MECHANISMS UNDERLYING THE ACTIVE BACTERIAL TOLERANCE RESPONSE AND DEVELOPMENT OF THERAPIES AGAINST CLINICAL BACTERIAL PERSISTERS

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Mechanisms Underlying the Active Bacterial Tolerance Response and Development of Therapies Against Clinical Bacterial Persisters

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

Bacteria tolerance are largely responsible for the recalcitrance of chronic infections with the underlying mechanisms remains incompletely revealed. Recent studies reveal that physiological dormancy alone is insufficient for maintaining a long-lasting phenotype and nutrient starvation is a known trigger of non-heritable phenotypic antibiotic tolerance in bacteria. In an attempt to investigate if active starvation-induced physiological responses underlie tolerance development, we applied RNA sequencing (RNA-Seq) to investigate whether bacteria actively adjusted gene expression patterns in response to starvation and showed that genes of the phage shock protein (psp) family were consistently over-expressed even under prolonged starvation conditions in an E. coli model. One product of this gene family, PspA, was found to play a pivotal role in maintenance of starvation-induced tolerance by preventing dissipation of the transmembrane proton motive force (PMF). Deletion of the pspA gene resulted in more rapid reduction in tolerance level during starvation process. We found that maintaining the transmembrane PMF is essential for formation of antibiotic tolerance in both Gram-negative and positive bacteria, including *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *S. aureus*. Rapid and complete eradication of the entire tolerant sub-population of the test strains could be achieved by complete disruption of PMF by the ionophore CCCP. Most importantly, we found that an FDA-approved antifungal drug, econazole, could also produce such effect in a non-toxic manner. We consider econazole-mediated PMF disruption a feasible strategy for prevention of occurrence of chronic and recurrent bacterial infections, especially among immunocompromised patients.

Apart from the investigation of the mechanisms underlying bacterial tolerance response, we aimed to discover nutrient compounds which reverted tolerant cells to become re-susceptible to conventional antibiotics by phenotype microarrays (PM) and found that *N*-acetyl-D-glucosamine (GlcNAc) or D-glucosamine (GlcN) altered persisters' β-lactam susceptibility. We demonstrated that cell structures completely collapsed after synergistic treatment of β-lactam, GlcNAc and cytoplasmic β-lactam amount was increased in the presence of GlcNAc. We found that the GlcNAc catabolism pathway was involved in the its resensitization effect as on one hand the amount of fructose-6-phosphate (Fru-6-P), GlcNAc catabolism product and the precursor of glycolysis, increased and another hand resensitization effect diminished after inhibition of NADH oxidation which is the major purpose of glycolysis. Additionally, we detected that GlcNAc triggered the produce of peptidoglycan precursor, UDP- *N*-acetyl-D-glucosamine (UDP-GlcNAc) and subsequently the re-activation of peptidoglycan biosynthesis which is the target of β-lactam. The mechanism underlying GlcNAc resensitization is complicated and may be the cooperation effect of GlcNAc catabolism and activated peptidoglycan biosynthesis. Our findings imply that GlcNAc or GlcN as adjuvants to β-lactam would be beneficial in the treatment of clinical chronic infections.

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Chapter One: Introduction

1.1 Background

Bacterial antibiotic persisters were first described in 1944 by Joseph Bigger, who observed that penicillin often failed to completely sterilize soldiers' wounds and recurrent infections often occurred after therapy [1]. Persistence was defined as the ability of bacteria to remain viable in the host or hostile environment for a prolonged period of time. Members of a subpopulation of slow-growing bacterial cells that have a decreased susceptibility to be killing by bactericidal does antibiotics within an otherwise susceptible clonal population, owing to a low target activity or low antibiotic uptake inducible by stress, are named persisters [2]. This phenomenon is thought to be ubiquitous among bacterial species and can be observed in *Salmonella enterica*, *Escherichia. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *enterica serovar Typhimurium* and *M. tuberculosis* [2-5].Persistence is recognized as one of the causes of antibiotic treatment failure and relapsing infections as persisters will resume growth after termination of antibiotic treatment.

Although persisters cannot be killed by a lethal dosage of antibiotics, they are totally different from resistant cells in terms of phenotypic characteristics, formation mechanism, and quantification parameters. Resistance is conferred by the inherited ability of microorganisms to grow at high concentrations of an antibiotics, irrespective of the duration of treatment [6]. Persistence is the ability which susceptible cells adjust metabolism, survive under hostile environment, become tolerant to transient antibiotics exposure and resume proliferation of a new generation which is susceptible to antibiotics. Persistence is quantified by the

minimum duration for killing (MDK), while resistance is depicted by the minimum inhibitory concentration (MIC). Persistence is formed upon cell metabolic adaptation without mutation and is therefore nonheritable. Resistance typically arises through mechanisms that block the interaction of drug with its target by genetic mutation of the target gene, enzymatic deactivation of drug or loss of an enzyme required to activate prodrug and is heritable **(Fig 1.1)** [7, 8]. Although the underlying mechanisms of persistence and resistance are different from each other, persistence boosts the chances for the emergence of resistance [9].This correlation is due to the fact that persisters act as a viable cell reservoir from which resistant mutants can generate by *de novo* chromosomal mutations or horizontal gene transfer [10]. Persistence also positively correlates with mutation rates which enhances the proportion of genetic resistance evolution [11]. Environmental stresses such as starvation, hypoxia, and antimicrobial action trigger the onset of SOS-induced mutagenesis, which is mediated by error-prone DNA polymerases and enhances the chance of resistance development. Apart from SOS mutagenesis, homologous recombination which switches from high-fidelity polymerase to error-prone polymerase is used by the bacterial cell during stress environment. Sub-lethal concentration antibiotics and carbon source starvation induce mutagenesis via RpoS-regulon mediated error-prone DNA polymerase IV [12]. Given the connection between persistence and resistance, persistence is recognized as a major cause of antibiotic treatment failure.

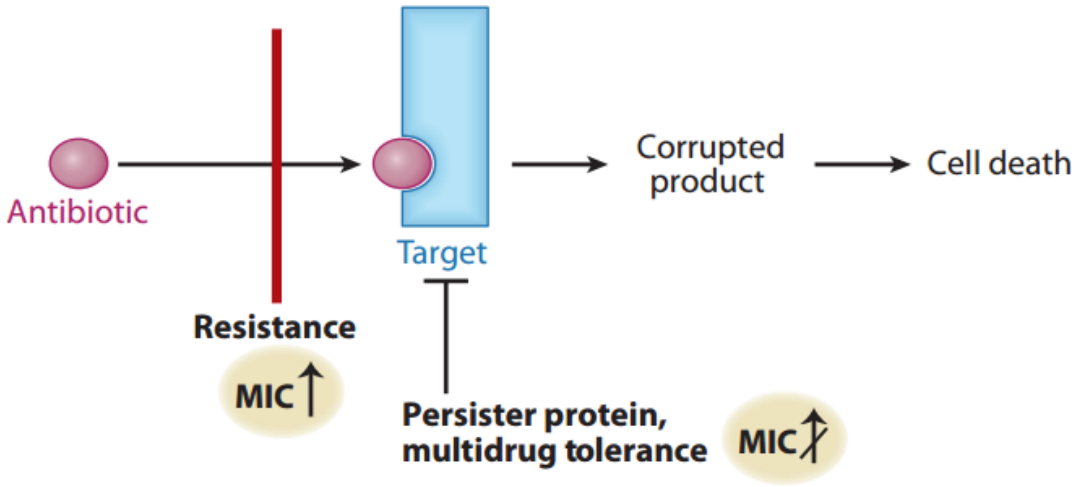

Fig 1.1 Resistance versus persistence.

Persister proteins act by blocking the target, so no corrupted product was produced. In contrast, resistance prevents antibiotic from binding to the target[8].

In clinical settings, patients often suffer from bacterial infections which require long-lasting and repeated antibiotics treatment, but often end up with treatment failure regardless of whether or not the infecting agent possess genetic mutations that confer a resistance phenotype. Most of patients with cystic fibrosis (CF) become chronically infected with *P. aeruginosa* which is associated with accelerated decline in lung function and therefore high mortality [13]. A state of asymptomatic typhoid fever, which is caused by *S. enterica* subsp. *enterica* serovar Typhi, occurs in 2-5% patients, in which a large population of bacteria are shed from the gallbladder, rendering it necessary to take four times longer to treat than acute typhoid fever, thereby substantially increasing the risk of gallbladder cancer [14].Tuberculosis is perhaps the most prominent case of a chronic infection as *M. tuberculosis* has a long proliferation period, and may form granulomas to escape immune response and drug killing. It is reported that 20% of individuals who were infected with latent *M. tuberculosis* following chemotherapy would relapse during their lifetime [15].

Typhoid fever is recurrent in 15% of patients after antibiotic treatment and often accompanied by a urinary tract infection caused by uropathogenic *E.coli* (UPEC); in addition 27% of female patients will suffer recurrent infection [16]. Half of all nosocomial infections are due to persistent infections associated with indwelling medical devices, the formation of biofilm in the surface of these devices are notoriously recalcitrant to drugs. Persister infections are deadly in immunocompromised patients undergoing cancer chemotherapy or infected with HIV. Persistent infections are increasingly prevalent and associated with increasing morbidity and mortality. It is urgent to understand mechanism of persistence formation and explore drugs to eradicate persisters.

1.2 Stochastic and deterministic factors control persistence formation

1.2.1 Stochasticity: bet-hedging strategy for bacterial survival

In the mid-exponential phase *E. coli*, a small sub-population (about 10^{-5} surviving ratio) survives from the lethal dose of antibiotic. By microfluidic observation of single *E.coli* cells, Balaban *et al* found that some cells entered into a state of non-growing persisters, which can form spontaneously in a favorable environment [17]. It was implicated that persisters are not entirely induced by stresses, but constitute drug-independent, pre-existing variants. This strategy is considered as a bet-hedging behavior, which maximizes the fitness of the population in periodically changing environments **(Fig 1.2A)** [18, 19]. Stochasticity also demonstrates the phenotypic heterogeneity of an isogenic population to protect cells against unexpected episodes of changing environments. The prominent mechanism of stochastic persistence is fluctuations in the concentrations of a small number of dedicated proteins which mediate persister formation [20]. The failure of becoming persisters in the early

exponential phase may result from the low level of persister protein production. However, fluctuations in protein expression alone is not enough to produce a large persister population unless it is amplified by other triggering processes, which are in turn elicited by other factors.

1.2.2Determinism: environmental factors affect persistence formation

Spontaneous persister formation is just one type of persister phenomena that does not adequately capture the total characteristics of persisters. Indeed, persister levels are largely affected by several environmental elements, implicating a deterministic aspect in the formation of persister. It seems that two processes together control persistence – a stochastic fluctuation in the level of persister proteins and the environmental factors, which control mean level of expression of these proteins, determine the size of the persister sub-population **(Fig 1.2)**.

1.2.2.1Nutrient limitation

The physiology of bacteria changes considerably if the majority of nutrients are exhausted. It has been shown that the persister fraction of a bacterial population increased abundantly upon nutrient deprivation [21]. It was reported that SHX-induced serine starvation resulted in persister formation as it stimulated (p)ppGpp production [22]. Apart from artificial deprivation of nutrients, stationary-phase and biofilms cells are also known to suffer from nutrient limitation spontaneously. It was reported that the stringent response (SR) was stimulated under starvation and played a role in mediating persister formation via decreasing the levels of reactive oxidative species (ROS), such as hydroxyl radical (▪OH) and HAQs [22]. Specifically, carbon source transitions trigger fluoroquinolone persister formation as it inhibits DNA gyrase activity by toxin-antitoxin (TA) module [23].

1.2.2.2Stationary phase

When a bacterial culture reached stationary phase, nutrients were exhausted with a drastic competition among the surviving cells and the population entered into a phase of slowing growth balanced with cell death. Apart from nutrient limitation and growth arrest, the age of inocula affects persister levels dramatically [24]. Consequently, stationary-phase cells harbor 10^3 to 10^4 times more persisters than exponential culture [25]. It is hypothesized that inactive metabolism, including reduction in drug target activities, underlies onset of stationary phase-induced persistence **(Fig 1.2B)**. Growth arrest confers persistence to β-lactams as cell wall synthesis process is decreased. Similarly, it requires highly active transcription or DNA replication for quinolones to exert bactericidal effect [26], but stationary phase cells are regulated by sigma factor RpoS, which changes the gene expression pattern and confers persistence [27]. SOS response which is essential for DNA damage repair is involved in stationary phase induced quinolone persistence as those persisters require it to eliminate the unresolved DNA nicks created by quinolones [27]. It seems these responses or proteins constitute a multiple and complicated network to alter the physiology of stationary phase cells and facilitate them to become tolerant to antibiotics.

1.2.2.3Biofilm

Biofilm structure can protect the bacteria cells residing inside as cells with biofilm-associated lifestyle become embedded in extracellular matrix polysaccharides, which protect cells from the bactericidal effect of antibiotics and hampers the host immune response. Within the biofilm, persister cells consist of a small subpopulation

which exhibits multidrug tolerance without genetic changes **(Fig 1.2E)**. Biofilm is common in the urinary tract infection and often attached to the indwelling devices. It is hard to eradicate and is strongly linked with chronic and recurrent clinical infections [28]. It was reported that matrix polysaccharides obstructed penetration of antibiotics, leading to failure in treatment of biofilm-associated infection. For example, tobramycin which is positively charged, was sequestered in the periphery of biofilms [29]. Kohanski *et al* reported that antibiotics such as β-lactam, fluoroquinolones and aminoglycosides triggered the generation of ROS, which damage cellular structures and led to cell death[30]. Biofilms cells contain less ROS under ceftazidime or piperacillin treatment, when compared to planktonic cells. Ciprofloxacin induces ROS in planktonic cells, but does not induce ROS in biofilms cells, due to the increasing concentration superoxide dismutase (SOD) and glutathione levels detected in biofilms [31]. The level of expression of efflux genes, such as those in the *mexAB-oprM* and *mexCD-oprJ*, was evaluated in biofilm-embedded cells. The fraction of cells tolerant to colistin was found to decrease significantly in the biofilm of the *mexAB-oprM* mutant [32]. Those physiological changes confer biofilm cells a much higher level of phenotypic tolerance than planktonic cells.

1.2.2.4Sublethal antibiotic treatment

Upon exposure to a sublethal dose antibiotics, the susceptibility of bacteria to the same antibiotic and other types antibiotics commonly decreased but this phenotype cannot be inherited by the next generation [33]. Persister fraction improves dramatically under the pretreatment with different types of antibiotics, such as ampicillin (halting cell wall synthesis), ciprofloxacin (haling DNA replication), rifampin (halting transcription) and tetracycline (halting translation) **(Fig 1.2C)** [33,

34]. In the presence of sub-MICs of antibiotics, cells produce more ATP and exhibit a higher translation capacity, with more β-galactosidase detectable when compared to untreated bacteria. Tryptophan catabolism, which coverts tryptophan and cysteine into pyruvate and then enters into the TCA cycle, is induced and results in amino acid starvation upon pretreatment with ampicillin. Those core hermetic stress responses induce the general stress response which is related to persistence formation. The stringent response is triggered by sublethal antibiotic concentrations, as the amount of (p)ppGpp increased under such conditions. RpoS regulon is also known to be involved in persistence form, and its expression is also initiated by pretreatment of antibiotics [35].

1.2.2.5Phagocytic vacuoles

Bacteria exhibit greatly increased tolerance to the bactericidal effect of antibiotic when they are inside host cell, as the phagocytic vacuoles of macrophages create a hostile environment, including nutrient deprivation, acidification and in-toxic metals **(Fig 1.2D)**. Helaine *et al* reported phagocytic vacuoles environment induced *Salmonella* phenotypic heterogeneity, leading to rapid formation of non-replicating cells which served as a reservoir for persisters, and 14 TA modules were involved in this process, such as *relBE, higAB, vapBC* [36]. Later, researchers found that even though most of nonreplicating *Salmonella* formed persisters, the majority of persister population was derived from the moderately growing, partially tolerant cells as the amount of nonreplicating *Salmonella* were small [5].Consistently, *mycobacterium tuberculosis* phenotypic heterogeneity was reported in host cell and host immunity factors including interferon-γ and anti-tuberculosis drugs such as isoniazid contribute to formation of non-growing bacteria [37]. Recently, Helaine *et al* found that dormancy alone was not sufficient to mediate persister formation in

phagocytic vacuoles and persisters reprogramed macrophages to inhibit proinflammatory innate immune response and trigger anti-inflammatory macrophage polarization, which facilitated persister cells to survive in host by the effectors secreted by *Salmonella* pathogenicity island 2 type 3 secretion system (T3SS) [38].

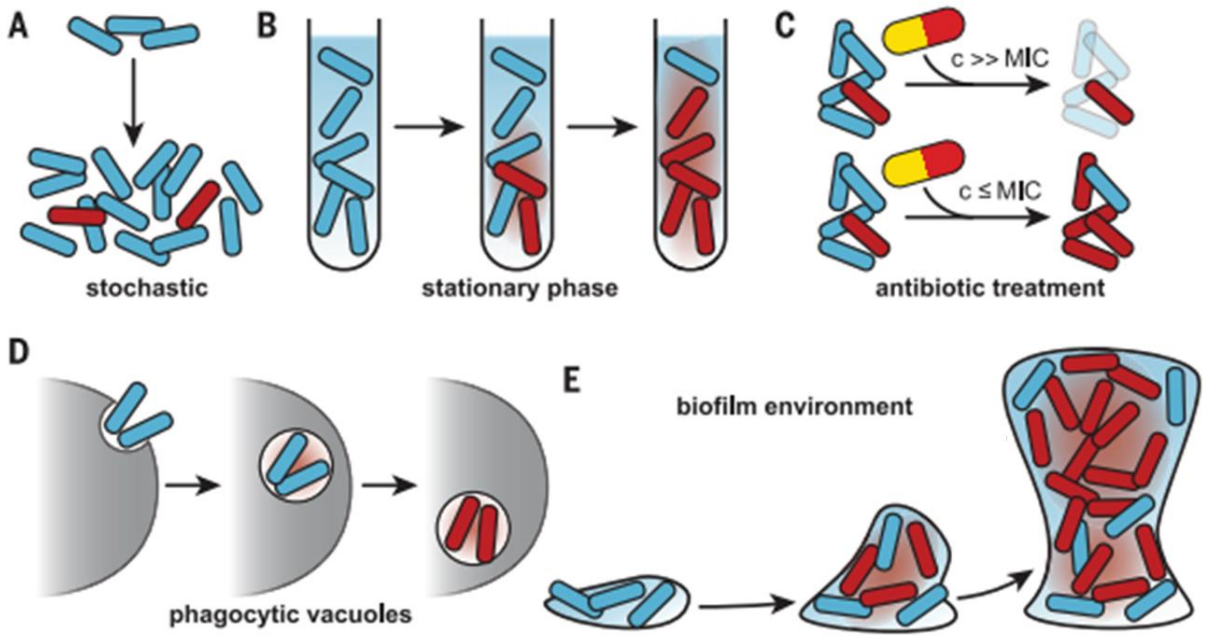

Fig 1.2 Environmental cues underlying persister formation.

(A) Stochastic persister formation occur spontaneously and involves a small sub-population. Different environmental conditions induce the formation of persister cells (red) from regular cells (blue), such as (B) stationary phase, (C) sublethal antibiotic treatment, (D) phagocytic vacuoles, (E) biofilm. c, concentration; MIC, minimum inhibitory concentration [39].

1.2.2.6Other inducers

Apart from the elements mentioned above, many other factors also induce persister formation, such as indole, heat and hypoxic conditions. Indole-induced persister fraction was monitored by time-lapse analysis of fluorescence in microfluidic device and it was shown that this phenomenon was caused by oxidative-stress and phage-shock pathway, as the persister sub-population diminished after deletion of the related genes such as *flu, oxyR, pspB* and *pspC* [40]. Heating induces persistence formation as heat shock proteins are involved in mediating persister formation [41, 42]. Hypoxic exposure also renders cells more tolerant to antibiotics, especially in *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*, and is related to proton motive force (PMF) maintenance and ATP generation [43, 44].Persistence is a complicated phenomenon in which the underlying mechanism remains poorly understood and the inducers are diverse. Some other unknown stresses maybe also have the capacity to lead to persistence and remain to be discovered.

1.3 Molecular mechanisms underlying bacterial persistence

Persister cells have typically been described to be dormant as dormancy protects cells from antibiotic killing because of target inactivity. Many reports indicate that DNA replication, transcription, translation and protein synthesis are inhibited in persister cells by modulation of (p) ppGpp, RopS, TA modules. However, dormancy cannot explain the physiological changes of persisters totally, as not all nonreplicating cells are persisters [45], stationary phase induced persisters show high metabolic oxidative activity [46], persisters inside phagocytic vacuoles maintain an active secretion system to survive against host immune response [38] and target inactivity is not enough to withstand fluoroquinolones as DNA repair is detected and demonstrated to be essential after treatment with fluoroquinolones [47]. Hence the mechanisms underlying persistence are complicated and have not been completely revealed.

1.3.1 Stringent response and (p) ppGpp signaling

Bacteria use the sensory systems to monitor the environment change and adapt themselves to stressful changes, including cyclic AMP (cAMP), cyclic-di-GMP (c-di-GMP) and guanosine pentaphosphate and guanosine tetraphosphate ((p)ppGpp). Bacterial persistence formation is induced by stochastic synthesis of (p)ppGpp in rare exponential cells and increasing production of (p)ppGpp under stationary phase, biofilm. The mechanism which lead to increase in the cellular amount of (p) ppGpp and reprograming of many cellular processes upon exposure to stresses is called the stringent response. The RelA-SpoT homologue (RSH) are the key enzymes regulating (p) ppGpp synthesis and hydrolysis. Persister fraction drastically declines in *relA spoT* mutants of *E. coli* and *P. aeruginosa* under stationary phase and biofilm-forming conditions [22, 48].

RelA synthesizes (p) ppGpp when sensing amino acid starvation and heat shock [49, 50]. It is a ribosome-associated protein, so during amino acid deprivation, it is drastically activated as deacylated tRNAs accumulate, and enter the A-site [51]. SpoT can sense various stress signals, such as fatty acid, iron and carbon source starvation [52-54]. It is a weak synthetic enzyme and strong hydrolytic enzyme for (p) ppGpp. The hydrolysis process is essential for the balance of (p) ppGpp in the presence of RelA as deletion of the *spoT* gene leads to cell death **(Fig 1.3)** [54]. Purification of full-length SpoT has not been successful, so the functional characteristics of SpoT remain unknown.

RNAP (RNA polymerase) activity is changed and then transcription is regulated along with (p) ppGpp accumulation. In *E.coli,* (p) ppGpp inhibits transcription by

directly interacting with RNAP to destabilize the open complexes at certain promoters, such as rRNA genes promoters [55]. DksA is an important enhancer of (p) ppGpp signal and it binds to the opposite side of RANP [56]. Apart from this, (p) ppGpp alters the preference of core RNAP to σ-factor. The alarmone dampens RNAP binding to σ^{70}-dependent promoters which mainly encode the genes of protein, lipid and DNA synthesis, and facilitates binding of the core RNAP to the other σ-factors, it then recognizes and specifically transcribes the genes related to stress responses **(Fig 1.3)** [57]. In *B.subtilis*, (p) ppGpp regulates transcription in an indirect way by reducing the GTP/ATP ratio, as it consumes GTP to produce (p) ppGpp and the alarone itself dampens GTP synthesis enzymes. Hence transcription which starts with guanosine is downregulated and the one which starts with adenosine is upregulated [58].

