerant persisters completely (Wan et al., 2021). It should be noted that most of the previous studies on bacterial tolerance did not assess the level of stress tolerance for more than 24 h. Using a starvation-induced antibiotic tolerance model, we found that a switch to physiological dormancy can only confer tolerance to antibiotics for approximately 48 h; after this period, bacteria need to maintain PMF to remain tolerant to antibiotics; without the ability to maintain a substantial level of PMF, the persisters died gradually, and the entire tolerant population could be eradicated by ampicillin, a β-lactam antibiotic, within 7 days. This finding that persisters need to actively maintain the tolerance phenotypes beyond a 48 h timeframe has important implications in the development of new drugs to target persisters, as it means that it is possible to kill persisters, presumably including those of antibiotic-resistant strains, by inhibiting the tolerance maintenance mechanisms. One approach our research used to identify the active tolerance mechanisms in persisters is by comparing the gene expression profile in persisters and that of the exponentially growing cells. This type of analysis is

only available in recent years following the introduction of the RNA sequencing (RNAseq) technique. Through this technique, we were able to identify a number of genes whose expression level in persisters that formed during nutrient starvation was even higher than that of the log-phase cells. This observation is significant because the expression of most of the genes is expected to be shut down or significantly reduced in persisters, genes that are over-expressed during starvation should be essential for enhancing survival fitness under adverse environmental conditions. In that study, the function of the genes that were over-expressed in persisters was tested by assessing the effect of deletion of the gene concerned. Based on the results of the analysis of the consistency between the gene expression and gene deletion data, we identified some of the key mechanisms concerned and proposed the concept of active tolerance maintenance in bacteria. We found that the PspA protein was responsible for maintaining the PMF during nutrient starvation and that persisters still actively generated PMF by undergoing a certain level of oxidative phosphorylation, even after they had encountered complete nutrient starvation for 24 h. In fact, we showed that *pspA* was one of the most up-regulated genes upon the onset of starvation-induced tolerance. The Psp response was previously found to play a role in regulating the pathogenicity of enterobacteria (Zhang et al., 2016) and is responsible for maintaining PMF under PMF-dissipating conditions (Engl et al., 2011), but this function has not been reported as being important for tolerance formation. If PMF is important for tolerance development, we postulated that bacteria do not employ PspA as the only PMF maintenance protein but utilize a range of mechanisms to ensure that a sufficient level of PMF is in force to maintain survival fitness under adverse growth conditions. In the literature, the Rcs regulon is another regulatory system documented to be responsible for maintaining PMF. The *rcs* operon contains a total of 19 genes and encodes the production of the colanic acid-rich capsular polysaccharide (Wall et al., 2018). Colonic acid is also known to play a role in maintaining PMF when the bacterial cell encounters specific stress. In a follow-up study, we tested whether specific genes in this operon were also responsible for PMF maintenance during starvation and showed that products of the genes *osmC* and *osmB* (Davalos-Garcia et al., 2001), which encode a periplasmic peroxidase and a lipoprotein, respectively, played a role in mediating functional linkage between the RpoS and Rcs regulon, and also contributed to maintenance of PMF in a manner similar to that of PspA. Another gene tested in this work was *bdm*, the expression of which is regulated by RcsB. This gene was reported to be involved in promoting flagellum synthesis in *Escherichia coli* (Francez-Charlot et al., 2005), but was never suggested to play a role in tolerance formation. Our data confirmed that the product of both the *bdm* and *rcsB* genes were also involved in PMF maintenance in persisters, as deletion of each of these two genes resulted in significantly lower tolerance level. All in all, these data suggest that bacteria need to actively synthesize a wide range of proteins to prevent dissipation of PMF even upon encountering nutrient starvation for up to 144 h. Conceivably, the reason why persisters utilize sophisticated mechanisms to prevent dissipation of PMF is because it is metabolically demanding to generate PMF under adverse conditions.

Apparently, preventing PMF dissipation alone is insufficient to maintain PMF in the long term, as we also found that persisters need to actively generate PMF by undergoing essential respiratory activities (Biquet-Bisquert et al., 2024; Kaila & Wikström, 2021). The ETC is responsible for the generation of PMF and ATP, but the activities of ETC components when bacteria encounter prolonged starvation and develop antibiotic tolerance have not been characterized. Our data showed that the enzymes NADH dehydrogenase I and NADH dehydrogenase II, which are the key components of the ETC encoded by the genes *nuoL* and *ndh* (Allison et al., 2011; Erhardt et al., 2012), play a key role in mediating tolerance formation (Wan et al., 2023). The functional role of other components of the ETC in generating PMF, and hence their role in maintaining the tolerance phenotype, was also confirmed. This finding is consistent with that of Fung et al. (2010), which showed that inhibitors of bacterial respiratory chain such as sodium azide exhibited strong killing effects on bacterial antibiotic persisters. Importantly, we also showed that deletion of genes encoding the ETC components results in the rapid and complete killing of persisters, which is never achievable by deleting a single putative tolerance gene in previously reported studies. When compared to the abolishment of PMF maintenance functions, deletion of the *nuoL* and *ndh* genes appeared to have a stronger tolerance suppression effect, indicating that utilization of multiple mechanisms to prevent dissipation of PMF is not sufficient and that persisters still need to constantly generate PMF to support essential physiological activities required for prolonged survival under antibiotic and other stresses.

The functional importance of PMF maintenance in persisters has been confirmed by other studies published more recently. A work by Mohiuddin, Massahi, and Orman (2022) identified another protein that helps maintain PMF in persisters. By performing high throughput screening of the promoter library, this research team found that deletion of the *waaG* gene resulted in the dissipation of PMF and caused a reduction in the size of the bacterial persister population at the stationary phase. The WaaG protein is a lipopolysaccharide glucosyltransferase known to play a role in shaping the outer membrane structure, which is presumably required for the effective maintenance of

PMF. In a related study, the same research team also found that phenothiazine drugs are highly effective in eradicating persisters by multiple actions, including inhibition of transcription and translation, perturbing energy metabolism and disrupting PMF (Mohiuddin, Massahi, & Orman, 2022a; Mohiuddin, Nguyen, & Orman, 2022b). These works prove beyond doubt that persisters generate and utilize much energy to support a range of defence and repair functions to survive against environmental stresses and the deleterious effects of antibiotics.

Consistently, we also identified a number of membrane-bound transporters that become functionally active during starvation and play a role in the formation of stress tolerance; amongst them, TolC-EmrKY and ChaAB are two major efflux systems that can exude antibiotics or various toxic metabolites out of the bacterial cell during starvation, enabling bacteria to survive in extreme environment (Wan et al., 2021). Apparently, this is one of the reasons why persisters need to maintain PMF even upon encountering starvation for a prolonged period, in addition to supporting the generation of energy required to support other essential defence functions. Nevertheless, these observations raise a number of new questions. First, it appears that persisters utilize specific efflux pumps to perform membrane transport activities. Efflux pumps have substrate specificity; a previous study also showed that persisters exhibited efflux activity towards drugs that were substrates for specific pumps (Bergmiller et al., 2017); why specific pumps are chosen in persisters is not clear. Second, the regulatory systems that are responsible for activating PMF maintenance functions as well as mediating changes in the level of synthesis of specific efflux pumps need to be identified.

The latest research findings on the importance of PMF in maintenance of antibiotic tolerance phenotypes in persisters are summarized and depicted in Figure 1. First, PMF is actively generated by ETC activities which also play a role in energy (ATP) production; second, dissipation of PMF in persisters is prevented by a number of proteins for which the expression level was up-regulated significantly during tolerance formation, such as PspA (b) and third, PMF is required to support other tolerance mechanisms, such as DNA synthesis and repair functions that reverse damages in nucleic acids inflicted by environmental stresses, as well as efflux activities that exude toxic metabolites and antibiotics in persisters. It should also be noted that PMF and ATP are both required to support the synthesis of various proteins that play key functional roles in the aforementioned tolerance mechanisms. The finding that persisters need to actively maintain PMF and efflux activity to survive under starvation conditions or other stresses provides us an opportunity to develop a new antimicrobial strategy that works by targeting bacteria, including the resistant strains, at their tolerance stage.