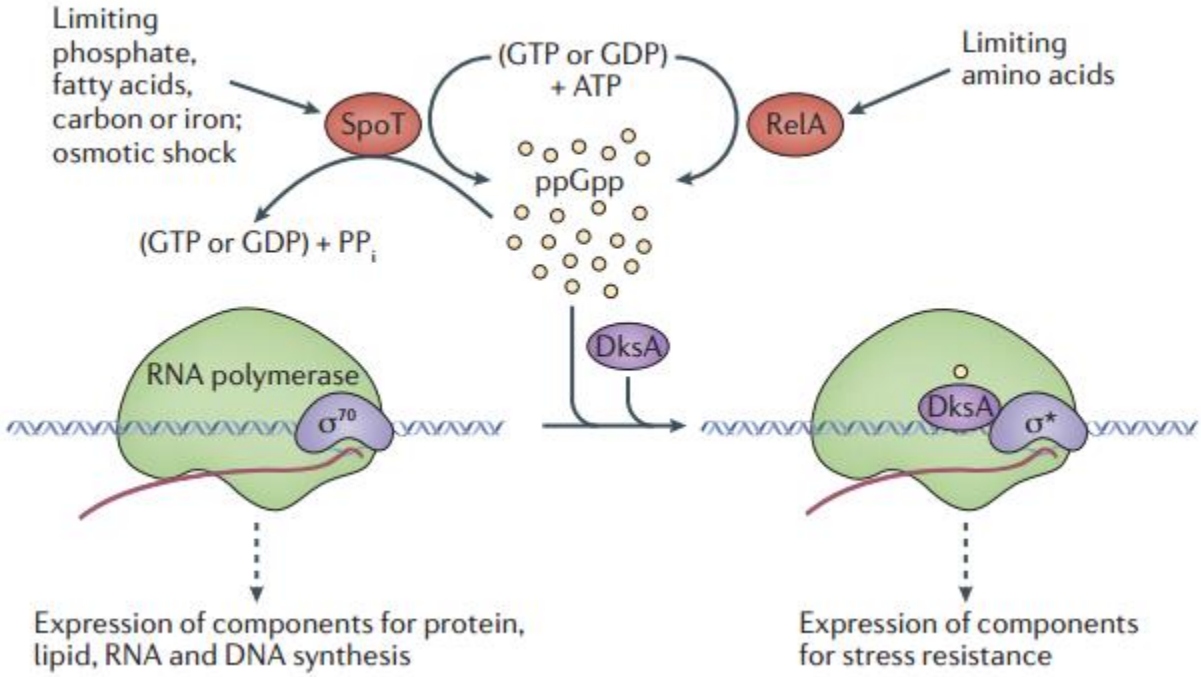

Fig 1.3 (p)ppGpp changes the RNAP promoter by altering σ-factors binding ability.

Under certain starvation conditions, RelA and SpoT synthesize ppGpp. Along with DksA, ppGpp directs transcription initiation at specific promoters by changing the binding preference of RNAP from σ^{70} to alternative σ-factor (σ*). PP_i, pyrophosphate [59].

TA modules encode mRNA endonucleases (mRNases), which contribute to formation of persistence as they control the cleavage of mRNA, inhibit translation and subsequently protein synthesis. It is known that TA-mediated persistence depends on (p)ppGpp [60]. TA-encoded mRNases which decrease bacteria growth rate are stable, while their corresponding antitoxins which inhibits toxin functions, are unstable as they are readily degraded by the Lon protease [61]. The effect of (p)ppGpp on TA modules is attributed to the increasing (p)ppGpp signal, which inhibits exopolyphosphatase (Ppx) activity and subsequently lead to accumulation of polyphosphate, which binds to Lon protease to enhance protease activity [62, 63]. The enhanced Lon protease degrades antitoxin, causing the corresponding toxin activation and increase in the prevalence of nongrowing cells, and eventually formation of persisters **(Fig 1.4)**.

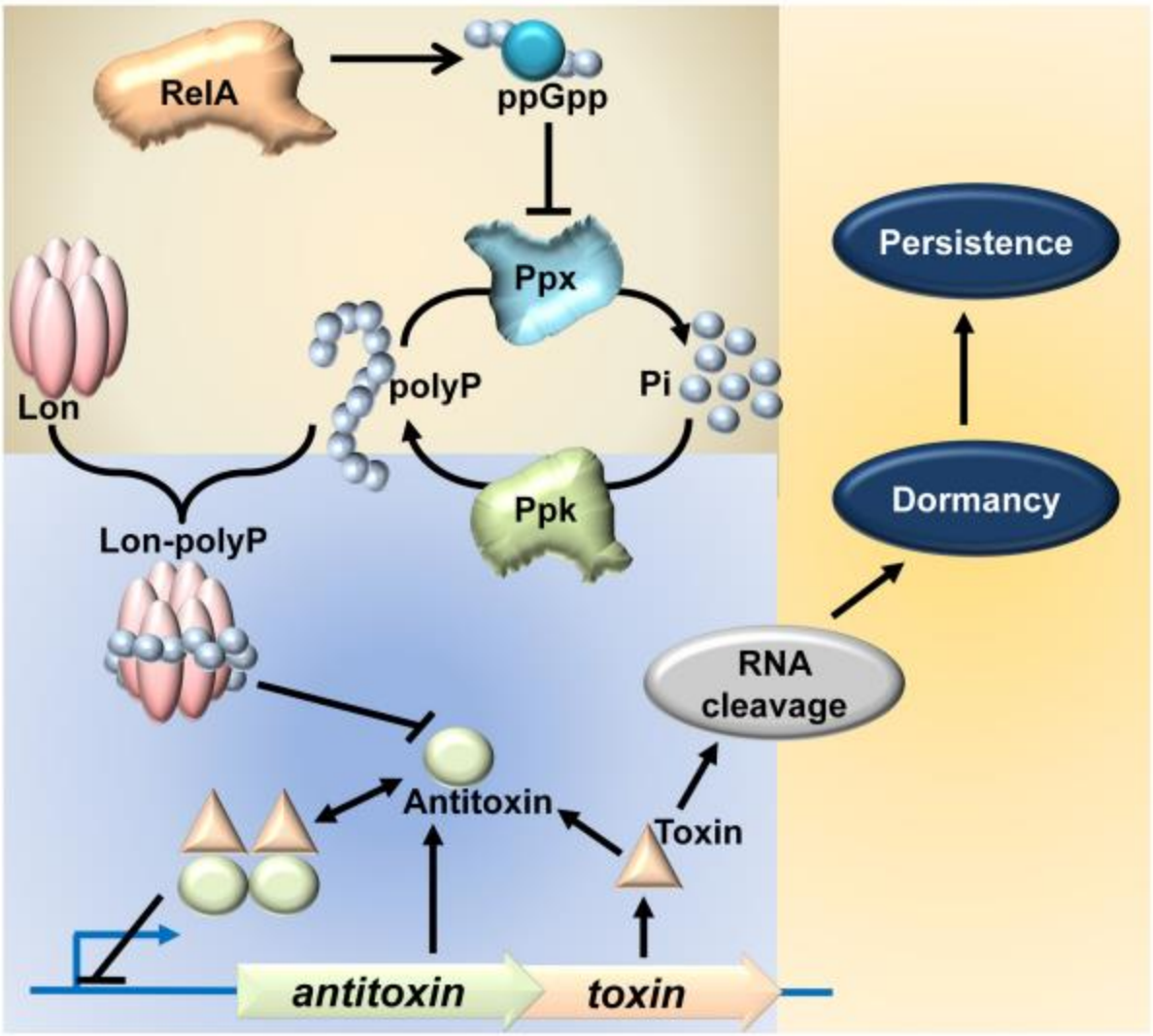

Fig 1.4 The process of (p)ppGpp-mediated TA-induced persistence.

Accumulation of (p)ppGpp inhibits exopolyphosphatase (Ppx) and leads to accumulation of polyphosphate (polyP), which is synthesized by polyphosphate kinase (PPK). PolyP binds to and stimulates Lon protease to degrade antitoxin, which renders the toxin free to confer persistence [64].

1.3.2 TA modules

TA loci consist of two genes, a stable toxin and an unstable antitoxin which inhibits the toxin. It is intuitive that activation of TA toxins can predispose formation of

persister. Persisters are cells with slow metabolic rate so that they can escape from antibiotic killing, as antibiotics typically target the cellular processes of actively growing and replicating cells. A few decades ago, a study showed that two mutants of *E.coli* in *hipA* gene generated persisters at a high rate [65]. Toxins inhibit the transcription, translation, post-translation and depolarize membrane to adjust cells to a dormancy state which is closely related to persistence formation. Six types of these elements have been characterized based on the way how antitoxin inhibits the toxin protein **(Fig 1.5)**. Among them, type I in which antitoxin is RNA molecule, binds to and inhibits toxin mRNA, and type II in which antitoxin is protein, binds to and inhibits toxin protein activity, commonly exist in the cells. These two types are most often connected with persistence formation. The degradation of type II antitoxin relies on protease (mainly the Lon protease and ClpX-ClpP), while as mentioned above, increasing (p)ppGpp activates Lon protease by facilitating polyphosphate accumulation.

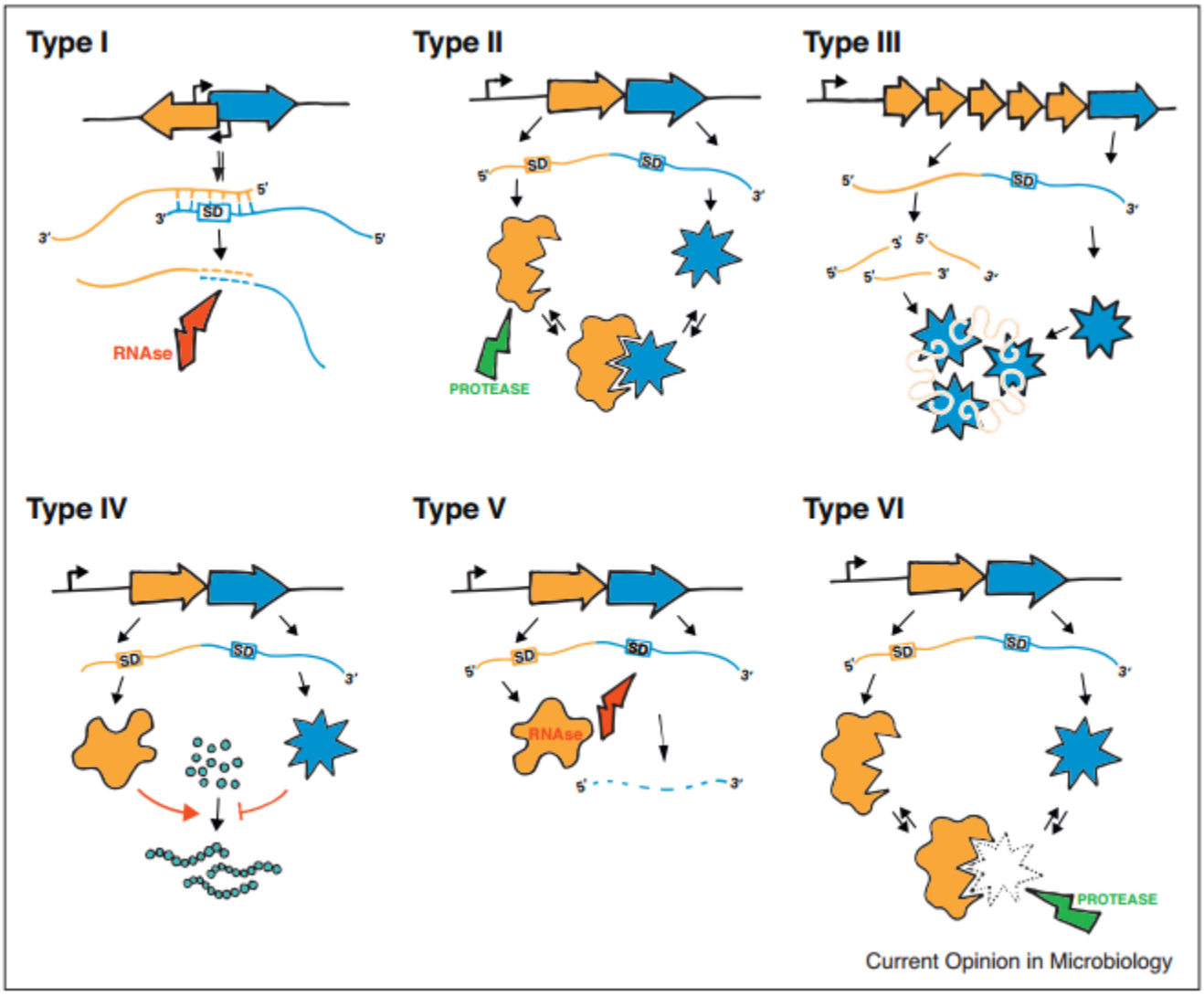

Fig 1.5 Types of TA module.

Type I: antitoxin is RNA which recognizes, binds to toxin mRNA and forms RNA duplex to lead to ribonucleases degradation. Type II: antitoxin is protein which recognizes, binds to toxin protein to repress its function. Type III: antitoxin is non-coding RNA which directly binds to and inhibit toxin protein. Type IV: antitoxin is protein which competes for the same binding site of toxin rather than direct interaction. Type V: antitoxin is RNAse which degrades toxin mRNA. Type VI: antitoxin is protein which binds to toxin and causes toxin degradation. Toxins are shown in blue, antitoxin in yellow [66].

TA toxins have diverse functions mediated by a variety of mechanisms, such as target degradation, phosphorylation, acetylation and membrane potential disruption. The precise mechanism of interaction between toxins and their targets is still not known but this interaction commonly results in corruption of essential cell metabolism such as DNA replication, translation, which finally leads to a halt in cell replication or even cell death. In many cases, toxins act as endoribonuclease to degrade mRNA, leading to complete translation shut down; cells then enter into dormancy state, examples are RelE and MazF **(Table 1.1)** [67, 68]. The *relBE* locus is activated by amino acid starvation, cleaves mRNA positioned at the ribosomal A-site in a ribosome-dependent manner [68-70]. MazF cleaves mRNA at ACA sites in an independence of ribosomes [71, 72]. Apart from mRNA, there are increasing research reports on toxin target on tRNA, which cause a layer of disruption and re-programming to protein translation. HipA is a kinase that phosphorylates and inhibits Glu-tRNA synthetase, prevents the acylation of this tRNA species and leads to translation stall and persistence formation [73]. VapC specifically targets the $tRNA^{fMet}$, an initiator of protein translation [74]. TacT acetylates the primary amine group of amino acid of charged tRNAs, which disturbs the formation of peptide bond between the blocked amino acid and the nascent peptide chain, leading to persistence [75]. Most type I TA modules target on membrane and result in membrane depolarization, such as *hokB/sokB*. The membrane potential disruption leads to ATP leakage and then formation of dormant persister cells [76].

Table 1.1 Common TA modules exhibiting different mechanisms.[66]

TA pair	**TA Type**	**Mode of action (RD/I=Ribosome dependent/independent)**	**Target**	**Ref**
hokB/sokB	1	Membrane depolarization	Membrane	[76]
istR/tisAB	1	Membrane depolarization	Membrane	[77]
par/fst	1	Membrane depolarization	Membrane	[78]
agrB/dinQ	1	Membrane depolarization	Membrane	[79]
hipAB	2	Phosphorylation	Glu-tRNA synthetase	[65, 80]
relBE	2	Endonuclease (RD)	mRNA/tmRNA	[68-70]
yafNO	2	Endonuclease (RD)	mRNA	[81]
vapBC	2	Endonuclease (RI)	tRNA/rRNA	[74]
mazEF	2	Endonuclease (RI)	mRNA/tRNA/rRNA/tmRNA	[72, 82]
mqsAR	2	Endonuclease (RI)	mRNA	[83]
kis/kid	2	Endonuclease (RI)	mRNA	[84]
hicBA	2	Endonuclease	mRNA	[85]
ficAT	2	Adenylylation (AMPylation)	GyrB/ParE	[86]
phd/doc	2	Phosphorylation	EF-Tu	[87]
ccdBA	2	Protein-Protein Interaction	DNA Gyrase (GyrA Subunit)	[88]

parDE	2	Protein-Protein Interaction (ATP-Dependent)	DNA Gyrase (GyrA Subunit)	[89]
epsilon/zeta	2	Phosphorylation	Uridine diphosphate-N-acetylglucosamine	[90]
ybaJ/hha	2	Transcriptional Regulator	DNA	[91]
tacTA	2	Acetylation	tRNA	[75]
abiQ/toxN	3	Endonuclease	mRNA	[92]
yeeUV	4	Protein-Protein Interaction	FtsZ and MreB	[93]
ghoST	5	Membrane depolarization	Membrane	[94]
socAB	6	Protein-Protein Interaction	DnaN (Beta-sliding clamp)	[95]

Several reports have shown that certain stresses specifically induce the activity of different type II mRNase TA modules in *E.coli,* for example, MqsA antitoxin is quickly degraded by Lon protease under oxidative stress, but is quite stable under other conditions [96]. A total of six TA loci in *E. coli* K-12 are involved in the SOS response, a transcriptional program induced by single-stranded DNA as this damaged DNA gradually inactivated the LexA repressor. These are *tisB/istR*-1, *dinQ/agrB, symE/symR* and *hokE/sokE* type I TA and the *dinJ/yafQ* and *yafNO* type II TA [97, 98]. Damaged DNA could induce activation of these six TA loci. For the type II TA, LexA boxes are located in front of the toxin and antitoxin gene operon, which means that both would be activated upon DNA damage and additional mechanisms are recruited to enforce antitoxin degradation. For type I TA, LexA only controls the transcription of toxin gene.

Despite these works, controversy of the TA loci's role in persister formation remains. Kenn Gerdes's group reported the role of (p)ppGpp, Lon protease and TA modules in persistence formation [99, 100]. However, they later reported that the crucial TA-deleted strains used in their experiment were infected by the notorious φ80 bacteriophage. They showed that the theory that decreasing persister fraction due to TA deletion in *E. coli* was incorrect as it was similar to the decreasing level caused by lysogenization with φ80, hence they retracted their publication. Nevertheless, the importance of (p)ppGpp and Lon protease in persister generation could be confirmed using reconstructed non-infected mutants [101]. They also retracted a paper which reported (p)ppGpp mediated activation of ten TA modules through a signaling pathway involving the polyphosphate and Lon, due to the contamination of the φ80 bacteriophage [102]. The inconsistent results of TA in persister generation was further addressed in other researches. Frederic *et al* reported that deletion of 10 TA systems in *E. coli* did not affect persistence to ampicillin or ofloxacin [103]. Conlon *et al.* reported that deletion of TA modules did not have effect on *S.aureus* persistence generation [104]. Hence persistence generation is the result of adjustment and cooperation of various responses. Merely TA-induced metabolic inactivation is insufficient to form persisters and the role of TA on persister formation still needs further confirmation.

1.3.3SOS response

SOS regulon is involved in DNA repair, and is induced in response to DNA damage by various stresses, such as antibiotic treatment, oxidative stress, and extreme pH [97]. Persister formation are usually dampened in the SOS-deficient mutants, especially upon treatment with DNA-damaging agents [25, 105, 106]. SOS regulon is inactive as LexA binds to the LexA box, which is typically located near or inside the RNA polymerase binding-site, thus repressing the transcription until single-

stranded DNA (ssDNA) generated either by double-strand DNA breaks (DSB) or double-strand ends (DSE) recruits and activates RecA [107]. The activated RecA induces LexA autocatalytic cleavage and then de-repress SOS regulon **(Fig 1.6)** [108].The SOS response is functionally related to DNA repair pathways, including homologous recombination (HR), nucleotide excision repair (NER) and translesion synthesis (TLS) [109]. HR and NER usually result in mutation-free repair, while TLS uses error-prone polymerases (PolB, DinB and UmuDC) and results in mutations which is harmful in most cases but may cause adaptive mutant to resistance in the hostile environment [110, 111].

The inability of de-repression of SOS response or the lack of LexA-regulated error-prone polymerase prevent the evolution of ciprofloxacin resistance [112]. Apart from resistance formation, SOS is also involved in stationary phase fluoroquinolone persistence formation as DNA repair is requested in the post-treatment recovery period to enable cells damaged by fluoroquinolone to survive and regrow [105]. It was reported that biofilm-specific high tolerance to fluoroquinolone was dependent on SOS response upon amino acids and carbon source starvation [106]. SOS response is also involved in cAMP-regulated β-lactam persistence generation in UPEC [113]. Persister population is usually considered as a bacterial reservoir that can evolve into resistant strains.

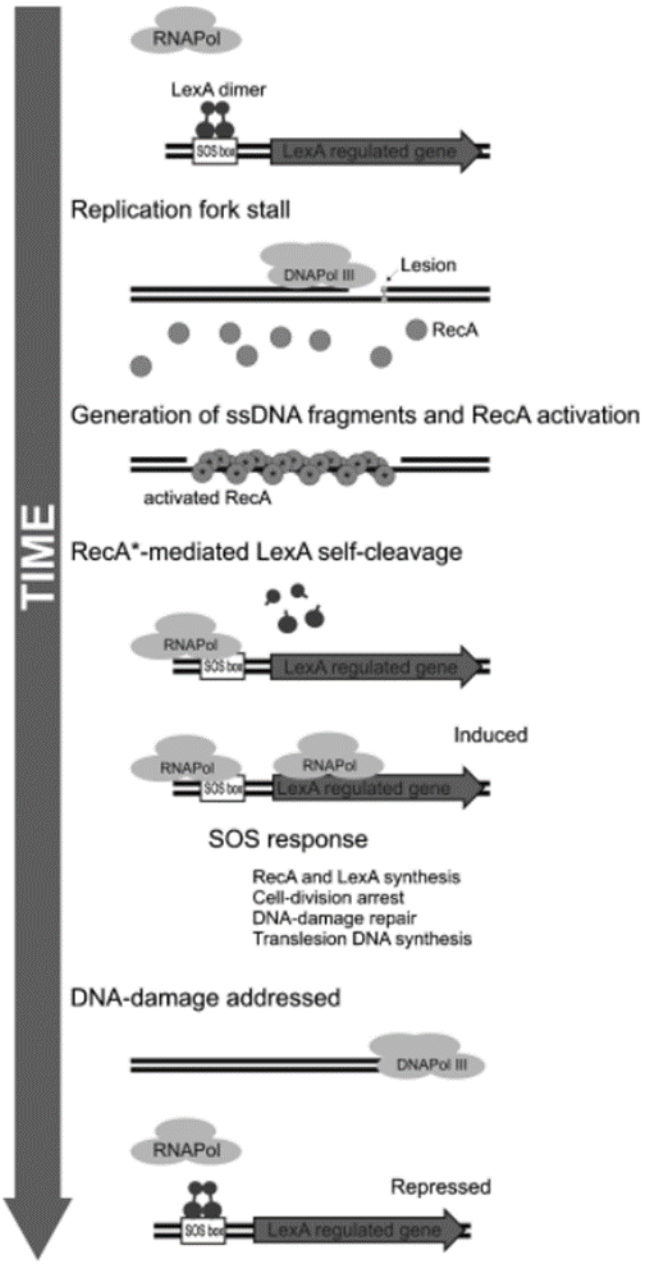

Fig 1.6 Schematic of the SOS induction process in *E. coli*.

LexA initially binds to the LexA box located in the upstream region of the SOS regulon, dampens RNA-polymerase binding and inhibits transcription. ssDNA recruits and activates RecA, which in turn triggers LexA self-cleavage and releases the binding site of RNA-polymerase [114].

During this process, SOS response is activated and results in enhancement of the mutation rate among those survived persisters [115]. Despite DNA damage repair, SOS mediates the generation of persisters via regulating TA loci which possess the LexA box as mentioned above [116].

1.3.4RpoS and the general stress response

The general stress response is the process in which bacteria express the default pathway to adapt to adverse environment. It mainly depends on the σ factor RpoS,

which controls transcriptional reprogramming. General stress response is active and is required for the shift of metabolism in persister cells [117]. The RpoS regulon regulates hundreds of genes involved in membrane permeability, cellular metabolism, cell shape and the capacity of cellular macromolecules repair, and is induced by nutrient limitation, heat shock, biofilm, oxidative stress, extreme PH and other cues [118]. Particularly, RpoS and SOS are considered complementary mechanisms expressed in response to DNA damage, as RpoS also regulates the repair of DNA breaks and mediates the reduction in replication fidelity, and generation of mutation [12, 119]. *P.aeruginosa* persister fraction declines ~70 times under stationary phase or heat stress in *rpoS* mutant [120]. The activation of general stress response also enable bacterial cells to become tolerant to acid, heat, salt and UV damage stress [121]. Biofilm associated persistence formation also required the participation of RpoS, as mutant deficient in *rpoS* results in a declined tolerance level to ciprofloxacin [122].

1.3.5 Inactivation of antibiotic targets

Dormancy is considered one major phenotypic characteristic of persistence because dormant cells have little or no translation, topoisomerase activity or cell-wall synthesis, hence antibiotics can bind to but cannot corrupt their target molecules [123]. Observation of single-cell persisters indicates that they are non-growing, transcriptome analysis of persisters demonstrates that they are under low metabolic activity and non-essential responses are shut down [17, 124]. Moreover, induction of low metabolic state by disturbing major cellular process consistently results in increasing persistence. As mentioned above, over-expression of toxins which prevent translation by cleaving mRNA or tRNA and inactivating mRNA-ribosome complex is involved in persister generation. It was reported that tolerance to ampicillin was achieved through (p)ppGpp-induced inhibition of peptidoglycan

synthesis [125]. Fluoroquinolone persistence is pronouncedly influenced by (p)ppGpp which dampened DNA replication, as well as transcription [126]. UmuDC which interprets DNA replication is also involved in modulation of fluoroquinolone persister formation [127, 128]. Ribosome hibernation is required for high tolerance to aminoglycosides in stationary phase, as aminoglycosides transfer active 70S ribosomes into inactive 100S particle so they cannot corrupt their original target to kill bacteria. It is consistent that aminoglycoside tolerance level decreases with the constant intracellular drug amount after deletion of gene encoding the hibernation-promoting factor (HPF) [129].

1.3.6 Drug efflux pumps

Efflux pumps are considered as one of the four general mechanisms of multidrug resistance generation as they not only export a broad range of antibiotics owing to their poly-substrate specificity, but also drive the acquisition of additional resistance mechanisms by decreasing intracellular antibiotic accumulation [2]. Moreover, efflux pumps are also involved in active mediation of persister formation by expelling the intracellular antibiotic out. Fluorescent antibiotic and single-cell microscopy observation indicate that the amount of intracellular antibiotic in persister is considerably lower than that of normal cells. Meanwhile, *tolC* together with other efflux genes is highly expressed in persister cells; in addition, survival rate in the presence of antibiotic was found to decrease after *tolC* deletion. Synergetic treatment of antibiotic and pump inhibitor PAβN eradicates persister cells effectively [130]. Efflux pumps are also required for macrophage-induced *mycobacterium* tolerance, as the pump inhibitor verapamil and reserpine contribute to a reduction in *Mycobacterium marinum* survival when used in conjunction with isoniazid or rifampicin. Verapamil also reduces tolerance frequency to rifampicin in *Mycobacterium tuberculosis*. Consistently, the tolerance level to rifampicin is

decreased in the efflux pump mutant of Rv1258c-deficient strain [4]. It was reported that biofilm formation was hampered in strains which efflux pump genes such as *emrD*, *emrE*, *emrK*, *acrD* and *mdtE* had been deleted; also, most of biofilm associated cells are tolerant to antibiotic [131]. The tolerance level induced by paraquat is largely abolished after deleting the *tolC* gene, which is known to be involved in extruding fluoroquinolones [132].

1.4 Therapeutic strategies for persister eradication

Since persisters are quite relevant to the clinical chronic infection and disease relapse, increasing researches study persister cells eradication approach and provide several effective strategies to eradicate persister fraction. It is roughly separated into four classes: (i) directly kill the persisters, target on the still active cell processes, (ii) potentiate conventional antibiotics, activate the antibiotic targets or stimulate antibiotic influx, (iii) combined usage of different type antibiotics, or (iv) disrupt or reduce the generation of persister **(Table 1.2)**. Sometimes it is hard to make a strict distinction among these three classes and some therapies might belong to two categories. Even though many compounds have been proven to exhibit the ability to kill persisters, more efforts are required to develop effective anti-persister strategies and test the clinical application potential of current anti-persister compounds.

Table 1.2 Compounds or therapies that kill persisters as described in the literature.

Compound	Target species	Mechanism and remark	Reference
Direct killing of persisters			
targeting membrane integrity and polarization			
2D-24	*P. aeruginosa*	a synthetic dendrimeric peptide which directly kills both planktonic and biofilm bacteria but are not toxic to mammalian cells despite being able to disrupt bacterial membrane; it exhibits synergistic antimicrobial effects with ciprofloxacin, tobramycin or carbenicillin	[133]
Antimicrobial peptides	*E. coli*	Trp/Arg-containing peptides directly kill planktonic and biofilm bacteria or enhance susceptibility to ampicillin or ofloxacin, possibly due to enhancement of membrane penetration.	[134]
QACs	*S. aureus*, *E. faecalis*	quaternary ammonium cations mimic antimicrobial peptides and disrupt membrane structure to kill persisters and eradicate biofilm	[135]
Carvacrol	*B. burgdorferi*	direct killing of both stationary phase and biofilm bacteria, probably due to its ability to cause membrane damage	[136]
Electrochemical scaffold	*P. aeruginosa*	Electrochemical scaffold re-sensitizes biofilm persister susceptibility to tobramycin and also directly kills biofilm-embedded persisters as it generates H_2O_2 which enhances membrane permeability and produces ROS	[137]
Electrical current	*P. aeruginosa*	proper electrical current ($70\mu A/cm^2$) causes reduction in the number of planktonic and biofilm persisters as it destroys membrane integrity and potentiates the killing effect of tobramycin	[138-140]
NH125	*S. aureus*	an imidazolium cation with a sixteen membered fatty tail, kills MRSA persisters by causing membrane permeabilization and disrupts biofilms at high concentration	[141, 142]
NH125 analogues	*S. aureus*	N-arylated NH125 analogues which eradicate stationary MRSA persisters, and target bacterial and fungal biofilms	[143, 144]
Lipidated lysine 9	*E. coli*, *S. aureus*	membrane-active agent that consists of one amino acid and two lipid tails, and causes depolarization and permeabilization of membrane	[145]

SPI009	*P. aeruginosa*, *E. coli*, *B. cenocepacia*, *S. typhimurium*	1-[(2,4-dichlorophenethyl) amino]-3-phenoxypropan-2-ol, directly kills persisters and synergistically enhances the efficiency of antibiotics as it interferes membrane integrity	[146, 147]
Colistin	*E. coli*	According to results of screening of compounds in the clinical drug library, colistin which damages membrane integrity exhibits the highest anti-persister activity in treatment of UPEC infection	[148]
NPIMA	*E. coli*, *S. aureus*, *P. aeruginosa*	5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride, kills both Gram-positive and Gram-negative persisters by damaging membranes and causing cell lysis	[149]
CD437; CD1530	*S. aureus*	synthetic retinoids exhibit high MRSA killing rates and synergistic antimicrobial effects with gentamicin in vitro and in vivo by penetrating and embedding in membrane lipid bilayers	[150]
NCK-10	*S. aureus*	membrane-active agent consists of aryl-alkyl-lysine and depolarizes and permeabilizes membrane, killing planktonic and biofilm persisters	[151]
TN-5	*P. aeruginosa*, *E. coli*	1,3,5-triazine (AMP) derivative, kills the planktonic persisters probably as a result of interaction with cell membrane	[152]
XF-70; CF-73	*S. aureus*	membrane-active agents that perturb membrane activity, kill slow-growing or non-dividing bacteria	[153, 154]
AM-0016	*M. tuberculosis*, *M. bovis*	specifically kill Gram-positive bacteria by causing rapid collapse of membrane potential	[155, 156]
Boromycin	*M. tuberculosis*, *M. bovis*, *E. faecalis* *S. aureus* *S. epidermidis*	disrupts membrane potential as a potassium ionophore and causes subsequent decrease in ATP level	[157]
HT61	*S. aureus*	a quinolone-derived compound that kills non-multiplying bacteria due to its depolarization effect on membrane and destruction of cell wall; it exhibits synergistic antimicrobial effect with gentamicin, chlorhexidine, mupirocin and neomycin	[158-160]

PAAG	*P. aeruginosa*	large molecule polycationic glycopolymer, poly (acetyl, arginyl) glucosamine rapidly eradicates persisters without exhibiting mammalian cell cytotoxicity as it causes membrane permeabilization and depolarization	[161]
LysH5; CF-301	*S. epidermidis, S. aureus*	The endolysin causes lysis of both growing and non-growing cells and so it eliminates persister cells and reduces biofilm formation	[162-164]
Art-175	*P. aeruginosa A.baumanii*	modified artilysin covalently combines with a bacteriophage-encoded endolysin to cause peptidoglycan degradation and then kill persisters. Other artilysins also exhibit persister elimination effect, such as Art-085.	[165, 166]
TCA1	*M. tuberculosis*	kills non-replicating Mtb in vitro and in vivo, has synergistic effect with rifampicin or isoniazid and causes down-regulation of genes involved in cell wall and molybdenum cofactor synthesis	[167]
targeting other mechanisms			
Engineered bacteriophages	*M. abscessus*	Three-phage cocktail therapy is applied to a patient with cystic fibrosis who was recurrently infected with *M. abscessus*, effectively inhibiting bacterial dissemination and curing the patient	[168]
5-Iodoindole	*S. aureus, E. coli*	eradicates both Gram-positive and Gram-negative persisters and inhibits biofilm formation without inducing resistance formation	[169]
ADC111-ADC113	*E. coli*	analogs of nitrofurantoin and tilbroquinol, were identified from the screening of 55,000 compounds. All demonstrate anti-persister effect	[170]
ADEP4	*S. aureus*	acyldepsipeptide antibiotic, activates ClpP protease and subsequently non-specifically cleaves proteins, causing self-digest of the cell. It kills both planktonic and biofilm persisters and completely eradicates *S. aureus* biofilms when used in combination with rifampicin	[171]

Cisplatin	*E. coli*, *S. aureus*, *P. aeruginosa*	anti-cancer drug, [*cis*-diamminedichloroplatinum (II)], forms intra-strand DNA crosslinks and kills cells through a growth-independent mechanism	[172]
mitomycin C	*E. coli*, *S. aureus*, *P. aeruginosa*, *B. burgdorferi* *A. baumannii*	anti-cancer drug, forms inter-strand DNA crosslinks and kills cells through a growth-independent mechanism	[173-175]
D157070/D155931 (rhodamines)	*M. tuberculosis*	inhibitor of dihydrolipoamide acyltransferase (DlaT), an enzyme required for tuberculosis generation a; DlaT is also related to resisting nitric oxide derived reactive nitrogen intermediates.	[176]
DG70	*M. tuberculosis*	It inhibits the function of the *menG* gene product which is involved in the final step of menaquinone biosynthesis and required for respiration then affect ATP biosynthesis. It has synergistic effect with isoniazid, rifampin and bedaquiline	[177]
Halogenated phenazines	*S. aureus*, *S. epidermidis*, *M. tuberculosis*	Halogenated phenazines 14 exhibits MRSA, MRSE and VRE biofilm eradication effect. Halogenated phenazines 13 shows bactericidal effect on non-growing *M. tuberculosis*	[178, 179]
Lassomycin	*M. tuberculosis*	It binds to *M. tuberculosis* specific ClpC1 ATPase complex and dramatically stimulates its ATPase activity without stimulating ClpP1P2-catalyzed protein breakdown, thus killing persister cells	[180]
Nitroxoline	*S. aureus*	Fe^{2+} and Zn^{2+} chelator, effectively eradicates biofilms and non-biofilm MRSA persisters	[181]
PA-824	*M. tuberculosis*	Bicyclic nitroimidazole, Rv3547 (deazaflavin-dependent nitroreductase) converts PA-824 into three metabolites, including des-nitroimidazole which generates reactive nitrogen species under anaerobic environment.	[182, 183]
Piscidin-3	*P. aeruginosa*	Belongs to host-defense peptides and the N-termini coordinates Cu2+ to cleave DNA, eradicating biofilm and persisters	[184]

Pyrazinamide	*M. tuberculosis*	pyrazinamide is intracellularly hydrolyzed to pyrazinoic acid, which then inhibits trans-translation and occupies ribosomes. It may be the reason why pyrazinamide can eradicate *M. tuberculosis* persisters	[185]
KKL-35	*M. tuberculosis*	inhibitor of trans-translation that targets helix 89 of the 23S rRNA can kill both aerobic and anoxic non-growing *M. tuberculosis*	[186]
Stevia whole leaf extract	*B. burgdorferi*	effectively eliminates *B. burgdorferi* persisters	[187]
Tosufloxacin	*E. coli*, *S. aureus*	from the screening of clinical drug library, the quinolone tosufloxacin has the highest anti-persister activity for treatment of uropathogenic *E. coli* infection. Tosulfoxacin also efficiently kills *S. aureus* persisters.	[148, 188]
Metronidazole	*M. tuberculosis*	It specifically kills anaerobic, dormant tubercle bacilli and exhibits synergistic antimicrobial effect with rifampin	[189]
Anthracyclines	*B. burgdorferi*	Six anthracyclines from the anti-cancer drug library were found to exhibit high activity against stationary phase *B. burgdorferi*	[190]
Clofazimine	*M. smegmatis*	reduces persister fraction by facilitating ROS production via a NADH-dependent redox cycling manner	[191]
Moxifloxacin and gatifloxacin	*E. coli*, *M. tuberculosis*	these two quinolones are effective against rifampin-tolerant and non-replicating bacteria	[192]

Re-sensitization of conventional antibiotics

reactivation of antibiotic targets

Spent medium	*S. aureus*	Spent medium resuscitates dormant *S. aureus* and then re-sensitizes them to antibiotics	[193]
cis-2-decenoic acid	*E. coli* *P. aeruginosa*	fatty acid signaling molecule, reverts dormant cells to a metabolically active state and subsequently renders them sensitive to ciprofloxacin, tobramycin, tetracycline by activating transcription and protein synthesis.	[194]

C10	*E. coli* *P. aeruginosa*	3-[4-(4-methoxyphenyl) piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate revert persisters to antibiotic-sensitive cells. Identified in a screening of norfloxacin potentiators	[195]
BF8	*P. aeruginosa*	(Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one, re-sensitizes both planktonic and biofilm-associated persisters to ciprofloxacin and tobramycin.	[196, 197]
GM-CSF	*P. aeruginosa*	host immune factor granulocyte macrophage-colony stimulating factor re-sensitizes both planktonic and biofilm persisters to diverse antibiotics, such as ciprofloxacin, tobramycin, tetracycline and gentamicin.	[198]
N-acetylcysteine	*M. tuberculosis*	It potentiates anti-tuberculosis drug activity as it activates respiration and metabolism. In addition, it promotes ROS generation.	[199]
increase of intracellular antibiotic amount			
Metabolites	*E. coli*, *S. aureus*	Glucose, fructose, pyruvate mannitol and alanine enhance gentamicin and kanamycin killing effect to persisters by enhancing production of PMF and thereby increasing gentamicin uptake. Its re-sensitization effect is observable both in vitro and in vivo	[200]
Mannitol	*P. aeruginosa*	Mannitol enhances clinical isolates and biofilm-associated *P. aeruginosa* susceptibility to tobramycin by enhancing PMF production	[201]
Glucose	*S. aureus*	enhances the killing effect of daptomycin in a PMF-independent manner	[202]
Hypo ionic shock treatment	*E. coli*	hypotonic shock enhances the killing effect of aminoglycoside, perhaps achievable by promoting entrance of aminoglycosides through mechanosensitive ion channels	[203]
L-serine	*E. coli*	L-serine re-sensitizes persisters to gentamicin, possibly due to inhibition of amino acid synthesis and enhancement of aminoglycoside influx. Additionally, NAD^+/NADH ratio is increased in the presence of L-serine and ofloxacin or moxifloxacin, causing a subsequent increase in ROS level	[204, 205]

L-arginine	*S. aureus*, *E. coli*, *P. aeruginosa*	it changes membrane pH gradient and potentiates aminoglycoside activity both in vitro and in vivo	[206]
efflux pump inhibitors	*E. coli*, *M. marinum*	efflux pump inhibitors PAβN and NMP cause increasing intracellular β-lactam concentration by inhibiting efflux pump activity and thus restoring carbenicillin susceptibility. Similarly, verapamil causes reduction in macrophage-induced tolerance and increase in the killing efficacy of rifampicin and isoniazid	[4, 207]
modification of antibiotic structure			
P14LR-kanamycin (P14KanS)	*S. aureus*, *P. aeruginosa*, *S. epidermidis*, *A.baumannii*	P14KanS is achieved by conjugation of kanamycin and an antimicrobial peptide (P14LRR). It disrupts the bacterial cell membrane and subsequently kills both Gram-positive and Gram-negative bacteria, and destroys biofilms.	[208]
Pentobra	*E. coli*, *S. aureus*	addition of 12 amino acids to tobramycin creates a modified tobramycin that spontaneously permeates membrane and exerts bactericidal effect	[209]
Combination of conventional antibiotics			
Colistin	*E. coli*, *P. aeruginosa*	a membrane active antibiotic that enhances the anti-persister activity of gentamicin and ofloxacin but not ampicillin, probably due to increased uptake of other antibiotics. Additionally, the macrolide erythromycin, together with colistin, eradicate tolerant biofilm cells as erythromycin shows motility and quorum sensing, which are essential for biofilm tolerance formation	[210, 211]
Daptomycin, cefoperazone and doxycycline	*B. burgdorferi*	Combination of daptomycin, doxycycline and cefoperazone results in eradication of stationary phase-induced *B. burgdorferi* persisters	[212]
ciprofloxacin and vancomycin	*S. epidermidis*	biofilm-associated persisters are killed by treatment with high concentration of ciprofloxacin, together with vancomycin	[213]

Polymyxin B and meropenem	*A. baumannii*	clinical *A. baumannii* was eliminated after treatment with polymyxin B and meropenem	[214]
Disruption of persister formation			
Benzamide compounds	*P. aeruginosa*	They bind and inhibit the global virulence quorum sensing transcriptional regulator, MvfR, resulting in reduced formation of persister cells; they also exhibit bacterial eradication effect when used in combintion with ciprofloxacin	[215, 216]
Cadaverine	*P. aeruginosa*	It was shown that gene PA4115 which produces cadaverine is related to persistence formation; exogenous cadaverine improves carbenicillin and ticarcillin killing of persisters	[217]
Engineered bacteriophages	*E. coli*	the engineered bacteriophage that suppresses the SOS network increases susceptibility to fluoroquinolones	[218]
Mesalamine	*E. coli*, *P. aeruginosa*	It causes rapid decrease of polyphosphate levels and subsequently alters bacterial sensitivity to oxidative stress, which contributes to attenuation of formation of persister cell and biofilm	[219]
MOPS or osmolytes	*E. coli*	MOPS or low concentration of osmolytes (trehalose, betaine, glycerol and glucose) inhibit protein aggregate accumulation and then persister formation	[220]
RelA inhibitors	*E. coli*, *B. subtilis*, *B. anthracis*, *Group A streptococci*	(p)ppGpp analogues competitively inhibit RelA and subsequently prevent stringent response and persister formation. Ralacin inhibits sporulation and biofilm formation but is only effective in Gram-positive strains. 2′-Deoxyguanosine-3′-5′-di (methylene bisphosphonate) is effective in both Gram-positive and Gram-negative strains.	[221, 222]
Nitric oxide	*E. coli*	inhibits respiration during stationary phase and then reduces the formation of type I persisters.	[223]
PKUMDL-LTQ-101-401	*E. coli*	A HipA inhibitor discovered from silico screening and exhibits anti-persister activity	[224]

1.4.1 Direct killing of persisters

Persisters exhibit low metabolic activity and the antibiotic targets in persisters are under the dormancy state, which attributes to treatment failure of conventional antibiotics. The remain essential enzymes [225].

1.4.1.1 Targeting membrane integrity and polarization

Hurdle *et al* proposed that bacterial membrane bilayer and the essential proteins related to membrane function such as membrane-bound ATP synthase were the promising targets for killing persistence cells, as integrity of membrane is still required for the survival of persister cells [225]. Researchers have screened the compound library to identify potential drugs which destroyed membrane structure and killed dormant persisters. Membrane-active agents such as NCK-10, XF-70 and lipidated lysine 9 disrupt the membrane integrity and change both permeabilization and potential, resulting in death of non-dividing cells [145, 151, 153]. Antimicrobial peptides (AMP) which exhibit penetration properties can damage membrane and are reported to be promising candidates of anti-persister drugs. In particular, 2D-24, a dendrimeric peptide, can directly kill *P.aeruginosa* persister and enhance conventional antibiotic effect as it disrupts membrane [152]. Trp/Arg containing peptides directly eradicate *E.coli* persisters and re-sensitize cells to ampicillin or ofloxacin [134]. TN-5,1,3,5-triazine derivative which belongs to AMPs also shows anti-persister effect and is supposed to interact with negatively charged cell membrane [37]. Some compounds with cations disrupt membrane structure and subsequently eradicate biofilm, killing persisters, examples are QACs, NH125 and NH125 analogues [135, 141-144]. The membrane-damaging polymyxin colistin is one of the most effective anti-persister antibiotics used for the treatment of UPEC infection [148]. It is reported that CD 437 and CD1530 (synthetic retinoids)

effectively killed MRSA persister and exhibited synergistic antimicrobial effect with gentamicin. The membrane-mimicking vesicle assay shows that CD 437 and CD1530 penetrate and become embedded into lipid bilayers, disrupting the integrity of membrane [150]. SPI009 (1-((2,4-dichlorophenethyl) amino)-3-phenoxypropan-2-ol) disrupts membrane integrity and directly kills both Gram-positive and Gram-negative persister cells. Moreover, SPI009 renders persisters susceptible to ofloxacin, amikacin and ceftazidime [147]. Electrical current and electrochemical scaffold also directly kill planktonic and biofilm persisters and re-sensitize persisters to tobramycin, the effect is partially due to the damage of membrane integrity [137-140].