APPLICATION POTENTIAL OF BACTERIAL ANTIBIOTIC TOLERANCE SUPPRESSION TECHNOLOGY

The incidence of fatal hospital infections caused by newly emerged resistant and highly virulent bacterial strains has increased significantly worldwide. There is an urgent need for the development of novel antimicrobial strategies for the purpose of infection control and clinical treatment of infections caused by the virulent and drug-resistant strains of various bacterial pathogens. The new findings regarding active tolerance mechanisms in bacterial persisters have important implications in the development of new antimicrobial strategies because bacteria of all types, including the multidrug-resistant and highly virulent organisms, often encounter various types of stresses in the environment as well as during the infection process, and exhibit variable degrees of stress tolerance. However, the development of a clinically feasible approach that can suppress the expression of the virulence and resistance-encoding genes must fulfil at least one of the following two criteria: (i) undermine bacterial survival under both environmental and host-inflicted stresses regardless of the virulence level and drug susceptibility status of the organisms concerned and (ii) suppress expression or export of both plasmid and chromosome-encoded virulence factors and antibiotic-degrading enzymes. Since persisters need to completely adjust their gene expression patterns so that only the essential cellular mechanisms, such as the PspA-mediated PMF maintenance functions, are expressed, they may have to suppress or even halt the expression and export of virulence factors and antibiotic-degrading enzymes. Hence, targeting PMF maintenance mechanisms or directly disrupting PMF should be an excellent antimicrobial strategy, regardless of the virulence and drug susceptibility status of the strains concerned. If suppressing PMF has the effect of not only reducing the survival fitness of persisters but also interfering with the extrusion of virulence factors and antibiotic-degrading enzymes, this antimicrobial approach can fulfil the stringent criteria of effective suppression of expression of hypervirulence and resistance-encoding genes in multidrug resistant/hypervirulent bacterial pathogens. Indeed, our recent works showed that disrupting PMF can, surprisingly, sensitize persisters to a wide range of antibiotics including the β-lactam, fluoroquinolone and aminoglycoside antibiotics, which inhibit a wide range of cellular functions such as DNA and protein synthesis (Wang et al., 2021). Although starvation-induced persisters are physiologically inactive, they are expected to maintain a minimum of essential functions, which are often energy-dependent (Chapman et al., 1971). In view of the fact that disrupting PMF has the effect of reducing efflux activities and

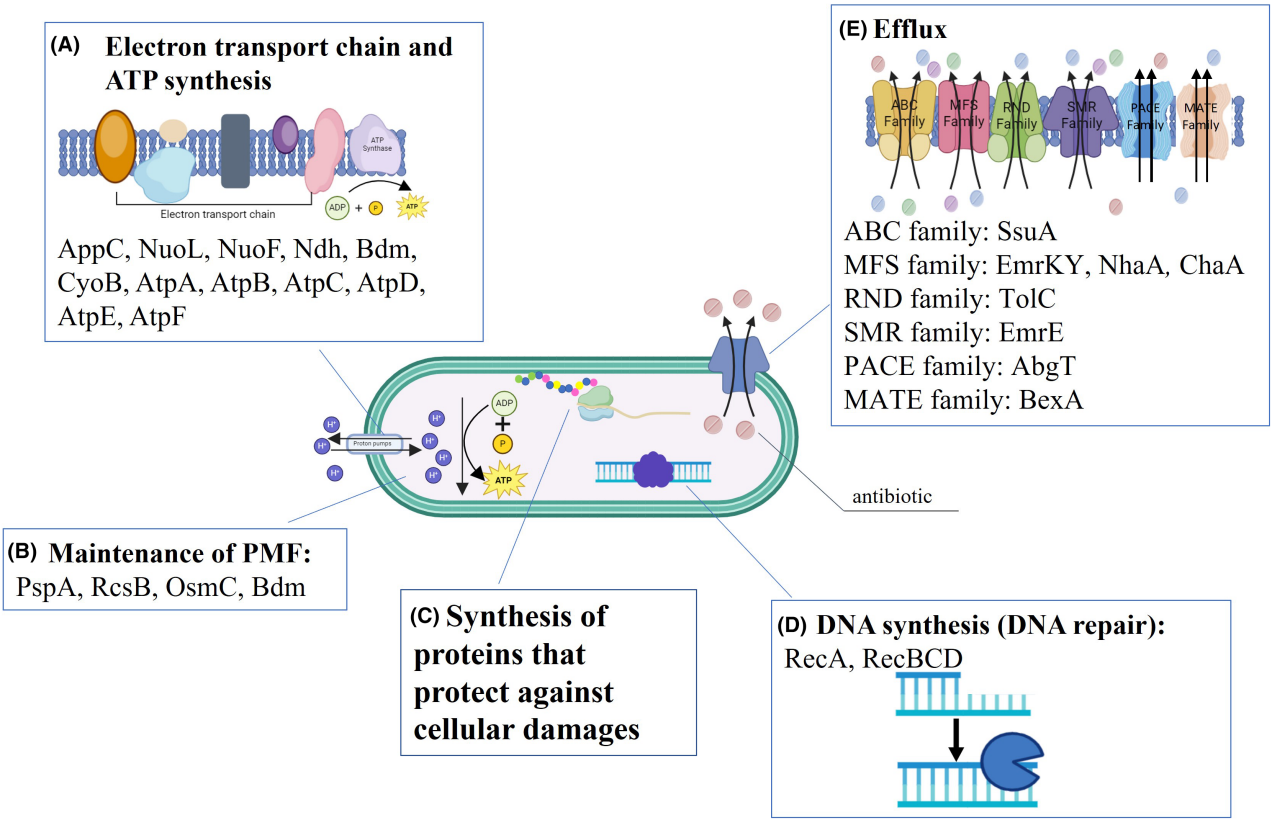

FIGURE 1 Proton motive force (PMF) is required to support a range of actively expressed cellular mechanisms that play a role in the maintenance of antibiotic tolerance phenotypes in bacterial antibiotic persisters. PMF is actively generated by electron transport chain activities which also play a role in energy (ATP) production (A) dissipation of PMF in persisters is prevented by a number of proteins including PspA (B) PMF and ATP-dependent tolerance mechanisms include protein synthesis functions that are activated to produce various proteins involved in protection against cellular damages (C) DNA synthesis and repair functions that reverse damages in nucleic acids inflicted by environmental stresses (D) as well as efflux activities that exude toxic metabolites and antibiotics in persisters (E). Representative proteins in each category of tolerance mechanism in bacterial antibiotic persisters are shown.

allowing accumulation of antibiotics in the intracellular compartment, we believe our preliminary findings actually imply that persisters undergo a substantial level of cell wall re-structuring, DNA repair and protein synthesis activities so that they may become sensitive to various antibiotics when the drug concentration increases to a certain level as a result of reduced efflux. This idea is consistent with the findings of Pu et al. and Völzing et al. as mentioned above, which show that persisters undergo DNA repair and efflux, respectively (Völzing & Brynildsen, 2015). If this is indeed the case, it means that PMF dissipators can also act synergistically with various other antibiotics to produce strong anti-persister effects, and that existing antibiotics including fluoroquinolones and aminoglycosides which inhibit DNA and protein synthesis may by themselves exert killing effects on persisters upon prolonged treatment, as these antibiotics can inhibit the essential DNA repair functions and the ability to synthesize proteins involved in mediating stress defence. One concern is that the uptake of aminoglycosides is dependent on PMF, hence dissipating PMF may gradually antagonize the effect of this class of antibiotics by hindering drug uptake. Nevertheless, persisters may not survive for long if PMF is weakened. It should also be noted that the types of cellular activities that remained turned on in persisters have to be identified, so as to devise the drug combination that can eradicate persisters most effectively.