Apart from targeting on membrane permeability, membrane potential depolarization is another promising strategy as it consequently results in decrease in ATP level, and may affect other cellular functions which depend on membrane potential, such as efflux pump activity. The membrane-active agents mentioned above, which effectively kills planktonic and biofilm persisters, exhibit both depolarization and permeabilization effects. The fluoroquinolone-derived HT61 which depolarizes membrane potential is reported to kill non-multiplying *S. aureus* and exhibit synergistic antimicrobial effect with gentamicin, chlorhexidine and mupirocin [158-160]. Boromycin (potassium ionophore) eradicates *S. aureus*, *E. faecalis* and *M. tuberculosis* persisters without detectable resistance by causing potential dissipation and subsequently ATP level reduction [157]. AM-0016 and PAAG (polycationic glycopolymer, poly (acetyl, arginyl) glucosamine) rapidly kill non-growing persisters via affecting membrane potential; these compounds induce little resistance [155, 161]. Even though persister cells are considered to be in a dormant state with most of metabolic pathway being shut down, membrane integrity is still critical for

the basic survival of cells and therefore the membrane is a feasible target for anti-persister therapy. The current discovered compounds or clinical drugs (2D-24, boromycin, colistin, HT61 and PAAG) which target membrane permeability and polarization prove that the strategy of disrupting membrane integrity or potential is an effective way to kill both Gram-positive and Gram-negative bacteria, with low propensity for resistance occurrence.

1.4.1.2 Targeting other mechanisms

Apart from cell membrane integrity, some other targets for developing anti-persister strategies have been discovered. One is protease which theoretically cleaves misfolded proteins in an ATP- and ClpX-, C- or A- dependent manner, another is inducer of DNA crosslinks which disturbs transcription and DNA replication. Generation of reactive nitrogen species and trans-translation disruption are also considered effective ways for persister eradication as these highly oxidative materials are very harmful for cells and regarded as the basis of their bactericidal effect. ADEP4, an acyldepsipeptide antibiotic, alters ClpP protease in an ATP-dependent manner, subsequently changes ClpP to a form which nonspecifically cleaves proteins and causes protein self-digestion in *S. aureus* [171]. Lassomycin binds to specific ClpC1 ATPase complex and activates Clp protease to kill *M. tuberculosis* persisters [180]. The integrity of DNA is necessary for cell survival and quinolones are conventional antibiotics which target DNA-related processes. The quinolone tosufloxacin has the highest anti-persister activity for UPEC and is also active against *S. aureus* persisters based on results of screening of clinical antibiotics [148, 188]. Several anti-cancer drugs are reported to exhibit the ability of persister elimination. The anti-cancer drug mitomycin C is effective against a broad range of bacteria persisters by exhibiting a growth-independent mechanisms both in vitro and

in vivo; the target organisms include *E. coli*, *S. aureus*, *P. aeruginosa*, *B. burgdorgeri* and *A. baumannii*. It is a prodrug and is activated intracellularly upon reduction, leading to inter-strand DNA cross-linking at 5'-GC-3' sites [173-175]. Similarly, the anti-cancer drug cisplatin, which induces intra-strand DNA crosslinks, and the anti-cancer drug anthracyclines, kill *E. coli*, *S. aureus*, *P. aeruginosa* and *B. burgdorferi*, respectively [172, 190]. Considering the strong side effect of the anti-cancer drugs, their potential for clinical application in eradicating persister remains to be determined. Nevertheless, they are good candidates for drug repurposing since known compounds without safety concern can quickly be utilized for clinical treatment. Kohanski *et al* reported that the highly deleterious oxidative stress was the major factor that leads to cell death both in Gram-positive and Gram-negative bacteria. Oxidative stress is commonly induced by all the three major types of antibiotics, regardless of drug-target interaction [30]. PA-824 kills anaerobic *M. tuberculosis* in vitro and in vivo since it triggers reactive nitrogen species generation as the lethal effects diminishes after addition of nitric oxide scavengers. PA-824 is a prodrug and is converted into three primary metabolites in the cytosol, including des-nitroimidazole which contributes to reactive nitrogen species generation [182, 183]. D157070 and D155931(rhodanines) largely reduce *M. tuberculosis* persister when used in synergy with conditions that enhance host immunity, such as hypoxia and nitric oxide, because they inhibit the activity of dihydrolipoamide acyltransferase, an enzyme resisting reactive nitrogen species, and subsequently cells are killed by reactive nitrogen species [176]. Inhibition of trans-translation which is critical for ribosome rescue will lead to the death of persisters, for example, pyrazinoic acid which is the intracellular metabolite of pyrazinamide, binds to and inactivate RpsA (a vital protein involved in ribosome-sparing of trans-translation) and subsequently kills non-dividing *M. tuberculosis* [185]. KKL-35 exerts anti-persister effect by targeting helix 89 of the 23S rRNA to inhibit trans-translation

[186]. Trans-translation is essential for *M. tuberculosis* survival, meaning that persister cells still need free ribosomes for basic protein synthesis to sustain fundamental metabolism. Thus, trans-translation is the remaining viable target for anti-persister strategies. Compounds that inhibit other processes, such as ATP biosynthesis inhibitor and Fe^{2+} or Zn^{2+} chelator, also effectively eradicate persister cells [177, 181].

Even though the mechanism of generation and maintenance of persistence is incompletely known and that dormancy is a widely accepted underlying basis of persistence, an increasing amount of evidence indicates that mere dormancy is insufficient for maintenance of the persistence phenotype [22, 38, 45, 130]. Active cellular responses may contribute to persistence formation and thus are the targets for persister eradication. Though it was reported that membrane potential was dissipated and ATP level was decreased in persister cells, the compounds which disrupt membrane potential or inhibit ATP synthesis show significant persister eradication effect [76, 155, 157, 159, 226]. We consider membrane potential and ATP above the baseline are still required for maintaining persister cell survival as significant reduction of ATP leads to the death of non-growing *M. tuberculosis* [43]. Meanwhile it is reported that inhibition of respiration, which is the major source for membrane potential and ATP, negatively impaired perister formation in *E. coli* [46]. Hence investigating the role of maintenance of membrane potential in persister formation may help us discover feasible anti-persister compounds; this can be achieved by screening inhibitors which perturb the active and essential response in persisters.

1.4.2 Re-sensitization of conventional antibiotics against persister cells

Tolerant cells are normally under dormant state, while conventional antibiotics target on active cell processes, such as peptidoglycan synthesis, DNA replication and translation. Apart from directly eradicating persisters, antibiotic potentiation is another way for anti-persister therapies. Compounds which (i) reactivate antibiotic targets, (ii) enhance antibiotic influx can lead to an increase in the killing effect of conventional antibiotics.

1.4.2.1 Reactivation of antibiotic targets

The compounds which revert persisters to metabolic active state are reported to re-sensitize persister cells to antibiotics. *cis*-2-decenoic acid (*cis*-DA) restores persister cells to a metabolically active state with increasing cell respiratory activities, and enhanced protein abundance and transcript expression levels. In the presence of *cis*-DA, persister cells become sensitive to different types of antibiotics such as ciprofloxacin, tobramycin and tetracycline [194]. Quorum sensing (QS) inhibitor BF8 re-sensitizes both planktonic and biofilm-associated persisters to ciprofloxacin and tobramycin in a QS-independent manner [196]. Further study shows BF8 re-sensitization is related to the increasing pH, suggesting a linkage between BF8 and cell membrane [197]. C10 and spent medium are reported to resuscitate dormant persisters and re-sensitize them to antibiotics [193, 195].

1.4.2.2 Increase of intracellular antibiotic amount

Some antibiotics require active transport systems to enter into bacteria cells, yet transport systems are in an inactive state in dormant cells, leading to low intracellular antibiotic concentration and final treatment failure [227]. Metabolites such as glucose, fructose, pyruvate and mannitol enhance the bactericidal effect of

aminoglycosides as they facilitate PMF production [200]. PMF is essential for cellular uptake of aminoglycosides, these metabolites enter glycolysis and TCA cycle and subsequently produce NADH which contributes to PMF generation via entering electron transport chain. This re-sensitization effect is diminished upon adding carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP, a PMF dissipator) **(Fig 2.6)**, which further indicates that the re-sensitization is achieved by PMF generation. Similarly, exogenous alanine and /or glucose re-sensitize persister cells to kanamycin by entering the catabolism pathway, producing NADH and then PMF [228]. Mannitol was also reported to enhance the susceptibility of clinically isolated *P. aeruginosa* to tobramycin [201]. L-serine and L-arginine also potentiate aminoglycoside activity by enhancing aminoglycoside influx [204, 206]. Silver ions enhance the killing effect of different types of antibiotics such as ampicillin, gentamicin and ofloxacin against the *E. coli* persisters, partially by membrane permeabilization [229]. It was reported that efflux pump system was actively involved in persistence formation by transporting antibiotic out of the cell. Hence efflux pump inhibitors are promising drugs for anti-persister therapies. The inhibitor PAβN and NMP re-sensitize carbenicillin susceptibility as they inhibit TolC efflux pumps and lead to an increase in intracellular carbenicillin accumulation in *E. coli* [130]. Verapamil causes an increase in the efficacy of rifampicin and isoniazid to kill macrophage-induced *M. marinum* persisters [4].

1.4.2.3 Modification of antibiotic structure

As the aminoglycoside tobramycin cannot penetrate bacterial membrane, it is ineffective against dormant bacteria; conjugation of tobramycin with 12 amino acids (named pentobra) was performed to facilitate the transportation of tobramycin. Pentobra reduces the fraction of *E. coli* and *S. aureus* persisters by 4-6 order of

magnitudes without exhibiting toxicity to mammalian cells [209]. P14KanS was developed by conjugation of kanamycin with P14LRR (an antimicrobial peptide) and it exhibits rapid antibacterial activity against both Gram-positive and Gram-negative persisters. Additionally, P14KanS demonstrates anti-biofilm and anti-inflammatory and protects *C. elegans* from lethal infection of pathogens [208]. These two conjugation drugs implicate a novel strategy for conventional antibiotic reutilization.

1.4.3 Combinations of conventional antibiotics

It has been reported that the tolerant cell fraction consists of several sub-populations, each of which exhibits tolerance to different antibiotics [230]. The combination of different antibiotics is regarded as an effective persister killing strategy. Indeed, several reports have shown that the combined use of conventional antibiotics has potent anti-persister activity. The macrolide erythromycin boosts *P. aeruginosa* biofilm eradication by colistin as erythromycin prevents QS and migration, which are essential for the initiation and formation of colistin-tolerant biofilm sub-populations. The combination of macrolide erythromycin and colistin first inhibits QS and migration, which play a role in biofilm formation, and then disrupts biofilm associated colistin tolerance [211]. Additionally, colistin enhances the anti-persister effect of gentamicin and ofloxacin, which may be due to an increase in uptake of these antibiotics after colistin induced the membrane damage process [210]. Similarly, clinical infection of *A. baumannii* is reported to be eliminated by combination treatment of polymyxin B and meropenem [214]. The combination of daptomycin, doxycycline and cefoperazone eradicates the most recalcitrant subpopulation of *B. burgdorferi* persistence cells which caused Lyme disease [212]. Researchers find that combination of high concentration of ciprofloxacin and vancomycin can kill biofilm-associated *S. epodermidis* persisters [213]. The

combination of different type antibiotics treatment therapy is easy to operate clinically and has the advantage of shortening treatment duration and reducing the chance of resistance occurrence. Meanwhile, this strategy revives or enriches the usage of conventional drugs, reducing the cost and time for treatment when compared with developing novel drugs.

1.4.4 Disruption of persister formation

Upon revelation of mechanisms underlying tolerance formation and maintenance, it is widely recognized that targeting those mechanisms is a high specific and effective way for persister eradication. HipA is widely recognized to be related with tolerance formation, meanwhile HipA inhibitor PKUMDL-LTQ-101-401 remarkably decreases persister population [224]. The engineered bacteriophage which suppresses SOS response by *lexA*3 expression dramatically kills *E. coli* persisters when used in combination with fluoroquinolones both in vitro an in vivo [218].Persister fraction is significantly decreased when stringent response is inhibited by (p)ppGpp analogues, as they competitively inhibit RelA activity and subsequently reduce (p)ppGpp production [218, 222]. Benzamide compounds completely eradicate *P.aeruginosa* persisters together with ciprofloxacin via inhibition of the QS regulator MfvR [215]. Inhibition of stationary phase respiration by NO also leads to decrease in type I persisters subpopulation in *E. coli* [223].

Numerous and diverse compounds are reported to show anti-persister ability via targeting different mechanisms and these novel therapies possess various advantages when compared with the current antibiotic therapies. The developed anti-persister strategy can eradiate the most recalcitrant bacteria such as those in biofilms and then restrict the evolution of resistance, relieving the suffering of patients [9]. However, attrition rates in clinical development of these anti-persister compounds are

dramatically high, particularly in phase 2 and phase 3 clinical trials. Therefore, additional efforts should be focused on novel drug screening and testing in animal models; pre-clinical assessment should be performed to increase the chance of successful translation to clinical trials.

1.5 Summary and objectives of the thesis

The work presents in this thesis focuses on mechanisms underlying tolerance formation, maintenance and exploration of novel drug therapies against persister-associated recalcitrant infection. Cell dormancy was the wide prevalent reason for tolerance formation while increasing evidences show that only dormancy is not necessary or sufficient for bacterial tolerance. We hypothesis some active metabolic processes, pathways or responses play a role in tolerance formation. We aim to find metabolisms which are essential for survival of tolerant cells.

Chapter I incudes the current understanding on the persistence phenomenon, which contains three parts:(i) the deterministic factors. Persister subpopulation is prevalent as a bet-hedging strategy for strain survival, but this population is quite small unless hostile environmental factors trigger metabolic changes. (ii) Current understanding of persister formation mechanism, including stringent response, TA modules, SOS response, general stress response, inactivation of antibiotic targets, efflux pump system. (iii) Therapeutic strategies against persister infection, which are divided into four classes: direct killing of persister, re-sensitization of conventional antibiotic, combinations of conventional antibiotics, disruption of persister formation.

Chapter II involves studies on active mechanism underlying persister formation. We found that active maintenance of PMF is essential for prolonged tolerance. Disruption of PMF by PMF inhibitor, CCCP or deletion of PMF generation genes can cause dramatic decrease in persister population. Moreover, we showed that econazole, an FDA-approved antifungal drug, could cause PMF dissipation in a non-toxic manner and effectively eradicated bacterial persisters when used in combination with β-lactams.

Chapter III describes the investigation of non-toxic compounds which can re-sensitize persisters to antibiotics. We found that *N*-acetyl-D-glucosamine (GlcNAc) or D-glucosamine (GlcN) altered persisters' β-lactam susceptibility and demonstrated that they could reactivate peptidoglycan biosynthesis, which is the target of β-lactam. Sugar catabolism pathways also became active with increasing NADH production and this process was involved in GlcNAc re-sensitization, as deletion of NADH dehydrogenases genes would diminish the GlcNAc re-sensitization effect.

Chapter IV summarizes the findings in the thesis and concludes that PMF was essential for long term maintenance of persistence and could be regarded as the target of persister eradication strategy. GlcNAc and GlcN exhibited persister eradication effect when used in combination with β-lactam and we demonstrated that such effect was due to the reactivation of metabolism induced by GlcNAc.

Chapter Two: Active Maintenance of Proton Motive Force Mediates Starvation-induced Bacterial Antibiotic Persistence

2.1 Abstract

Nutrient starvation is a known trigger of non-heritable phenotypic antibiotic persistence in bacteria. Recent studies reveal that physiological dormancy alone is insufficient for maintaining a long-lasting phenotype. In an attempt to investigate the range of starvation-induced physiological responses underlying persistence development, we found that active maintenance of the transmembrane PMF is essential for prolonged expression of phenotypic antibiotic persistence in both Gram-negative and Gram-positive bacteria. Rapid eradication of antibiotic tolerant sub-population could be achieved by disruption of PMF using the ionophore CCCP, or through suppression of PMF maintenance mechanisms by simultaneous inhibition of the phage shock response and electron transport chain complex activities. Most importantly, we identified an FDA-approved antifungal drug, econazole, which could cause PMF dissipation in a non-toxic manner and act in combination with ceftazidime to effectively eradicate bacterial persisters in mouse infection models. We consider econazole-mediated PMF disruption a feasible strategy for prevention and treatment of chronic and recurrent bacterial infections, especially among immunocompromised patients.

2.2 Introduction

First reported by Joseph Bigger in 1944, bacterial antibiotic persistence is loosely defined as the ability to withstand the deleterious effects of antibiotics at concentrations that can otherwise be lethal, without exhibiting a change in antibiotic susceptibility upon re-growth under favorable conditions [231]. Such non-resistant bacterial sub-populations are also known as persisters. Recent studies reveal that re-growth of antibiotic-tolerant persisters that reside in the human body for a prolonged period is responsible for causing a wide range of chronic and recurrent infections, especially among immuno-compromised patients [7, 232]. Hence delineating the cellular mechanisms that underlie onset and long-term maintenance of a stable antibiotic persistence phenotype in bacteria is more clinically relevant than studying mechanisms governing emergence of transient antibiotic persisters in exponentially growing population. Research studies in the past decade generally suggest that persisters are predominantly dormant, with a number of toxin-antitoxin (TA) modules being postulated to play a role in mediating metabolic shutdown [233]. For instance, the HipA protein was found to drive cells into dormancy by phosphorylating the residue Ser239 in the ATP-binding site of glutamyl-tRNA-synthetase [73]. However, the role of TA modules in regulating formation of persisters have recently been refuted [101, 226]. Recent studies suggest the existence of redundant cellular mechanisms underlying tolerance formation, and that dormancy alone is insufficient for long-term maintenance of the tolerance phenotype. Nguyen *et al*. showed that starvation-induced antibiotic tolerance involves curtailing production of pro-oxidant metabolites and increasing antioxidant defenses [22]. Intriguingly, it was found that persisters could be derived from the fastest-growing bacterial sub-population, and that dormancy is neither necessary nor sufficient for formation of persisters [45]. Currently, a number of bacterial stress responses that

are actively induced by environmental cues are known to play an essential role in formation of antibiotic persistence in bacteria. The stringent response, which involves production of the alarmone (p)ppGpp and is inducible by nutrient starvation and sub-lethal antibiotic treatment, can modulate bacterial tolerance via adjustment of target protein activities and transcriptional reprogramming [35, 234]. A recent study also showed that efflux activities played an active role in conferring the bacterial persistence phenotype through up-regulation of the expression level of a range of multidrug efflux genes, such as *tolC, acrA, acrB, acrD, emrA, emrB, macA* and *macB* [207]. In this previous study, time-lapse imaging of antibiotic persisters containing fluorescence-labelled TolC confirmed that high expression level of *tolC* directly led to formation of antibiotic-tolerant sub-population. However, the molecular basis of efflux-mediated tolerance remains unclear and deserves further study. In view of these findings, we propose to investigate the full spectrum of regulatory and active defense mechanisms involved in formation of bacterial antibiotic persistence.

In order to unravel the range of active cellular mechanisms required for eliciting and maintaining prolonged phenotypic persistence to antibiotics, nutrient starvation was chosen as an induction factor in this work because of the following reasons: (i) starvation was previously shown to induce a significantly stronger persistence phenotype than compounds that inhibit bacterial growth under nutrient-rich conditions [21], suggesting that starvation-induced persistence probably involves inducible defensive mechanisms and deserves more in-depth and systematic exploration; (ii) nutrient starvation is commonly encountered by bacteria during the infection process, hence starvation-induced tolerance responses should be investigated comprehensively at the transcriptional and physiological level; (iii) it is a readily manipulated test condition, which facilitates investigation into the key

mechanisms underlying formation of antibiotic persistence [22, 106, 235]. In this work, we found that active maintenance of PMF is essential for starvation-induced tolerance, and that disruption of PMF resulted in complete eradication of the entire antibiotic-tolerant sub-population. These findings confirm that bacteria actively encode protective cellular mechanisms for expression and maintenance of antibiotic persistence phenotypes, providing the molecular basis for devising new approaches to eradicate antibiotic tolerant sub-population through PMF disruption.

2.3 Materials and Methods

2.3.1 Strains, media, culture conditions and chemicals.

Escherichia coli K-12 strains, derivatives thereof and other strains used listed in **Table 2.1** were used in all experiments. Luria-Bertani (LB) broth was used for all cultures unless stated otherwise. Standard LB agar (Difco, Leeuwarden, The Netherlands) was used in testing the proportion of the bacterial population that survived in the antibiotic persistence assay. Ampicillin and carbonyl cyanide 3-chloropheylhydrazone (CCCP) were purchased from Sigma (St. Louis, MO). $DiSC_3(5)$ was purchased from Invitrogen. BOCILLIN^{TM} FL Penicillin and Nile Red were obtained from ThermoFisher and Meilunbio, respectively.

Table 2.1 Strains used in this study

Strain	**Source**	**Identifier**
Escherichia coli:BW25113	Coli Genetics Stock Center	CGSC#7636
BW25113 Δ*pspA*::kan^r	Coli Genetics Stock Center	CGSC#9162
BW25113Δ*pspB*:: kan^r	Coli Genetics Stock Center	CGSC#9163
BW25113Δ*pspC*:: kan^r	Coli Genetics Stock Center	CGSC#9164

BW25113Δ*pspD*:: kan^r	Coli Genetics Stock Center	CGSC#9165
BW25113Δ*pspE*:: kan^r	Coli Genetics Stock Center	CGSC#9166
BW25113Δ*pspF*:: kan^r	Coli Genetics Stock Center	CGSC#9161
BW25113Δ*nuoI*:: kan^r	Coli Genetics Stock Center	CGSC#9834
BW25113Δ*ndh*:: kan^r	Coli Genetics Stock Center	CGSC#11791
BW25113Δ*ndhnuoI*:: kan^r	This work	N/A
Klebsiella pneumoniae	ATCC	ATCC13883
Staphylococcus aureus	ATCC	ATCC29213
Acinetobacter baumannii	ATCC	ATCC19606
Salmonella typhimurium PY01	ATCC	ATCC14028s
Pseudomonas aeruginosa PAO1	DSMZ	DSM 22644

2.3.2 Determination of Minimal Inhibitory Concentrations (MICs)

The MIC of ampicillin or econazole against *Acinetobacter baumannii* ATCC19606, *Klebsiella pneumoniae* ATCC13883, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ATCC29213 and *E. coli* K-12 BW25113 and its gene knockout derivatives were determined by incubating freshly grown cultures (Mueller Hinton Broth (MHB)) (BD Difco, America) with various concentrations of ampicillin or econazole for 16 hours, recording the concentration that inhibited bacterial growth and resulted in a lack of turbidity. Results were based on the average of at least three independent experiments and interpreted according to CLSI guideline [236].