PMF DISSIPATORS AS POTENTIAL ANTI-TOLERANCE AGENTS

Known PMF dissipators are often too toxic to be used as persister-killing agents because mammalian cells are also susceptible to such agents (Farha et al., 2013). Inhibitors of respiratory chain components should also be highly effective in disrupting the maintenance of PMF in persisters but are extremely toxic and cannot be applied clinically. One strategy is to use a non-toxic level of PMF dissipator which is not able to kill persisters but may be sufficient to inhibit the ability of the organism to export virulence factors or antibiotic-degrading enzymes. Another approach is to use an adjuvant compound to enhance the effect of PMF dissipators. Our

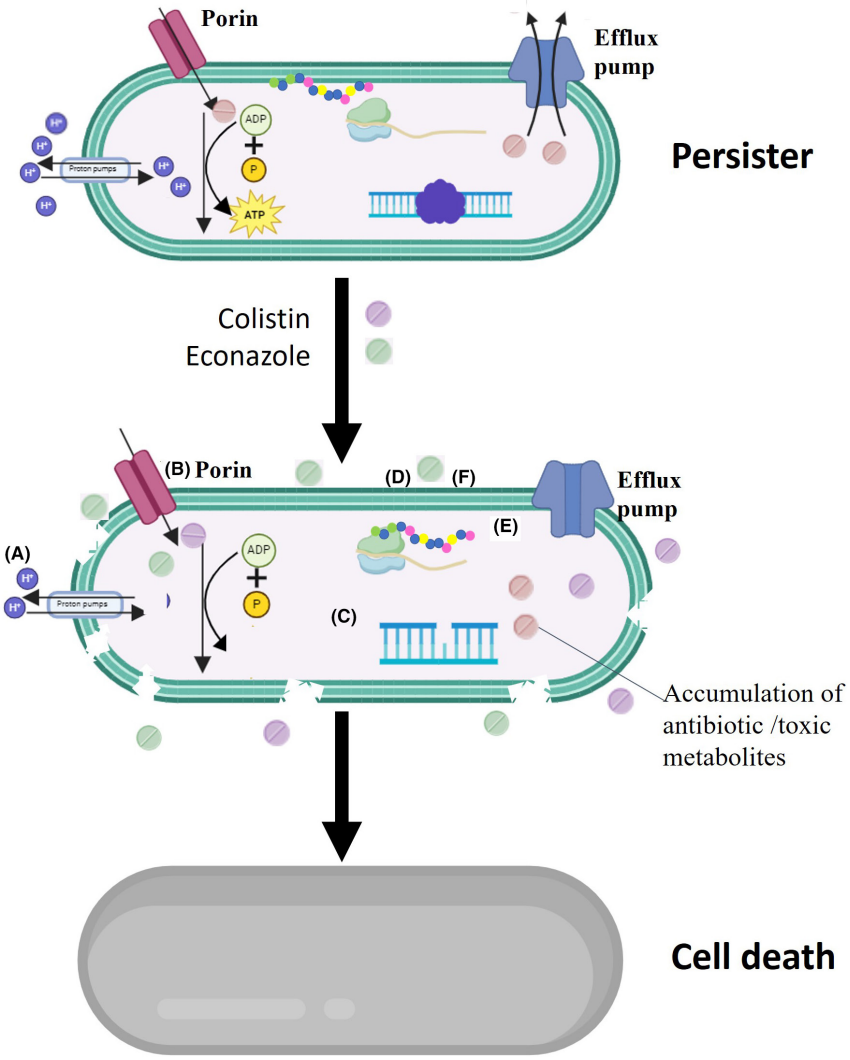

FIGURE 2 A feasible approach to inhibit the ability of bacterial persisters to express phenotypic antibiotic tolerance by suppressing active cellular mechanisms involved in the maintenance of the tolerance phenotype. Combined usage of a PMF dissipator such as econazole and the antibiotic colistin allows a much lower dosage of each agent to be applied for treatment as the two agents act synergistically to cause PMF dissipation in the following manner. Colistin can cause membrane damage and hence dissipation of PMF; membrane damage can lead to the dislocation of electron transport chain components, thereby affecting the ability to generate PMF and produce ATP in persisters (A). Leakage in the membrane would also accelerate the influx of both the PMF dissipator and colistin, eliciting a vicious cycle of membrane damage and PMF dissipation (B). These events would affect energy production and therefore impede the ability of persisters to repair DNA damage (C) and synthesize the wide range of proteins involved in the maintenance of the tolerance phenotypes (D). Disruption of PMF would also affect efflux and cause accumulation of toxic metabolites and antibiotics, the latter may further inhibit DNA repair and synthesis of the wide range of proteins required to maintain the survival fitness of persisters (E). Lower PMF and ATP levels may also impede the synthesis and export of virulence factors and resistance enzymes in persisters of virulent and antibiotic-resistant strains, respectively (F).