2.3.3 Persistence assay

Upon reaching the exponential phase, bacteria were washed and resuspended in saline (0.85% NaCl), incubated at 37℃ under constant shaking (250rpm/min) for 24 hrs, followed by treatment with ampicillin at concentration of 10 times of MIC **(Table 2.3)** for 144 hrs (6 days), supplementing fresh ampicillin every 48 hrs. Standard serial dilution and plating on LB agar was performed after ampicillin treatment for 4hrs, 2 days, 4 days and 6 days to determine the fraction of the test population that survived at different time points during the course of treatment [21].

2.3.4 RNA Sequencing and analysis

Fresh *E. coli* K-12 BW25113 colonies were inoculated into LB medium and grown overnight at 37℃ under constant shaking (250 rpm/min). The overnight culture was diluted 100-fold in LB broth and cultivated for about1hr until the OD_{600} value reached 0.2 (exponential phase). Aliquots of this exponential phase culture were washed and resuspended in saline, followed by incubation at 37℃ for 24 hrs under constant shaking (250rpm/min) to create a population which experienced prolonged starvation stress. Total RNA of bacteria collected from the exponential phase and starvation phase was extracted by the RNeasy Mini Kit (Qiagen, Germany); transcriptome sequencing was performed at the Beijing Genomics Institute (Hong Kong).

2.3.5 Western blot analysis

Upon starvation for 24hrs, bacteria were harvested by centrifugation and solubilized in sample buffer for 10 mins at 100℃. Total cellular proteins were separated by SDS-PAGE and electroblotted onto PVDF membrane (BIO-RAD 0.2 μM) using a semi-dry electroblotting apparatus (BIO-RAD). Membranes containing fractionated

samples were first probed with anti-PspA or anti-GAPDH antibodies and then washed with tris-buffered saline and Tween 20 (TBST). Washed membranes were re-blocked and probed with anti-rabbit antibodies simultaneously. Target protein bands were detected by measurement of chemiluminescence exhibited by the HRP substrate (EMD Millipore); relative band intensities of Western blots were calculated by ImageJ [237].

2.3.6 Membrane permeability assay

The membrane permeability or integrity of the test organisms was measured using SYTOX, which can enter the cell through damaged cell membrane and bind to nucleic acid, releasing fluorescence signal. *E. coli* BW25113 and its Δ*pspA* derivative at a concentration of OD_{600} of 0.2, which had been subjected to 24 hrs starvation, were collected by centrifugation (6000g, 2mins), washed twice and resuspended in saline. SYTOX was then added to give a final concentration of 1μM, followed by incubation for 30min in the dark at room temperature. The relative fluorescence signal in the wild type and Δ*pspA* strain was measured by a Cary Eclipse Fluorescence Spectrophotometer (Agilent), with an excitation wavelength of 488$\pm$10 nm and an emission wavelength of 523$\pm$10 nm [238, 239].

2.3.7 Growth rate detection

The overnight cultured wild type strain was diluted 1:100 by LB Broth, followed by adding 100 μM PAβN with adding saline as negative control. OD_{600} value was tested on different time point.

2.3.8 Membrane potential assay

The transmembrane electrical potential was measured by using a membrane potential-sensitive probe, $DiSC_3(5)$. Population of bacteria in either the exponential phase or under 24 hrs starvation were collected by centrifugation (6000×g, 2mins), washed twice and resuspended in PBS (pH 7.4). KCl and $DiSC_3(5)$ were added until final concentration of 100mM and 1μM was respectively reached, followed by incubation at room temperature for 25mins in the dark to allow the dye to penetrate through the outer membrane and produce a quenching effect. Valinomycin (1μM) was then added to the positive control group to transport K^+ into cytoplasm, which resulted in depolarization. The fluorescence reading was monitored by using a Clariostar Microplate Reader (BMG LABTECH) at an excitation wavelength of 622±10 nm and an emission wavelength of 670±10 nm for 10mins. Upon depolarization, the dye was rapidly released into the medium, resulting in dequenching and facilitating detection fluorometrically [240]. Single-cell imaging was also conducted for testing the difference of the membrane electrical potential between wild type strain and the Δ*pspA* mutant. Bacteria were imaged by the Leica TCS SP8 MP Multiphoton Microscope with a 60×oil-immersion objective. $DiSC_3(5)$ was excited by 638 nm laser and fluorescence was detected by HyD detector at emission wavelength 675±25 nm. The images were acquired and analyzed by the Leica Application Suite X (LAS X) software.

2.3.9 Assessment of effect of proton ionophore, sodium azide and econazole on starvation-induced persistence

To explore the role of PMF in maintenance of persistence, the uncoupling agent CCCP, sodium azide or econazole was added to bacteria, which had been subjected to starvation for 24 hrs, to give a final concentration of 100 μM,5mM or 40μM

respectively, followed by incubation for up to 144 hrs and treatment with ampicillin at 100 μg/ml for 144 hrs. Standard serial dilution and plating on LB agar were performed after ampicillin or CCCP treatment.

2.3.10 Antibiotic accumulation assay

Bacteria which had been subjected to 24hrs starvation were incubated with BOCILLIN™ FL Penicillin (10 μg/mL) for 30min at 37℃, with shaking at 250 rpm/min. Upon washing twice with PBS, fluorescence signal was measured by a Clariostar Microplate Reader at an excitation wavelength of 488±10 nm and an emission wavelength of 512±10 nm. Cells were also observed by the Leica TCS SP8 MP Multiphoton Microscope and images were analyzed by the LAS X software.

2.3.10 Antibiotic efflux assay

A10-mL portion of bacterial population which had been subjected to 24 hrs starvation was centrifuged at 6000g for 5min at room temperature. The pellet was resuspended in PBS containing 1mM $MgCl_2$ (PPB) and adjusted to OD_{600} 0.2. Nile red in the form of a stock solution of 5mM in DMSO was added to produce a final concentration of 5 μM, followed by incubation at 37℃ for 30 mins, with 250rpm/min shaking. CCCP was then added to produce a final concentration of 100 μM; fluorescence was measured for a period of 30 mins by a Clariostar Microplate Reader at an excitation wavelength of 544±10 nm and an emission wavelength of 650±10 nm [241].

2.3.11 Electron microscopy analysis

E. coli which had been subjected to starvation for 24hrs was treated with econazole alone, ampicillin alone and a combination of econazole and ampicillin for 24hrs,

followed by examination under scanning electron microscopy (SEM). Cells treated with saline were included as negative control. Briefly, bacterial cells were fixed in 0.4% polyoxymethylene overnight and then in Osmium tetroxide (OsO_4) for 2hrs, followed by washing for three times with PBS. Dehydration was performed post fixing using pure ethanol. Cells were then infiltrated and embedded in Spurr resin for examination by SEM.

2.3.12 Mouse deep-seated thigh infection model

Six-weeks-old NIH male mice (body weight, ~20g) purchased from the Guangdong Center for Experimental Animals were used in animal experiments. The mice were made neutropenic by administering 150mg/kg cyclophosphamide at 3 days and 1 day before infection. An inoculum of 1×10^6 CFU of *E. coli* BW25113 were injected to the right thigh of the mouse. At 24hrs post-infection, the mice in each group received antibacterial treatment (i.p.) every 12hrs for 72hrs. Mice were euthanized and the infected thighs were aseptically excised, homogenized in PBS and the number of *E. coli* was enumerated by serial dilution, spreading on LB plates, and incubation at 37℃ overnight. The population size of bacteria that survived different treatments were recorded, compared and analyzed using unpaired t-test and presented by Graph Pad Prism. All experimental protocols followed the standard operating procedures of the Biosafety level 2 animal facilities approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

2.3.13 Mouse peritonitis model

NIH male mice of about six-weeks-old with body weight of ~20g were used. After 5 days of quarantine, different amounts of *S.* Typhimurium strain PY01 were inoculated into the animals via intraperitoneal injection. The mice were subjected to

antibacterial treatment (i.p.) 24hrs after inoculation, at 12hrs intervals. After 72hrs treatment, the mortality rate of the test mice was recorded. Live mice were euthanized and subjected to peritoneal washes which involved injection of 2 mL of saline in the intraperitoneal space, followed by abdominal massage. The abdomen was then open and 200 μL of peritoneal fluid were collected and serially diluted in PBS. A 100 μL portion of each dilution was spread on LB plates, followed by incubation overnight at 37°C. Colonies were counted to determine the bacterial load in the test samples, which were expressed as CFU/ml. Statistical analysis and ethic approval were the same as described above.

2.3 Results

2.3.1 Genes in the *psp* family are over-expressed during starvation

In order to explore the range of physiological responses that play a role in development and maintenance of antibiotic persistence during starvation, we first performed RNA-Seq to identify genes whose expression level was significantly up-regulated after the test organisms had experienced a prolonged starvation episode. Since metabolic activities are reduced to a minimum when nutrients are depleted, the expression level of most functional genes is expected to be kept at a minimum, with the exception of essential proteins which may modulate adaptive physiological responses. Such proteins are therefore expected to contribute directly or indirectly to formation of starvation-induced persistence. Based on the RNA-Seq data, we identified a total of 56 genes which, when compared to exponentially growing cells, were expressed at a level three-folds or more after the test organisms had encountered starvation stress for 24hrs in saline **(Table 2.2)**. These genes included those encoding transcriptional regulators, membrane transporters, oxidative enzymes, DNA repair proteins and starvation stress sensors, among them, a

functionally important gene cluster in which the expression level of all members was significantly elevated was identified. This gene cluster is the phage shock protein (*psp*) family which includes the *pspA*, *B*, *C*, *D*, *E* and *F* genes, the expression level of which was up-regulated 124, 101, 123, 28, 8 and 11 folds respectively after encountering starvation for 24hrs **(Table 2.2)**. We then focused on investigating the role of this gene cluster in mediating bacterial starvation tolerance response.

Table 2.2 A total of 56 shortlisted genes whose expression level was found to be upregulated by three folds or more in RNA-Seq upon starvation for 24 hrs.

Gene	**Fold-upregulated***	**Gene product**
mtr[1]	213.5	tryptophan-specific transport protein
emrK[1]	122.5	multidrug resistance efflux pump protein K
nanT[1]	66.3	MFS transporter, SHS family, sialic acid transporter
emrY[1]	54.3	multidrug resistance efflux pump protein Y
eamB[1]	46.7	cysteine/O-acetylserine efflux protein
mdtN[1]	15.5	multidrug resistance protein MdtN
eamA[1]	13.2	O-acetylserine/cysteine efflux transporter
argT[1]	12.8	lysine/arginine/ornithine transport system substrate-binding protein
zraP[1]	11.6	zinc resistance-associated protein
cusC[1]	10.5	Cu(I)/Ag(I) efflux system outer membrane protein CusC
cusF[1]	9.5	Cu(I)/Ag(I) efflux system periplasmic protein CusF
mdfA[1]	9.4	MFS transporter, multidrug/chloramphenicol efflux transport protein
aaeA[1]	7.9	p-hydroxybenzoic acid efflux pump subunit AaeA
cusB[1]	6.5	Cu(I)/Ag(I) efflux system membrane protein CusB
marC[1]	6.1	multiple antibiotic resistance protein
mdtK[1]	6.0	multidrug resistance protein, MATE family
artM[1]	5.4	arginine transport system permease protein
mdtH[1]	5.2	MFS transporter, DHA1 family, multidrug resistance protein

kefG[1]	5.0	glutathione-regulated potassium-efflux system ancillary protein KefG
mdtP[1]	4.4	multidrug resistance outer membrane protein MdtP
mdtO[1]	4.0	multidrug resistance protein MdtO
artP[1]	3.6	arginine transport system ATP-binding protein [EC:3.6.3.-]
aaeB[1]	3.4	p-hydroxybenzoic acid efflux pump subunit AaeB
rmf[2]	199.9	ribosome modulation factor
mqsA[2]	126.8	HTH-type transcriptional regulator / antitoxin for MqsR toxin
puuR[2]	56.3	HTH-type transcriptional regulator, repressor for puuD
mazF[2]	13.1	mRNA interferase
pspA[3]	123.5	phage shock protein A
pspC[3]	122.8	phage shock protein C
pspB[3]	100.8	phage shock protein B
hspQ[3]	40.2	heat shock protein HspQ
pspD[3]	28.4	phage shock protein D
papD[3]	24.9	chaperone protein PapD
fimC[3]	21.1	fimbrial chaperone protein
ecpD[3]	16.0	chaperone protein EcpD
pspF[3]	10.6	phage shock protein F
pspE[3]	7.8	phage shock protein E
yphA[4]	13.6	putative oxidoreductase
zraP[4]	6.7	Cu/Zn superoxide dismutase [EC:1.15.1.1]
recG[5]	19.3	DNA repair protein RecG
dinJ[5]	18.4	DNA-damage-inducible protein J
umuD[5]	16.0	DNA polymerase V [EC:3.4.21.-]
sbmC[5]	15.4	DNA gyrase inhibitor
rpoE[5]	13.6	RNA polymerase sigma-70 factor, ECF subfamily
recN[5]	10.4	DNA repair protein RecN (Recombination protein N)

umuC[5]	9.9	DNA polymerase V
rpoD[5]	3.6	RNA polymerase primary sigma factor
dnaQ[5]	3.4	DNA polymerase III subunit epsilon [EC:2.7.7.7]
rpoH[5]	3.2	RNA polymerase sigma-32 factor
dinB[5]	3.2	DNA polymerase IV [EC:2.7.7.7]
uspB[6]	13.4	universal stress protein B
rspB[6]	11.4	starvation sensing protein RspB [EC:1.1.1.-]
rspA[6]	6.1	starvation sensing protein RspA
uspD[6]	5.7	universal stress protein D
dps[6]	4.8	starvation-inducible DNA-binding protein
cstA[6]	4.2	carbon starvation protein

*Fold difference in expression level of the test genes in *E. coli* population which had been starved for 24 hrs, with exponentially growing population of identical cell density as control.

[1] Efflux and membrane protein genes

[2] Transcriptional regulator genes

[3] Psp system and chaperone genes

[4] Oxidative enzyme genes

[5] DNA repair genes

[6] Starvation stress sensing genes

2.3.2 The role of Psp response in mediating starvation-induced persistence

To test whether products of the up-regulated *psp* genes play a role in formation of antibiotic persistence, we monitored and compared the change in level of starvation-induced persistence of specific gene knockout mutants to the wild type strain within a six days period and noticed that, although the level of tolerance in both the wild type and Δ*pspA* strain was similar at the initial phase of treatment, the proportion of ampicillin persisters in the Δ*pspA* mutant dropped at a significantly faster rate over

the six days period (~3.4×10^5 cells/mL) while population size of non-ampicillin treated Δ*pspA* mutant is ~7.3×10^6 cells/ml, so the survival rate after ampicillin treatment in Δ*pspA* is 4.6%. The population of ampicillin persisters in wild type after six days period is ~3.8×10^6 cells/ml and that of non-ampicillin treated wild type is ~2.5×10^7 cells/ml, which makes the survival rate after ampicillin treatment in wild type is 15.6% , much higher than that in Δ*pspA* mutant **(Fig 2.1A)**. The size of the persisters population in knockout mutants of the other genes in the *psp* family, however, was similar to that of wild type throughout the experiment as the survival rate after ampicillin treatment in Δ*pspC* ,Δ*pspD* and Δ*pspF* is 8.1%, 29.6% and 37.3%, respectively **(Fig 2.1B)**. It should be noted that the size of the antibiotic tolerant sub-population in the Δ*pspA* strain dropped to ~3×10^5 cells/mL after 6 days of ampicillin treatment, which was only 10% of that of wild type (~4×10^6 cells/mL). These findings imply that the *pspA* gene product is not essential for persistence formation but required for long term maintenance of the persistence phenotype. We confirmed that the phenotypes of the tolerant sub-population were not due to genetic mutations that conferred drug resistance because upon removal of antibiotic stress, as the tolerant sub-population was able to regrow as antibiotic susceptible organisms, and the minimum inhibitory concentration (MIC) of knockout mutants of the *psp* gene family remained the same as that of wild type (8μg/ml) **(Fig 2.2, Table 2.3)**. PspA is the key functional protein among members of the Psp family and known to play a major role in Psp response [242]. We next investigated its role in maintenance of the persistence phenotype and tested whether an increase in gene expression level of *pspA* actually resulted in a corresponding increase in protein level. Western blotting was performed, with results showing that PspA was barely detectable in the exponential phase, yet an abundance of this protein was synthesized upon encountering starvation for 24hrs, the level of which was 9.4 folds that of the exponential phase control **(Fig 2.1C)**. In addition to maintenance of antibiotic

persistence, the *pspA* gene product was also found to be essential for bacterial survival during starvation in the absence of antibiotics. Throughout a period of starvation for six days without ampicillin treatment, the population size of the *pspA* knockout strain shrank gradually to a level of ~7.3×10^6 cells/ml, whereas that of wild type remained relatively constant at ~2.5×10^7 cells/ml **(Fig 2.1A)**.

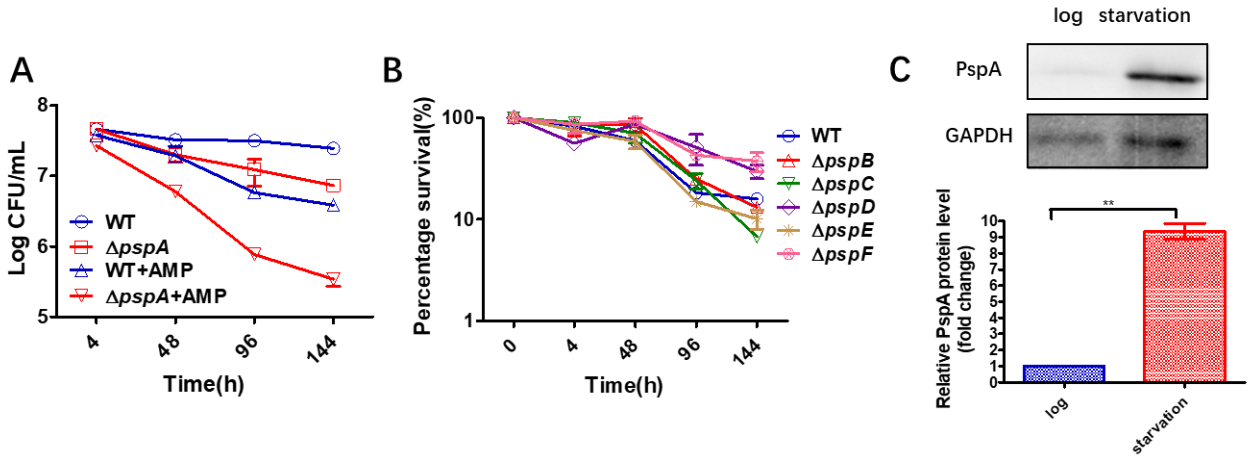

Fig 2.1 Activated Psp response during nutrient starvation is essential for bacterial survival and regulation of antibiotic tolerance.

(A) The wild type strain and Δ*pspA* mutant was starved for 24 hrs starvation, followed by treatment with ampicillin at 100 μg/ml for 144 hrs with nondrug treatment as negative control, variation in CFU recorded at different time points is shown. (B) Relative tolerance ratio of the wild type strain and *psp* mutants calculated on the basis of the survival ratio of bacterial population that survived treatment with ampicillin at 100 μg/ml for 144 hrs upon starvation for 24 hrs, as compared to those subjected to 24 hrs starvation only. (C) Western blot analysis of the PspA protein in bacterial population at a cell density of OD (600nm) 0.2 and bacterial population at cell density of OD (600nm) 0.2 which then was subjected to starvation for 24 hrs, with the endogenous protein GAPDH as internal control.

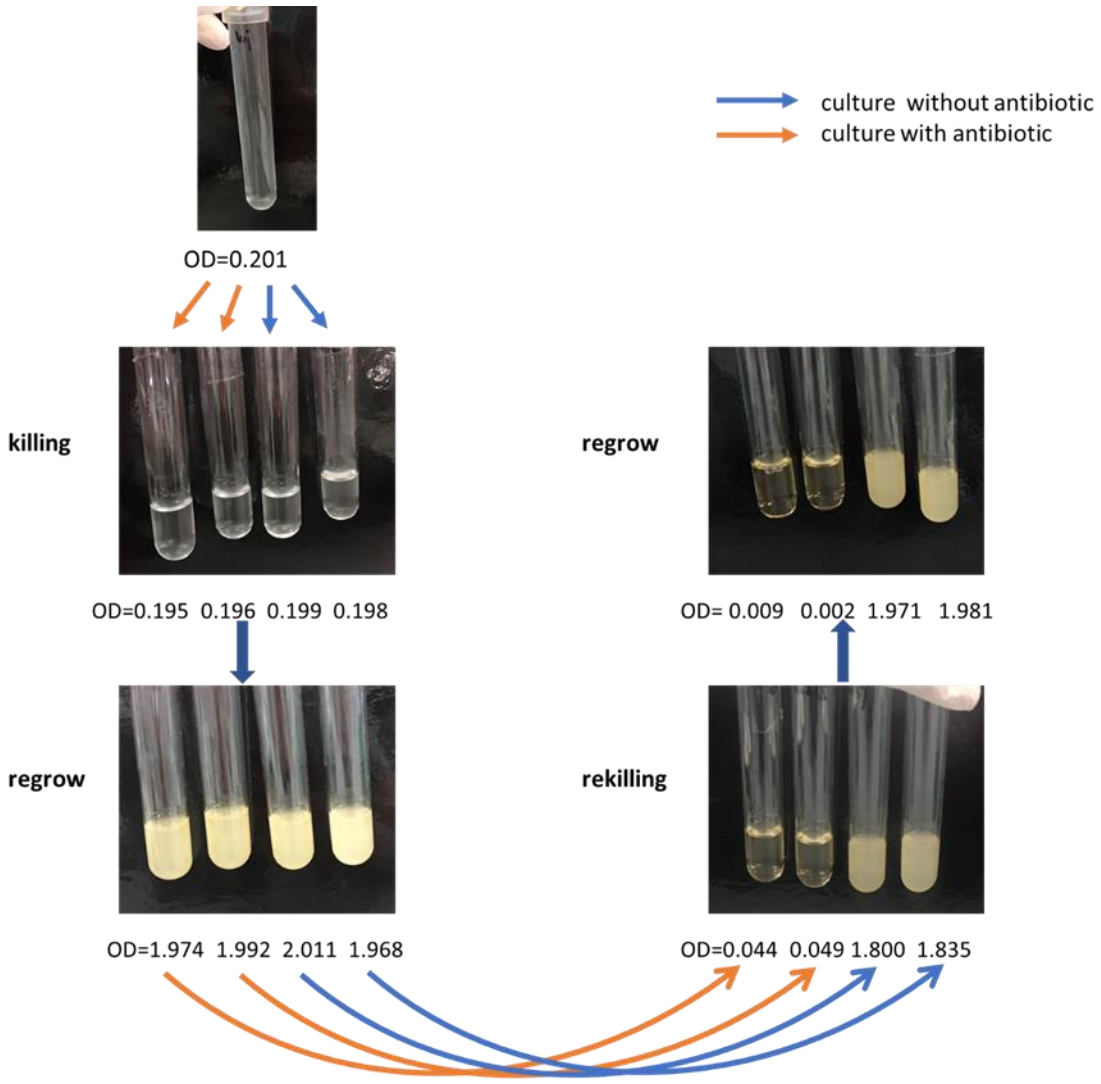

Fig 2.2 Assessment of antibiotic susceptibility of bacterial sub-population exhibiting starvation-induced antibiotic persistence.

Wild type bacteria subjected to nutrient starvation for 24hrs were split into two portions, one was treated with 100μg/mL ampicillin for 4hrs to obtain antibiotic tolerant sub-population, and the other without antibiotic was set as control. The tolerant sub-population was then collected by centrifugation, followed by re-suspension and dilution in fresh LB and incubation at 37°C to induce regrowth. Fresh bacterial culture derived from this tolerant sub-population was subjected to antibiotic

susceptibility tests, with results confirming that offsprings of such sub-population remained susceptible to the test agent.

Table 2.3 MIC of ampicillin and econazole for different bacterial strains.

Bacterial strains	**MIC**	
	Ampicillin (μg/ml)	**Econazole (μM)**
E. coli BW25113	8	>160
S. aureus ATCC29213	2	40
K. pneumoniae ATCC13883	512	>160
A. baumannii ATCC19606	128	>160
P. aeruginosa PAO1	1024	>160
S.typhimurium PY01	2	>160
Δ*pspA*	8	
Δ*pspB*	8	
Δ*pspC*	8	
Δ*pspD*	8	
Δ*pspE*	8	
Δ*pspF*	8	
Δ*tolC*	4	
Δ*nuoI*	8	
Δ*ndh*	8	
Δ*ndhnuoI*	8	

2.3.3 Psp proteins play a role in maintaining PMF during starvation

It was reported that Psp proteins are involved in a wide range of membrane functions, with the PspBC complex being located in the inner membrane, interacting with PspA to prevent alteration in inner membrane permeability and cytoplasmic shrinkage [243]. We therefore hypothesized that deleting the *pspA* gene may undermine membrane integrity, especially under adverse environmental conditions, leading to membrane leakage. By using the dye SYTOX to test membrane permeability during starvation, however, we showed that the amount of dye taken up by the wild type and Δ*pspA* strain during starvation was similar **(Fig 2.3A)**, indicating that membrane permeability was not significantly altered in the Δ*pspA* mutant. Likewise, although colistin treatment was found to cause membrane damage and an eventual increase in membrane permeability, the degree of changes in membrane permeability in both wild type *E. coli* and Δ*pspA* mutant after colistin treatment were similar **(Fig 2.3 A)**, suggesting that the Psp response conferred little protective effect against this membrane destabilizing agent.

One major role of the PspA protein is to maintain bacterial PMF [244]. Oligomers of PspA other than the PspBCA complex were found to bind to membrane and prevent proton leakage [245]. We then postulate that the reason why increased PspA expression could help maintain phenotypic tolerance is that it helps preserve PMF during starvation. The membrane potential is normally created by establishing a proton (H^+) gradient across the cell membrane [246]. We therefore used the dye $DiSC_3(5)$ to test the extent of changes in bacterial cell membrane potential upon entry into the starvation mode. In the exponential phase, the fluorescence intensities of the wild type and Δ*pspA* strains were found to be similar and exhibited the same

degree of changes upon addition of valinomycin. A sharp increase in fluorescence was observable after valinomycin had caused dissipation of the membrane potential in both strains **(Fig 2.3B)**. Strong quenching of $DiSC_3(5)$ fluorescence will be observed when it accumulates into cells via membrane potential gradient, while adding valinomycin to dissipate membrane potential, $DiSC_3(5)$ will be rapidly released into the medium resulting in dequenching with the fluorescence intensity enhancing. Upon encountering starvation for 24hrs, however, the fluorescence intensity of the Δ*pspA* strain was significantly higher than that of the wild type; such difference was found to be widened further upon addition of valinomycin, indicating that the amount of dye accumulated intracellularly was much lower in the *pspA* mutant during starvation, presumably due to the lack of ability to maintain H^+ gradient in the absence of the *pspA* gene product **(Fig 2.3C)**. On the other hand, the fluorescence intensity of the wild type strain also remained at a level similar to that of the exponential phase at 24hrs starvation and exhibited a similar degree of change in intensity upon addition of valinomycin, thereby confirming that the proton gradient of the wild type could be maintained at a level equivalent to that of the exponential phase during starvation **(Fig 2.3D)**. These findings were consistent with results of the confocal microscopy experiment, in which only the wild type strain could be stained by $DiSC_3(5)$ upon starvation for 24hrs. The dye apparently could not enter the Δ*pspA* cells because the membrane potential was too low. The same phenomenon was observable in the wild type strain in the presence of valinomycin, indicating that deleting the *pspA* gene resulted in the same consequence as that mediated by valinomycin, that is, failure to maintain the proton gradient under starvation **(Fig 2.3E)**. Based on the confocal images, we calculated the fluorescence intensity of each group and found that the intensity of the wild type strain (100 RFU/cell) was approximately seven folds that of the Δ*pspA* mutant strain (15

RFU/cell); such finding further confirmed that knocking out the *pspA* gene would cause rapid dissipation of the proton gradient during nutrient starvation **(Fig 2.3F)**.

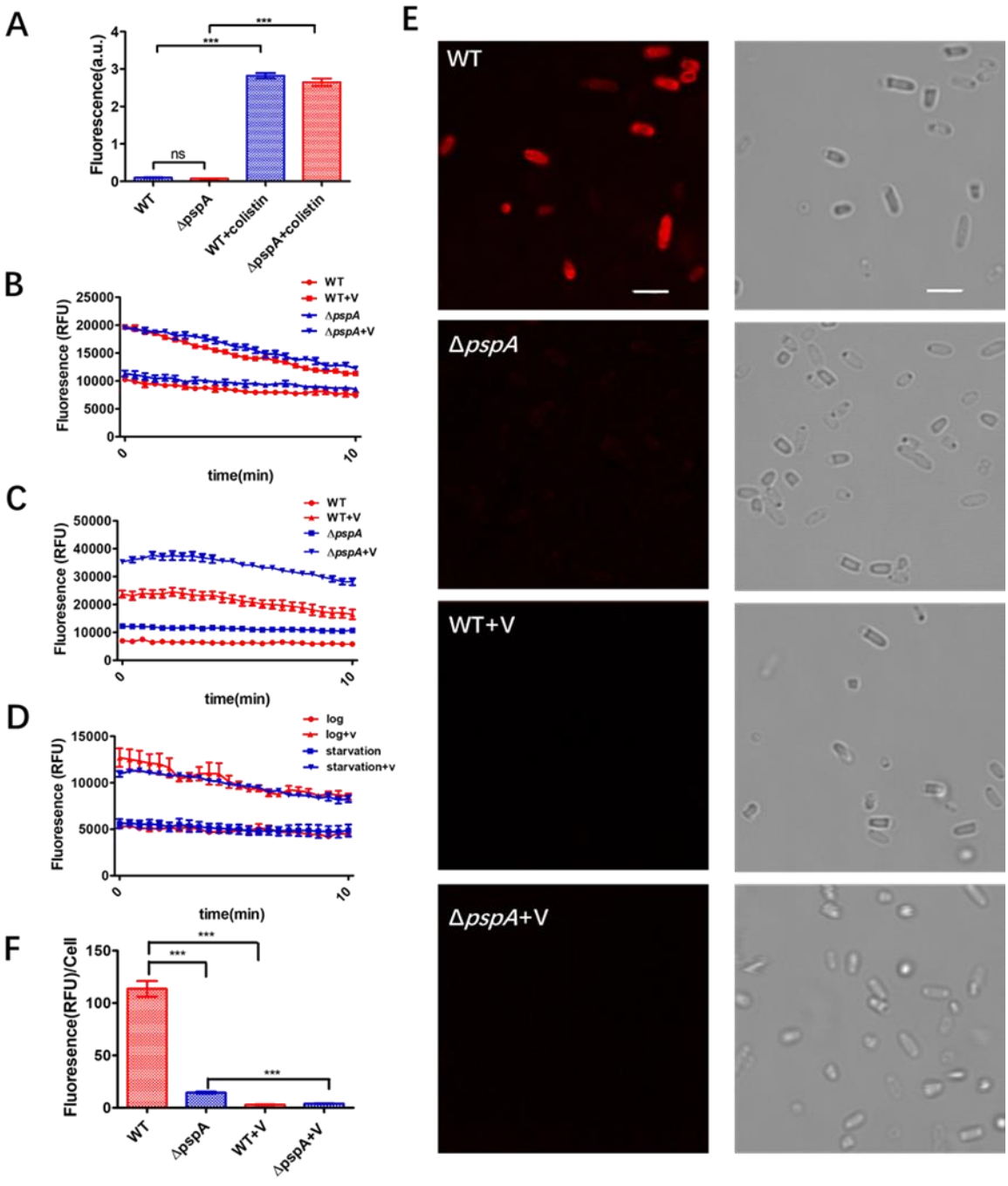

Fig 2.3 Psp response helps maintain PMF during starvation in *E. coli*.

(A) Fluorescence intensity of SYTOX, which was used to detect membrane permeability of the wild type and Δ*pspA* strains, showed that the cell membrane in

the Δ*pspA* strain remained intact; strains treated with colistin were included as positive control. (B) Comparison between the fluorescence intensity of $DiSC_3(5)$-stained cells in the exponential growth phase of both wild type and Δ*pspA* strains reveals the same initial intensity and a similar degree of changes in membrane potential upon addition of valinomycin (labelled as V). (C) Comparison between the fluorescence intensity of $DiSC_3(5)$-strained wild type and the Δ*pspA* strain which had been subjected to starvation for 24 hrs depicts a much higher fluorescence intensity and hence a much lower proton gradient in the *ΔpspA* mutant. (D) Comparison between the fluorescence intensity of $DiSC_3(5)$-strained exponentially growing wild type population and those which had been subjected to 24 hrs starvation reveals a similar initial fluorescence intensity and also a similar degree of changes in fluorescence intensity, and hence membrane potential, upon addition of valinomycin. Data are the average of at least two independent experiments performed with three biological replicates ($n \geq 6$). Error bars represent standard deviations. (E) Confocal microscopy images of DiSC3(5)-stained cells which have been subjected to 24 hrs starvation in the absence and presence of valinomycin. The left and right panels are the fluorescence and bright field images respectively (scale bar:4 m). (F) The mean $DiSC_3(5)$ fluorescence intensity of confocal microscopy image, which was calculated by the LAS X software.

2.3.4 Maintenance of PMF is essential for long term survival of starvation-induced persisters

Upon identifying the PMF maintenance role of the *pspA* gene product and confirming the functional importance of PMF in actively maintaining phenotypic persistence, we hypothesize that merely preventing dissipation of pre-existing PMF is not sufficient for long term maintenance of the persistence phenotype, and that bacteria subjected to starvation stress may still undergo a low level of oxidative

phosphorylation to generate a basal level of PMF to support the ability to import exogenous nutrients and extrude toxic metabolites and antibiotics from the cytosol. To test this possibility, we determined whether sodium azide, which inhibits cytochrome C oxidase and hence the ability to generate PMF, could also cause significant reduction in the persistence level of bacterial population subjected to prolonged starvation. Our results showed that the population size of the wild type strain was only slightly reduced upon treatment with sodium azide, regardless of whether ampicillin was present or not **(Fig 2.4A)**. The effect of sodium azide alone on the Δ*pspA* mutant, which lacks the ability to maintain PMF, was similar to that of the wild type strain, exhibiting a slight bactericidal effect. In the presence of ampicillin, however, it was able to eradicate the entire tolerant sub-population beyond 96hrs **(Fig 2.4B)**. These findings therefore confirm that, under nutrient-deficient conditions, the transmembrane proton gradient is essential for prolonged maintenance of the persistence phenotype, as simultaneous inhibition of both PMF production and maintenance, by sodium azide treatment and deletion of the *pspA* gene respectively, led to complete eradication of ampicillin persisters that formed under prolonged starvation conditions. To further confirm the role of PMF in starvation-induced tolerance response, the uncoupling agent carbonyl cyanide-m-chlorophenylhydrazone (CCCP), which is known to cause immediate and complete dissipation of PMF, was used to test its effect on *E. coli* persistence response. When CCCP was added to *E. coli* cells which had been starved for 24hrs, the size of the surviving population dropped from ~2.5×10^7 cells/mL to ~5×10^5 cells/mL within 24 hrs, and to ~2×10^4 cells/mL if ampicillin was added **(Fig 2.4A)**. The entire antibiotic tolerant population was then eradicated by 48hrs with or without ampicillin treatment, indicating that starvation induced persisters could no longer survive for a prolonged period upon collapse of PMF **(Fig 2.4A)**. It should be noted that addition of CCCP to an exponential-growing population did not result in alteration of the

population size **(Fig 2.4C)**, indicating that CCCP only exhibits strong suppressive effect on starving population. The persistence suppressing effect of CCCP was also observed in the Δ*pspA* mutant **(Fig 2.4B)**. The fact that CCCP was more effective in eradicating persister cells than *pspA* gene deletion alone, sodium azide alone, or a combination of these two factors, was likely due to its strong and direct effect in causing the collapse of PMF, whereas the combined effect of *pspA* gene deletion and sodium azide treatment only resulted in slow dissipation of the existing proton gradient. Taken together, our data proved that active maintenance of PMF is key mechanism underlying prolonged expression of phenotypic antibiotic persistence during nutrient starvation.

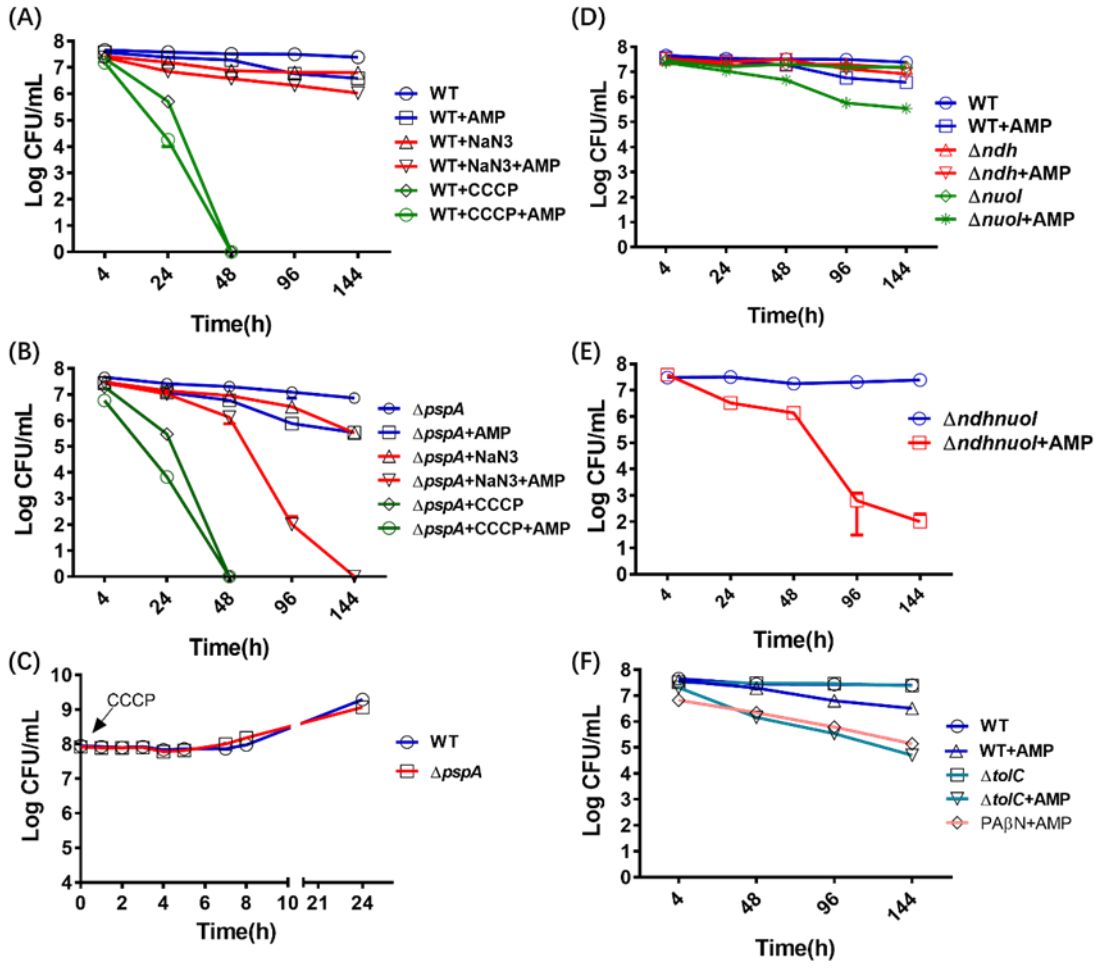

Fig 2.4 PMF is essential for starvation-induced persister formation.

(A)The CFU detected at different treatment time point by ampicillin, NaN_3, CCCP or their combination upon wild type which have been starved for 24hrs. (B) The CFU detected after CCCP added to exponential phase (OD_{600}=0.2)wild type and Δ*pspA*, setting the time point of adding CCCP as 0hrs.(C) The CFU detected at different treatment time point by ampicillin, NaN_3, CCCP or their combination upon Δ*pspA* which have been starved for 24hrs. (D,E) the wild type strain and Δ*ndh*, Δ*nuoI*, Δ*ndh*Δ*nuoI* mutant was starved for 24hrs starvation, followed by treatment with ampicillin at 100 μg/mL for 144hrs, CFU was detected at different time point. (F) The CFU detected at different treatment time point by ampicillin upon cells which have been starved for 24 hrs.

To confirm if active maintenance of PMF indeed plays a key role in expressing phenotypic persistence in bacterial persisters, we further tested if disruption of genetic pathway governing PMF formation could affect persister formation. The respiratory electron-transport chain plays an important role in generating a transmembrane proton gradient. Two enzymes, namely NADH dehydrogenase I (*nuoI*)and NADH dehydrogenase II (*ndh*), are key players in this pathway [247]. Upon starvation for 24hrs and then six days of ampicillin treatment, the population size of Δ*nuoI* was found to drop to ~3.5×10^5 cell/mL, which represented ~72 fold reduction when compared to the wildtype *E. coli* strain **(Fig 2.4D)**. But the persister fraction in Δ*ndh* kept similar to wildtype. Importantly, the population size of persisters of Δ*nuoI*Δ*ndh* strain, in which both genes were simultaneously deleted, dropped sharply to ~200 cell/ml, an 1.4×10^5 fold reduction, upon treatment with ampicillin for six days, suggesting that inhibition of activities of electron transport chain components severely affects production and maintenance of bacterial PMF, and hence the long term survival of persisters that are exposed to ampicillin **(Fig 2.4E)**. To our knowledge, this is the strongest persistence suppression effect

recorded upon deletion of putative persistence gene(s). Nevertheless, our data showed that inhibition of electron transport chain activities alone was not as effective as simultaneous deletion of the *pspA* gene and sodium azide treatment in eradicating ampicillin persisters, suggesting that collapse of PMF could be slowed down significantly by the PspA protein during starvation even if the ability to generate a proton gradient via aerobic respiration is diminished in bacteria.

2.3.5 Active Efflux Driven by Proton Gradient Contributes Partially to maintenance of Starvation-Induced Persisters in *Escherichia coli*

PMF is involved in numerous cellular functions; in particular, it plays an essential role in maintaining efflux activities. Bacterial efflux could lead to decrease in antibiotic accumulation, thereby facilitating the cells to form persisters and survive antibiotic treatment [4, 207]. We therefore tested if the role of PMF in maintaining the antibiotic tolerance phenotype was due to its effect on promoting efflux activities. We used a fluorescent β-lactam antibiotic known as BOCILLIN^{TM} FL Penicillin (BOCILLIN) to depict the degree of accumulation ofβ-lactam antibiotic in the presence and absence of CCCP. We first confirmed that CCCP had little effect on the overall fluorescence signal as the fluorescence level exhibited by CCCP alone was ~250 RFU, or ~180 times less than that of BOCILLIN (~45000 RFU) **(Fig 2.5A)**. In this experiment, we used fluorescence spectrophotometry to test the intensity of the accumulated BOCILLIN and performed confocal microscopy to assess the degree of accumulation of BOCILLIN with or without CCCP treatment. Wild type bacterial cells which have been subjected to starvation for 24hrs, followed by CCCP treatment, were generally well-stained by BOCILLIN (~40000 RFU), whereas the fluorescence level in the absence of CCCP was significantly lower (~20000 RFU), indicating that the amount of β-lactam antibiotic accumulated intracellularly increased upon PMF dissipation **(Fig 2.5B,C)**. We then determined whether

accumulation of BOCILLIN associated with artificial dissipation of PMF during starvation was due to failure to undergo efflux. The dye Nile red, a common substrate of efflux pumps, was used in investigation of bacterial efflux activities. In this experiment, Nile red was incubated with the 24hrs-starvation population for 30mins, followed by addition of CCCP and fluorescence measurement. The results showed that collapse of proton gradient upon addition of CCCP correlated well with an increase in fluorescence signal. Specifically, the fluorescence intensity increased from ~6500RFU to ~10000 RFU within 30mins, indicating a diminished efflux efficiency in the absence of proton gradient **(Fig 2.5D)**.

Persister formation was previously shown to negatively correlate with intracellular β-lactam accumulation [207]. To further determine if efflux activities were indeed involved in starvation-induced antibiotic tolerance, we tested whether deleting the *tolC* gene, the product of which constitutes a key component of several major efflux systems such as AcrAB-TolC and EmrAB-TolC, resulted in reduction in the size of antibiotic-tolerant population during starvation. Under our assay condition, the size of the tolerant population in *E. coli* Δ*tolC* mutants (~5×10^4 cells/mL) was much smaller than that of wild type (~2.5×10^7 cells/mL) upon treatment with ampicillin for six days, suggesting that efflux pumps played a role in expression of the antibiotic tolerance phenotype **(Fig 2.4F)**. This idea was further confirmed by testing the effect of PAβN, an efflux pump inhibitor, which was also found to cause a significant drop in the size of tolerant population (~1.5×10^5 cells/mL) **(Fig 2.4F)**. We showed that PAβN did not exert any negative effect on bacterial growth **(Fig 2.5E)**, indicating that the tolerance suppression effects conferred by this compound was not due to its bactericidal effect. These data suggested that efflux pump activity, which might be maintained through the PMF, contributed to expression of phenotypic antibiotic persistence during nutrient starvation.

Through comparison between the effect of PMF dissipation and efflux suppression on the survival of starvation-induced persisters, we found that the disruption of PMF exhibited a much stronger effect on tolerance suppression than inhibiting efflux activity. The entire persister population in the wild type strain was eradicated within 48hrs upon CCCP treatment; In the case of *pspA* knockout, treatment with sodium azide and ampicillin could cause complete eradication by 96hrs. On the other hand, the size of the persister population in the wild type strain remained at ~5×10^4 cells/ml upon deletion of *tolC* or treatment with efflux pump inhibitor PAβN. These data strongly suggest that maintenance of PMF by active generation of an electrochemical gradient and preventing proton leakage are key mechanisms underlying maintenance of starvation-induced bacterial antibiotic persistence, and that the functional role of PMF probably lies in regulation of efflux and other important membrane transportation activities, which warrant further investigation.