laboratory has recently identified an FDA-approved drug known as econazole that can act as a non-toxic PMF dissipator when we screened for compounds that can act synergistically with the membrane-targeting antibiotic colistin to kill colistin-resistant strains (Xie et al., 2022; Xu et al., 2020). The reason why we tested the enhancement effect of PMF dissipator on colistin activity is that colistin itself acts specifically on the cell membrane of Gram-negative bacteria to suppress PMF formation, hence PMF dissipators and colistin should act synergistically to disrupt PMF. Our data indeed showed that the reason why econazole can be used clinically to cause dissipation of bacterial PMF without exerting an unbearable level of toxicity on human is that its PMF-suppressing effect can be enhanced by colistin, thereby allowing the use of a much lower dose of this agent to exert its PMF suppressing effect. In fact, the opposite is also true, that is, the presence of PMF dissipator allows a much lower or non-toxic dose of colistin to exert its antimicrobial effect. This finding is clinically important as colistin-resistant mutants have emerged and become prevalent in the past few years

(Zafer et al., 2019). It should also be noted that colistin may inflict membrane damage in bacteria and potentially cause dislocation of the ETC components in the inner membrane of Gram-negative bacteria, thereby undermining the ability of bacterial persisters to generate PMF (Xu et al., 2024). Hence the persister killing effect of a colistin and PMF dissipator combination is particularly strong. Econazole is usually used topically, whether a non-toxic dosage of econazole or other PMF dissipators can restore the clinical value of colistin in the treatment of various infections remains to be determined. In view of the vast potential of PMF-suppressing agents and their ability to enhance the antimicrobial activity of currently used antibiotics, more works need to be done to further investigate whether PMF dissipators, when used alone or in combination with colistin, can effectively eradicate antibiotic persisters or suppress the ability of persisters of multidrug-resistant/hypervirulent bacterial pathogens to express their virulence and resistance phenotypes by interfering with the ability to export virulence factors and resistance enzymes.

A number of compounds that exhibit the potential to kill bacterial persisters have been identified in recent years (Defraine et al., 2018). The mechanism of action of some of the key compounds involves disrupting bacterial cell membrane and collapsing membrane potential, highlighting the functional importance of PMF in the maintenance of phenotypic tolerance. Feasible approaches for inhibition of the ability of persisters to maintain antibiotic tolerance phenotypes are shown in Figure 2. These include inflicting membrane damage to dislocate ETC components and suppress the ability to generate PMF and produce ATP in persisters, and causing leakage in the membrane to accelerate the influx of both the PMF dissipator and colistin and elicit a vicious cycle of membrane damage and PMF dissipation. These events would completely inhibit energy production in persisters and impede their ability to repair DNA damage and synthesize proteins involved in the maintenance of the tolerance phenotypes. Disruption of PMF would also affect efflux and cause accumulation of toxic metabolites and antibiotics, thereby further inhibiting DNA repair and synthesis of proteins required to maintain the survival fitness of persisters. Lower PMF and ATP levels may also impede the synthesis and export of virulence factors and resistance enzymes in persisters of virulent and antibiotic-resistant strains, respectively. Further research in these directions should generate sufficient scientific data that allow us to explore novel ways of utilizing PMF dissipating agents, alone and in combination with appropriate antimicrobial agents such as aminoglycosides, fluoroquinolones and polymyxin, to treat drug-resistant, chronic, recurrent and other potentially fatal bacterial infections by eradicating bacterial cells which inevitably enter the tolerance mode from time to time during the infection process.

AUTHOR CONTRIBUTIONS

Yingkun Wan: Writing - review & editing, Writing - original draft. Jiaqi Zheng: Writing - review & editing. Edward Wai-Chi Chan: Writing - review & editing, Writing - original draft, Supervision. Sheng Chen: Writing - review & editing, Writing - original draft, Supervision, Funding

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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