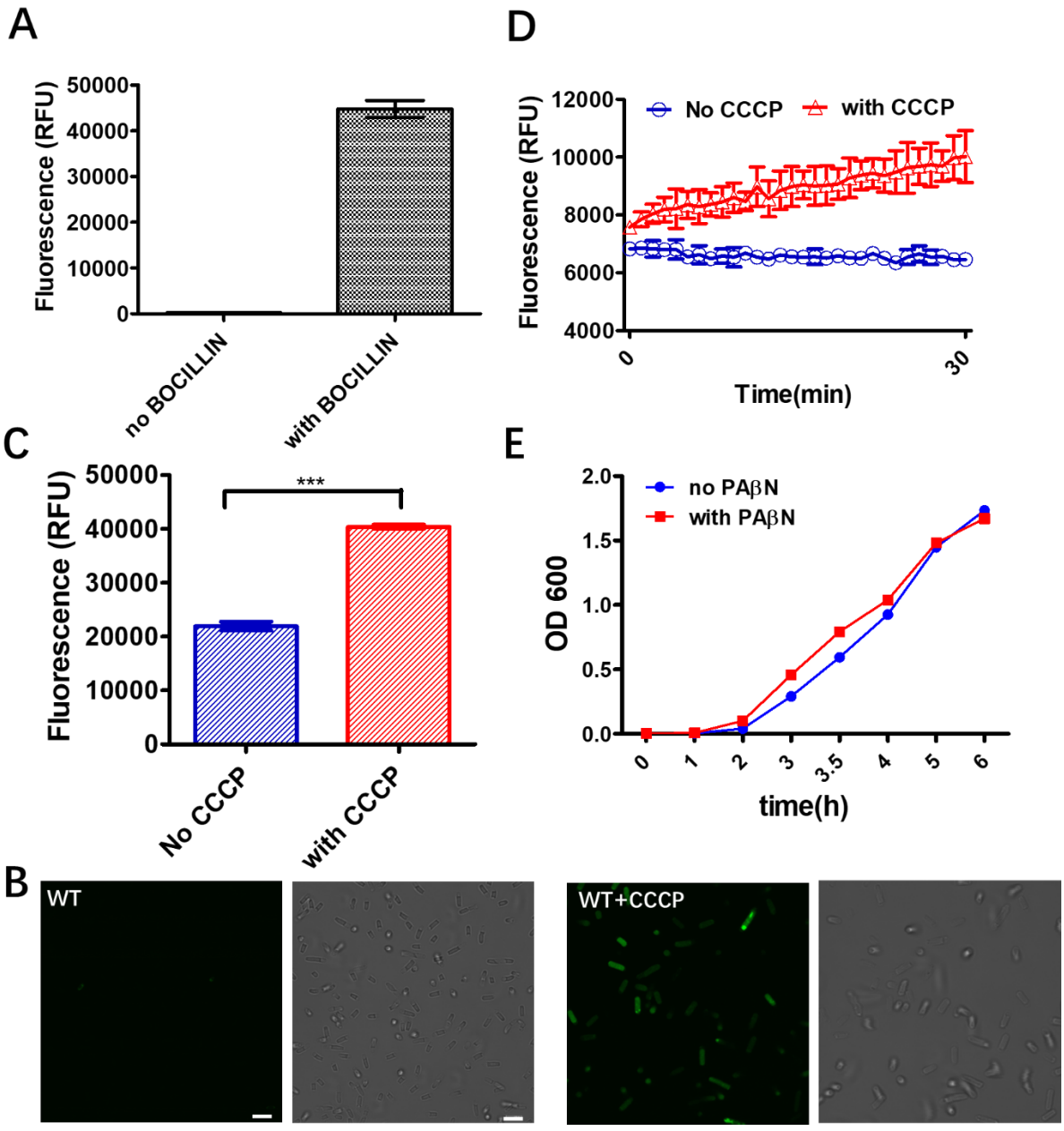

Fig 2.5 Active efflux driven by proton gradient contributes partially to starvation-induced persister formation.

(A) The fluorescence signal of bacterial population treated with CCCP only (no BOCILLIN) was measured and compared with those treated with both CCCP and BOCILLI. CCCP was not found to exhibit a detectable level of fluorescence under the excitation/emission wavelength used to detect BOCILLIN. (B) Confocal microscopy images of fluorescent antibiotic accumulation (BOCILLIN, 10 μg/mL)

in bacteria subjected to 24hrs starvation in the presence and absence of CCCP. The left and right panels are the bright field and fluorescence images respectively (scale bar: 4μm). (C) Accumulation of fluorescent antibiotic (BOCILLIN) inside wild type cells subjected to 24hrs starvation in the presence and absence of CCCP. (D) The fluorescent efflux substrate Nile Red was used to stain wild type bacterial population which had been subjected to 24hrs starvation in the presence and absence of CCCP. (E) Growth rate of wild type strain in the presence and absence of PAβN (100 μM).

2.3.6 Maintenance of PMF is key persistence mechanism in both Gram-negative and Gram-positive bacteria

To determine if maintenance of PMF is an active cellular mechanism universally employed by various bacterial species to promote persistence maintenance, we tested whether CCCP could eradicate starvation-induced persisters of major bacterial pathogens. Our data confirmed that CCCP could eradicate persisters of *K. pneumoniae*, *A. baumannii*, *S. aureus* and *S.typhimurium* in 24hrs, and persisters of *P. aeruginosa* in 96hrs even in the absence of ampicillin, confirming that PMF maintenance is universal mechanism underlying prolonged expression of the bacterial antibiotic persistence phenotype **(Fig 2.6)**.

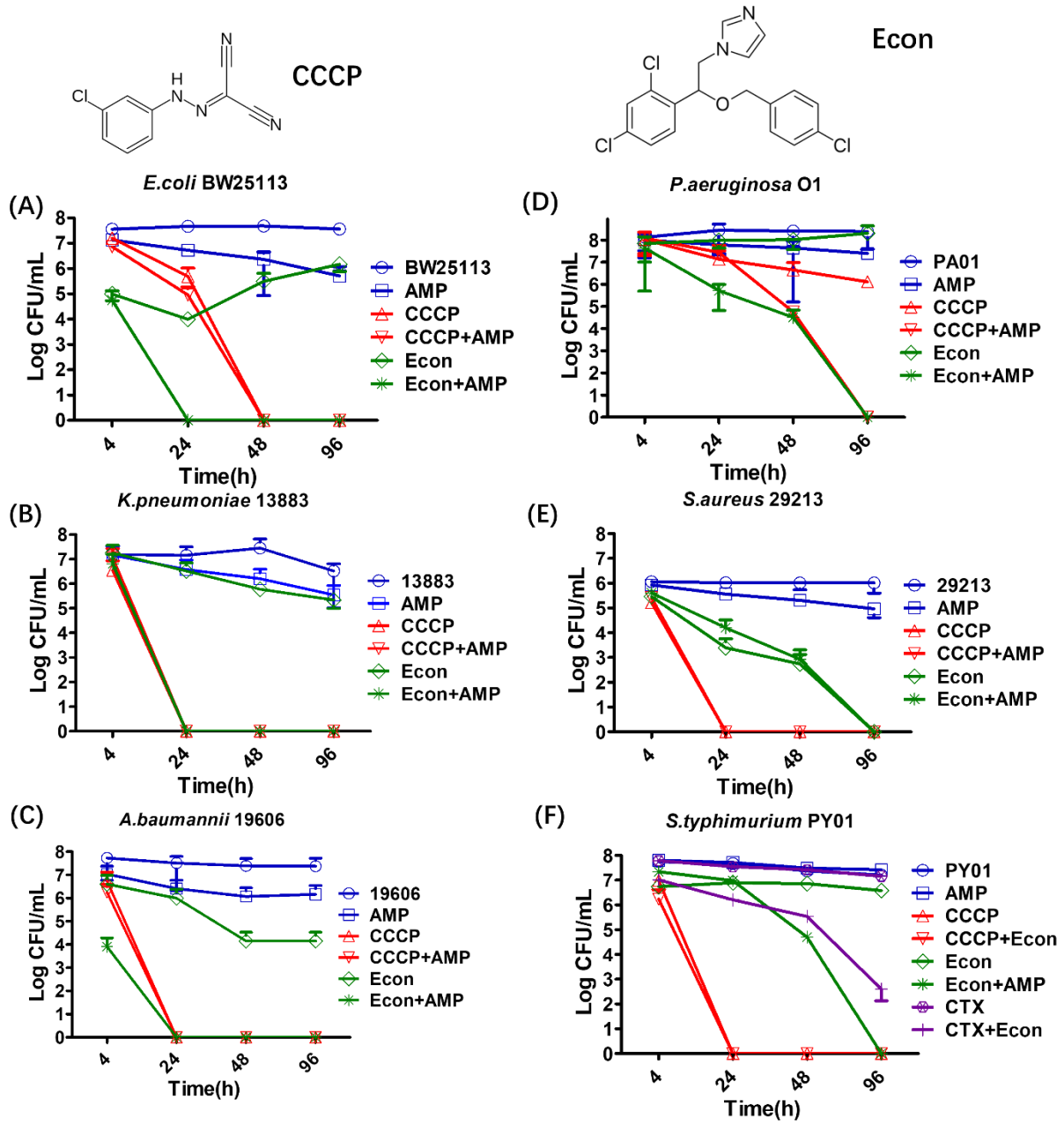

Fig 2. 6 PMF maintenance is essential for starvation-induced tolerance formation in major Gram-negative and Gram-positive bacterial pathogens.

Changes in the size of antibiotic-tolerant sub-population in *E. coli* (A), *K. pneumoniae* (B), *A. baumannii* (C), P. aregenosa (D), *S. aureus* (E) and *S.typhimurium* (F) which had been starved for 24hrs; variation in CFU/ml recorded at different time points upon treatment with ampicillin alone, CCCP alone, Econ alone, CCCP in the presence of ampicillin, as well as Econ in the presence of

ampicillin is shown. Data are the average of at least two independent experiments performed with three biological replicates ($n \geq 6$)

2.3.7 An anti-persister therapy that involves targeting bacterial PMF

In view of the human toxicity of CCCP and other PMF dissipators such as valinomycin, nigericin, gramicidin and dicyclohexylcarbodiimide (DCCD), which prevents these compounds from being used as PMF disrupting agent to eradicate bacterial antibiotic persisters, we searched for non-toxic substitutes which could be utilized clinically to treat or minimize the chance of occurrence of chronic and recurrent infections, especially among immunocompromised patients. In a separate work carried out in our laboratory, we identified an FDA-approved antifungal drug, econazole, which was able to effectively disrupt bacterial cell membrane PMF at a concentration of 40μM **(Fig 2.7)**. Like CCCP, econazole did not exhibit antibacterial activity on all major Gram-negative bacterial species tested, with MIC being > 160μM in all test strains except *S. aureus*, for which a MIC of 40 μM was recorded **(Table 2.3)**. Contrary to CCCP, however, this imidazole type of antifungal drug is FDA approved and has been proven safe for use as a therapeutic agent, including systematic administration in human. We then tested if econazole could also eradicate bacterial antibiotic persisters in a manner similar to CCCP. Our data showed that 40μM econazole alone only exhibited slight killing effect on persisters of Gram-negative bacteria such as *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*, but eradicated persisters of Gram-positive bacteria (*S. aureus*) within 48hrs. When used in combination with ampicillin, however, econazole exhibited significant killing effect on persisters of *E. coli*, *K. pneumoniae* and *A. baumannii*, eradicating the entire persister population within 24hrs. For *P. aeruginosa*, combined usage of econazole and ampicillin could eradicate persisters by 96hrs **(Fig 2.6)**.

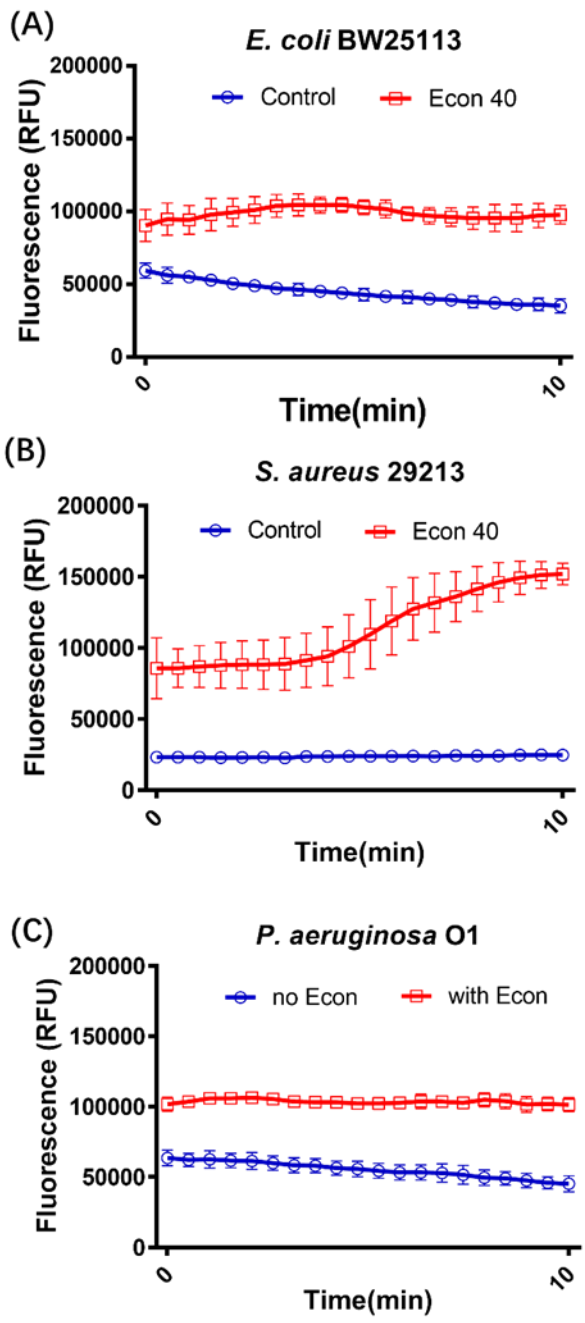

Fig 2.7 Econazole (Econ) causes dissipation of bacterial PMF.

Fluorescence intensity of $DiSC_3(5)$, which measures membrane potential of bacteria, were shown to increase significantly over a period of 10 min time after treatment with Econ in *E. coli* (A), *S. aureus* (B) and *P. aeruginosa* (C) when compared to non-treatment control, suggesting that Econ could dissipate membrane PMF of these organisms.

The effects of combined treatment of econazole and ampicillin on *E. coli* persisters were further investigated by SEM. Upon treatment with a high dose of ampicillin (100μg/ml and 1000μg/ml), persister cells exhibited slight shrinkage in the pole areas, but the microscopy image of the membrane remained as sharp and smooth as cells treated with saline **(Fig 2.8A,B,C)**. However, persister cells treated with 20μM econazole exhibited a rough cell surface, as well as leakage of intracellular material characterized by an increasingly transparent cytosol **(Fig 2.8D)**. When treated with a combination of econazole and ampicillin, the cell membrane structure was severely damaged, leading to swelling and death of the cells under SEM **(Fig 2.8E)**. It should

also be noted that econazole was not found to cause any detectable membrane damage or morphological changes in exponentially growing cells which actively undergo aerobic respiration to generate a strong PMF (data not shown), suggesting that its deleterious effects on starvation-induced antibiotic persisters were due to PMF dissipation. These findings are consistent with the prolonged killing data in that bacteria under starvation were initially tolerant to ampicillin, but could be completely eradicated by the econazole and ampicillin combination.

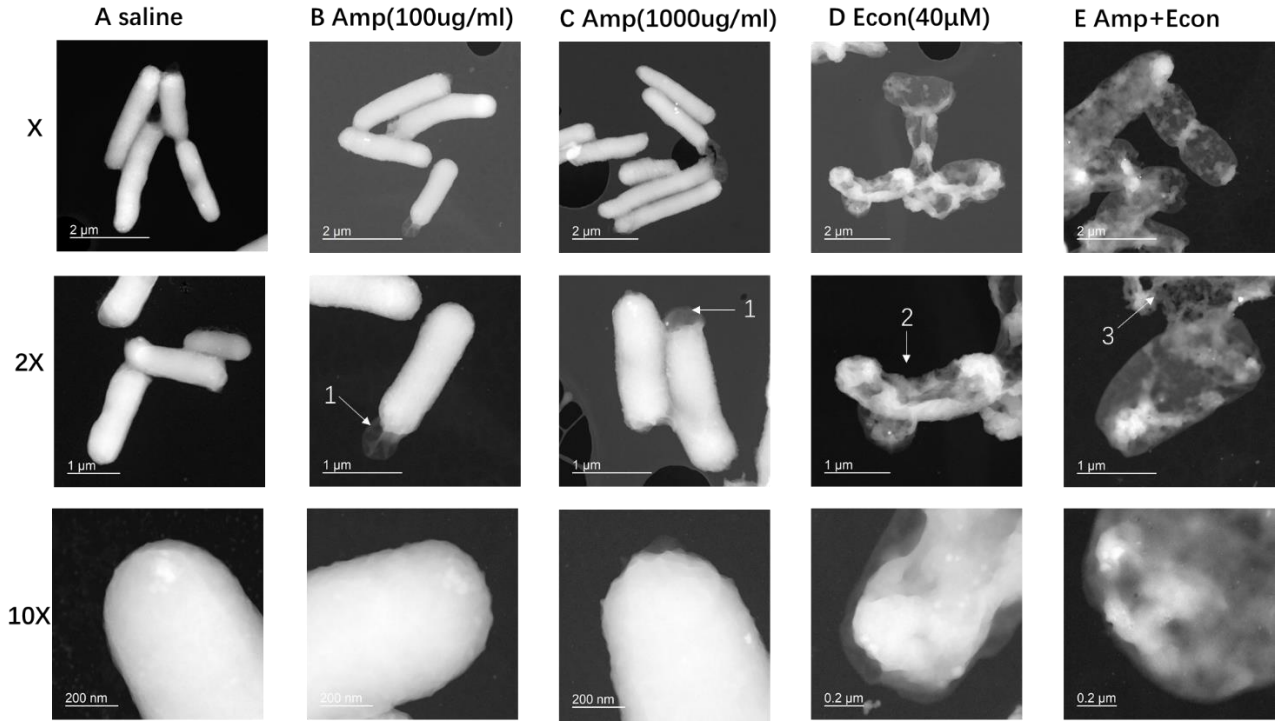

Fig 2.8 SEM images of *E. coli* cells.

(A)Intact membrane were observed in cells without any treatment; (B, C) upon exposure to ampicillin (100μg/ml and 1000μg/ml), smooth surface could still be seen, but there was slight shrinkage at the poles of the cell (arrow 1). (D) Treatment with 40μM econazole (Econ) alone resulted in severe structural damages in cellular membrane (arrow 2). (E) Treatment with econazole (40 μM) and ampicillin (100

μg/ml) caused further severe membrane damage with swelling and death of cells (arrow 3). Arrows depict areas where cell membrane was damaged.

The efficacy of the β-lactam and econazole combination in eradicating bacterial persisters was further tested in mouse infection models, with ceftazidime, an antibiotic commonly used in clinical treatment of bacterial infection, being the test agent. First, a deep-seated thigh persister model using *E. coli* BW25113 as the test organism was established; our data showed that treatment with econazole alone (20μmol/Kg), or a combination of econazole (20μmol/Kg) and ceftazidime (20mg/Kg), resulted in significantly more (P=0.026 and P=0.031 respectively) efficient eradication of *E. coli* persisters than treatment with ceftazidime (20mg/Kg) alone **(Fig 2.9A)**. Second, we tested a peritoneal infection model involving *S.* Typhimurium PY1 and found that both ceftazidime and a combination of ceftazidime and econazole could protect the test animals from being killed by persistent infection elicited by inoculation of 7.6×10^5 CFU of *S.* Typhimurium PY1, with the combination therapy being slightly more effective **(Fig 2.9B)**. After treatment for 72hrs, CFU of *S.* Typhimurium PY1 in the surviving animals were determined, with results showing that the combination therapy caused significantly higher (P<0.0001) rate of eradication of PY1 persisters in mice **(Fig 2.9C)**. Similar effects were seen in mice model infected with a lower dose (2.8×10^5 CFU) of *S.* Typhimurium PY1 **(Fig 2.10)**. When infected with a relatively high dose of *S.* Typhimurium PY1 (1.5×10^6 CFU), however, an 80% survival rate was recorded among mice treated with a combination of econazole (20μmol/Kg) and ceftazidime (20mg/Kg), whereas treatment with ceftazidime alone (20mg/kg) could only rescue 10% of the infected mice **(Fig 2.9D)**. These findings confirm that econazole could significantly enhance the efficacy of ceftazidime in eradicating *Salmonella* persisters *in vivo*.

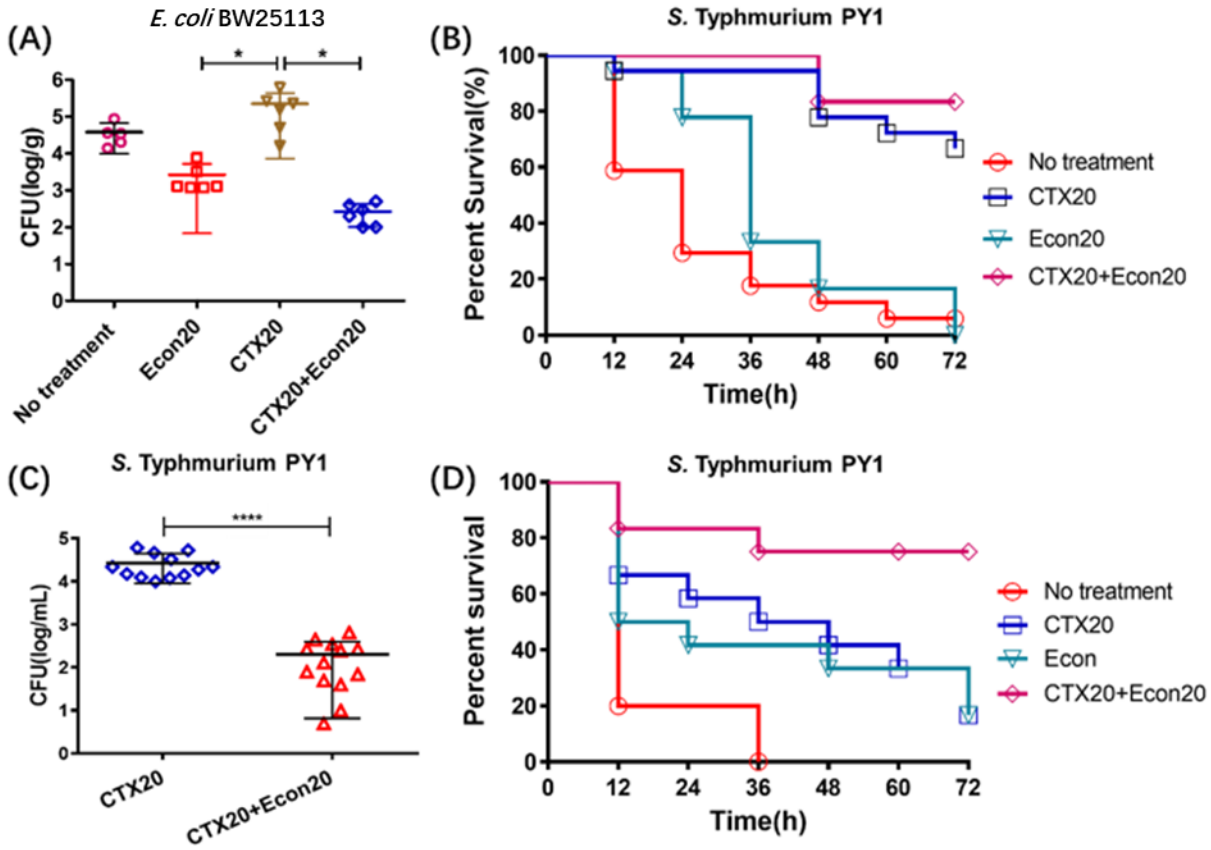

Fig 2.9 Ceftazidime and econazole combination therapy could effectively eradicate bacterial persisters in *in vivo* mice models.

(A) *E. coli* BW25113 mice deep-seated thigh infection model. 1×10^6 CFU *E. coli* BW25113 were injected to the right thigh of the test animal. (B) *S.* Typhimurium PY1 persister peritonitis model. Mice were intraperitoneally injected with 7.6×10^5 CFU *S.* Typhimurium PY01. (C) *S.* Typhimurium survival fraction. Mice that survived in (B) were euthanized, peritoneal washes were performed by injection 2 mL of saline into the intraperitoneal space. The abdomen was then opened and 100 μL of peritoneal fluid were collected for determination of bacterial cell count. (D) *S.* Typhimurium PY1 persister peritonitis model; same as (B) with the only difference being inoculation with a higher amount of *S.* Typhimurium, 1.5×10^6 CFU. Since most of the test animals were dead, bacterial survival assay was not performed.

Econ20, econazole (20 μmol/Kg); CTX20, ceftazidime (20 mg/Kg). * P<0.05; **** P<0.0001.

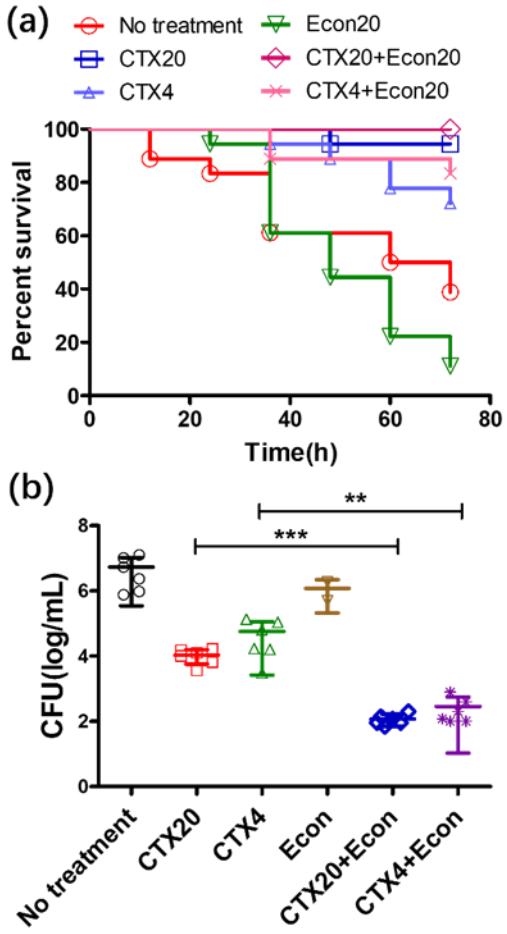

Fig 2.10 Ceftazidime and econazole combinational therapy could effectively eradicate bacterial persisters in *in vivo* mice models.

(a) *S.* Typhimurium PY1 persister peritonitis model. Mice were intraperitoneally injected with 2.8×10^5 CFU *S. Typhimurium* PY01. (b) S. Typhimurium survival fraction. Mice that survived in (a) were euthanized, peritoneal washes were performed by injection 2 mL of saline in the intraperitoneal followed by a massage of the abdomen. The abdomen was then cut open and 100 μL of peritoneal fluid were collected to determine CFU. Mice treated with ceftazidime (4mg/kg or 20mg/kg) exhibited significantly slower (P=0.028 and P=0.004 respectively) eradication of *S. Typhimurium* PY1 persister than treatment with the combination of econazole and ceftazidime (4mg/kg or 20mg/kg).

Based on these findings, we propose a model of PMF-mediated development of starvation-induced persistence **(Fig 2.11)**. First, maintaining PMF is essential for prolonged survival of starvation-induced persisters. Efflux activities driven by PMF extrude β-lactam and facilitate persister formation; other unknown responses which

presumably involve import / export of nutrients / specific metabolites during starvation are mediated by PMF and are also important for maintaining a persistence phenotype. Compounds such as CCCP and econazole cause dissipation of bacterial membrane PMF and inhibition of ATP production, which in turn affect the aforementioned energy-dependent cellular functions that are involved in maintenance of the persistence phenotype, suppressing the survival fitness of persister cells. Persisters are eradicated more effectively in the presence of β-lactam upon dissipation of PMF since a lack of PMF results in inactivation of antibiotic efflux activities and accumulation of antibiotic in the periplasm of persisters. Accumulation of β-lactam antibiotics inhibits some hitherto poorly characterized cell wall activities in persisters, leading to cell death.

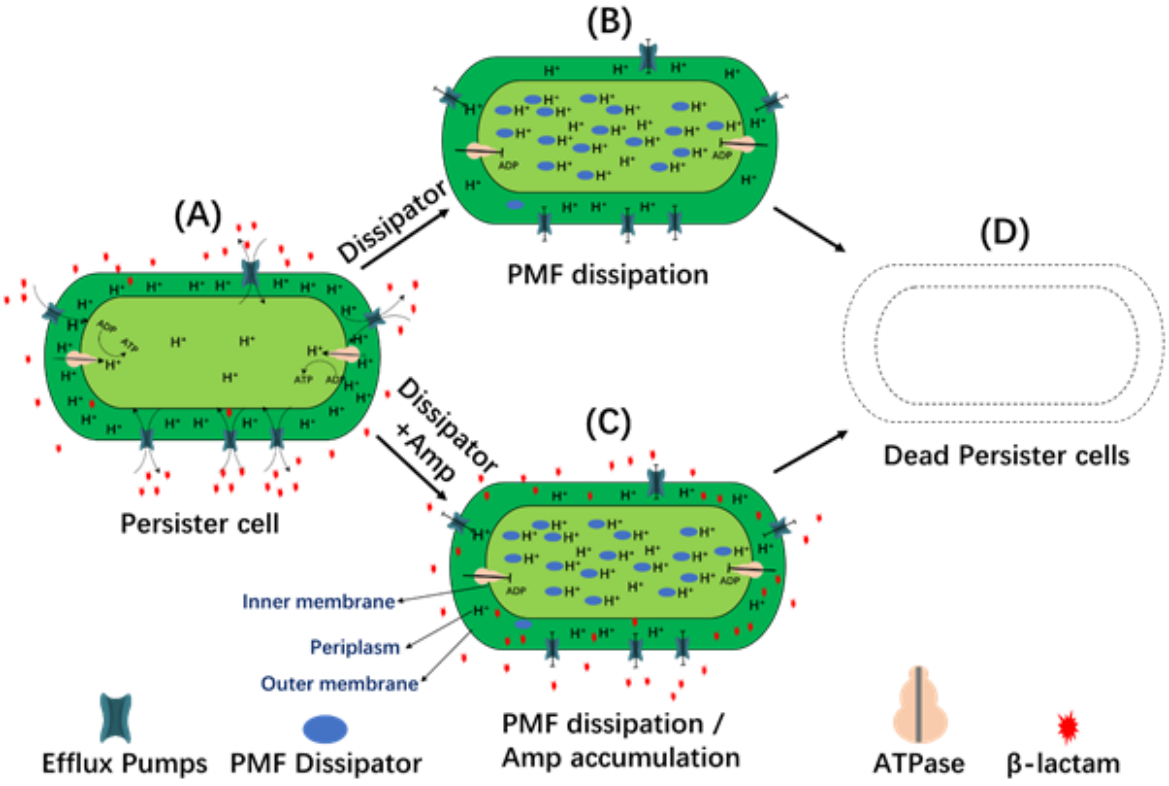

Fig 2.11 Proposed model of PMF-mediated development of starvation-induced tolerance.

(A) Maintaining PMF is essential for prolonged survival of starvation-induced persisters. Efflux activities driven by PMF extrude β-lactam to facilitate persister formation; other unknown responses which presumably involve import / export of specific metabolites / nutrients are mediated by PMF and are also important for maintaining persistence phenotype. (B) Effect of PMF dissipator such as CCCP and econazole on persister killing. PMF dissipator causes dissipation of bacterial membrane PMF, leading to disruption of ATP production, which in turn affects a series of cellular functions that are involved in maintaining the persistence phenotype, leading to killing of persisters. (C) Effect of PMF dissipator and ampicillin on persister killing. Persisters are eradicated more effectively in the presence of β-lactam if PMF cannot be maintained under starvation stress. Dissipation of bacterial membrane PMF inactivates antibiotic efflux activities, leading to accumulation of antibiotic. The accumulation of antibiotics and arrest of other cellular functions lead to more effective killing of persisters. (D) Dissipation of PMF with or without the presence of antibiotic could both lead to persister killing. The cellular basis of re-sensitization of persisters to β-lactams upon PMF dissipation remains to be elucidated.

2.4 Discussion

Antibiotic persisters are often regarded as bacterial sub-population that exhibits a dormant physiological state, which is presumably conferred by a network of redundant yet poorly defined mechanisms [17, 248-250]. In this work, we aimed to delineate the active persistence mechanisms in bacteria. Through systematic analysis of the gene expression profile of bacteria subjected to prolonged starvation, we showed that products of the *psp* gene family act by preventing dissipation of PMF, thereby facilitating proper functioning of specific efflux and transportation systems

which are in turn essential for maintenance of survival fitness of the antibiotic tolerant sub-population. Discovered by Peter Model in 1990 [251], the PspA protein was first shown to be induced in *Escherichia coli* upon infection by the filamentous phage f1. Psp proteins have since been postulated to play a role in regulating bacterial virulence, maintenance of PMF and mediation of envelope stress response [242]. The Psp response was also found to be involved in regulation of indole-induced tolerance, with the indole-induced persister sub-population size being reduced dramatically in the *pspBC* mutant [40]. It has also been shown that PspA was over-expressed in stationary phase bacterial population, and under alkaline conditions (pH 9), organisms lacking the *pspABC* genes exhibited significantly lower survival rate than wild type, suggesting that the Psp response can enhance bacterial survival under hostile conditions [252]. However, the functional importance of the Psp response in mediating persistence formation appears to be overlooked. This work is the first which describes the essential role of PspA in modulating starvation-induced persistence response through PMF maintenance.

During the exponential phase, generation of the proton gradient is required for energy production. However, when substrates for oxidative phosphorylation are exhausted, PMF must be maintained by preventing dissipation of the existing proton gradient. In this work, the reason why we monitored changes in persistence level over a six-days period is that we believe the effect of lack of PMF maintenance function cannot be seen immediately. In fact, various previous studies showed that disrupting PMF and diminishing ATP level could actually lead to formation of persisters in exponentially growing population, presumably by triggering dormancy [253]. Our findings show that PMF is required for prolonged survival of bacterial persisters. A lack of ability to maintain PMF, as in the case of *pspA* knockout, results in gradual reduction in the size of antibiotic tolerant sub-population when compared

to the wild type strain. Inhibition of the ability to generate PMF by treatment with sodium azide also mildly affected persistence. Importantly, when the ability to generate and maintain PMF was simultaneously inhibited, by treating the *pspA* knockout mutant with sodium azide, persistence level was found to drop drastically, but the rate of killing was still slower than that recorded for starvation population treated with CCCP, which could eradicate persisters within 48 hours by rapidly disrupting PMF. These observations therefore suggest that active generation of a basal level of PMF is required for expression of phenotypic persistence during nutrient starvation. Consistently, we confirmed that knocking out key components of the respiratory electron transport chain (Δ*nuoI* / Δ*ndh*), which plays a role in generating PMF, resulted in a dramatic drop in persistence population. Our findings regarding the functional importance of PMF in maintenance of persistence are consistent with that of Ma *et al* (2010), who showed that inhibition of energy production by introducing mutations in the *sucB* and *ubiF* genes would affect persister survival[254]. Taken together, it is highly likely that a basal level of metabolism is maintained in persisters for ATP production and preservation of the transmembrane proton gradient, possibly through actively scavenging cellular materials released from dead cells as carbon sources. Consistently, our gene expression data showed that expression of various membrane-bound transporters was up-regulated upon prolonged starvation **(Table 2.2)**. It is likely that such scavenging processes also involve PMF-dependent transportation activities.

Our work further showed that PMF maintenance was coupled to efflux activities which were also inducible to enhance bacterial survival fitness during starvation **(Table 2.2)**. These efflux activities are presumably involved in export of intracellular antibiotics or toxic metabolites during starvation or other stresses [255], reducing the amount of antibiotic accumulated intracellularly and enabling

organisms under starvation to become antibiotic tolerant. We showed that efflux systems would lose the driven energy and exhibit decreased efflux efficiency if PMF collapsed. Consistently, Wu *et al.* showed that structural defect of the AcrAB-TolC pump was associated with reduced antibiotic persistence [132]. Recently, Pu *et al.* reported that efflux activities were involved in stationary phase-induced persistence by pumping the intracellular antibiotic out [207]. We confirmed that the efflux system played a role in maintaining phenotypic drug persistence under prolonged starvation conditions. Our findings therefore help bridge this knowledge gap and have important implications in future exploration of starvation-induced persistence mechanisms and development of anti-persistence strategies.

Our data confirm that the role of PMF is not limited to supporting efflux activity, as PMF dissipation as a result of treatment with CCCP alone or sodium azide plus ampicillin leads to rapid eradication of persisters, whereas deletion of efflux genes or treatment with efflux pump inhibitor only resulted in moderate reduction in the size of persister population. PMF is essential for proper functioning of a wide range of membrane proteins, including the aforementioned nutrient scavenging transportation proteins; PMF-dependent mechanisms underlying maintenance of persister phenotype, such as the type of efflux activities involved, remain to be identified. Nevertheless, due to its functional importance in maintaining viability of persisters, we consider PMF as an excellent target for eradication of persisters. Complete eradication of persisters can rarely be achieved by inhibiting one specific cellular function. There were two previous reports of complete eradication of antibiotic persisters in Gram-positive bacteria, which involved the use of the retinoid and acyldepsipeptide antibiotic to inflict membrane damage and activate casein lytic proteases respectively [150, 171]. However, these antibiotics are not effective on Gram-negative organisms. Disrupting bacterial PMF can completely eradicate

persisters of both Gram-positive and Gram-negative bacteria. Nevertheless, targeting PMF is increasingly being regarded as a feasible antimicrobial strategy. Attempts that were made to screen for FDA-approved drugs or novel drug that can cause dissipation of bacterial PMF without exhibiting toxicity in human have identified several anti-mycobacteria drugs which exhibit a PMF disrupting effect as strong as CCCP. In this work, we successfully identified an FDA-approved antifungal drug, econazole, that can cause dissipation of bacterial PMF and effectively eradicate persisters of *S. aureus* when used alone, and persisters of Gram-negative bacterial pathogens when used in combination with ampicillin. The combined usage of econazole and ceftazidime was further shown to effectively eradicate bacterial persisters in animal infection models. Our findings are highly clinically relevant as they imply that most bacterial species in the persistence status can be eradicated by PMF-suppressing agents alone or by combined usage of such agents and β-lactam within a 5-days treatment course.

To summarize, our study shows that PMF is essential for prolonged expression of starvation induced antibiotic persistence phenotype in both Gram-positive and Gram-negative bacteria. Findings in this work represent a significant advance in understanding the cellular basis of the phenomenon of bacterial antibiotic persistence: emergence of sub-population antibiotic persisters is due to the combined effects of metabolic shutdown and activation of a range of PMF-dependent defense mechanisms in response to variation in environmental conditions, with the latter being particularly important for long-term maintenance of the persistence phenotype. Identification of an FDA approved antifungal drug, econazole, which could cause dissipation of bacterial PMF, shows that eradication of bacterial persisters of both Gram-positive and Gram-negative bacteria by a non-toxic PMF-disrupting agent is highly feasible.

Chapter Three: Exogenous *N*-acetyl-D-glucosamine plus β-lactam Eliminates Tolerant Bacteria

3.1 Abstract

Bacterial persistence to antibiotics is known to result in reduced efficacy in antimicrobial treatment and recurrent infections. In an attempt to test if specific nutrient can abolish the persistence phenotype, we identified *N*-acetyl-D-glucosamine (GlcNAc) as a potent persistence-suppressing agent. We showed that GlcNAc could strongly re-sensitize a tolerant population to ampicillin without supporting bacterial growth and caused total collapse of the cellular structure if β-lactam is present. Such re-sensitization effect was attributable to two physiology-modulating effects of GlcNAc. First, activation of glycolysis by products of GlcNAc catabolism was found to play an indispensable role in mediating the re-sensitization effect of GlcNAc, as the amount of fructose-6-phosphate (Fru-6-P) was increased and re-sensitization effect was diminished after inhibition of NADH dehydrogenation. Second, uptake of GlcNAc by the tolerant population triggered formation of the peptidoglycan precursor UDP- *N*-acetyl-D-glucosamine (UDP-GlcNAc) and subsequently re-activated the peptidoglycan biosynthesis process, rendering the organism susceptible to β-lactam antibiotics. Our findings imply that GlcNAc may serve as an adjuvant of β-lactam in enhancing the efficacy of treatment of otherwise hard-to-treat chronic and recurrent bacterial infections caused by phenotypically antibiotic tolerant bacterial persisters.

3.2 Introduction

Current antimicrobial regimens are often ineffective in completely eradicating the infecting agents in treatment of bacterial infections, predisposing occurrence of relapsed and chronic infections. Antibiotic-tolerant sub-populations of strains, or commonly known as persisters, are recalcitrant to the bactericidal effects of even a high dose of antimicrobials, and are therefore responsible for failure of antimicrobial therapy[2]. Antibiotic persistence is a reversible physiological phenotype initiated by multiples stresses and characterized by a slow or non-growing state which renders antimicrobial action infective; yet such tolerant organisms can rapidly resume active growth when environmental conditions become favorable [10]. Multiple cellular mechanisms underlying persistence formation have been proposed since the first description of this phenomenon in 1944[231]. One common theory is that bacterial toxin-antitoxin (TA) systems, which are inducible by the stringent response alarmone (p)ppGpp, are involved in formation of antibiotic tolerant persisters by inhibiting DNA replication, transcription and protein synthesis, which are common cellular processes targeted by conventional antibiotic[2, 39].

Based on the current understanding of persistence formation mechanisms, several strategies have been proposed to eradicate antibiotic persisters. The first approach involves direct killing of metabolically inactive persisters. Two synthetic retinoid compounds, CD437 and CD1530, were designed and found to exhibit killing effects on methicillin-resistant *S.aureus* (MRSA) persister by disrupting membrane bilayers[150]. Yet these compounds, which were previously used as anti-tumor agents, are highly toxic and hence unsuitable for treatment of immunocompromised patients who suffer from serious bacterial infections [256, 257]. Another compound, ADEP4, which actives Clp proteases, was found to activate non-specific protease

and subsequent protein digestion, killing persistent *M. tuberculosis* and *S. aureus* organisms[171]. The second approach aims to resuscitate persisters or re-sensitize them to antibiotics. *Cis-2*-decenoic acid, a fatty acid signaling molecule, was found to revert the dormant persister cells to a metabolically active state which is susceptible to antimicrobial agents[194]. Metabolites such as glucose, fructose, pyruvate and mannitol were also reported to re-sensitize persisters to gentamicin by promoting generation of PMF, which is required by aminoglycoside uptake[200]. The third approach is combination usage of several conventional antibiotics to kill persisters as they are heterogeneous. Macrolide erythromycin boosts biofilm-associated *P. aeruginosa* persister eradication by colistin [211]. The fourth approach is to interfere with the persister formation process. Relacin, a novel (p)ppGpp analogue, prevents (p)ppGpp accumulation and therefore inhibits the stringent response, reducing the survival fitness of persisters[221]. Likewise, phage-encoded expression of LexA3 inhibits SOS response-mediated DNA repair, rendering both planktonic and biofilm-associated persister cells susceptible to quinolones[218].

In this work, we screened for nutrients that can suppress starvation-induced persistence response so as to develop a non-toxic and clinically feasible anti-persister therapeutic approach. Currently, compounds that directly kill antibiotic persisters are often toxic to mammalian cell. We found that GlcNAc and D-glucosamine (GlcN) could strongly re-sensitize persisters to β-lactams. GlcN·HCl was previously reported to exhibit a strong antimicrobial effect on *S.saparophytics* and *Micrococcus luteus* [258]. GlcNAc is also known to induce persister death in *C.albicans* by triggering ROS[259]. GlcNAc is a major component of the bacterial cell wall or peptidoglycan, which is a matrix composed of alternating GlcNAc and *N*-acetylmuramic acid (MurNAc) molecules cross-linked by peptide bridges, and the

target of β-lactams. GlcNAc was shown to induce overexpression of *mdtEF*, the multidrug exporter genes which are involved in persister formation[260]. Both GlcNAc and GlcN are converted to glucosamine- 6-phosphate (GlcN-6-P) upon transportation into the cytosol [261, 262]. GlcN-6-P could enter the hexosamine pathway to generate UDP-GlcNAc, the precursor for synthesis of peptidoglycan, and could also be catabolized to Fru-6-P, a substrate of glycolysis[263, 264].

We hypothesize that the mechanism of GlcNAc-induced persister re-sensitization to β-lactams is as follows: (i) GlcNAc triggers activation of peptidoglycan synthesis by generation of UDP-GlcNAc, so that the target of β-lactams becomes functionally active. Hurdle *et al.* proposed that the bacterial membrane bilayer or proteins which are integral to membrane function was an ideal target for treating persistent infections as even persisters need to maintain an integrated membrane and cell wall in order to remain viable [225]. Bacteriophage-derived lysin CF-301 exhibits pronounced MRSA killing effect as it causes cell lysis through eliciting peptidoglycan degradation[163]. GlcNAc does not cause peptidoglycan degradation, instead it activates the peptidoglycan synthesis processes in persisters, thereby re-sensitizing them to β-lactams. (ii) GlcNAc catabolism produces Fru-6-P, which in turn promotes onset of glycolysis, enhances PMF, revive oxidative phosphorylation and subsequently reverts the dormant cells to a metabolic active and β-lactam-sensitive state.

3.3 **Experimental Procedures**

3.3.1 Strains, culture conditions and chemicals

Escherichia coli:BW25113, BW3110 and clinically isolated *E. coli* 2 strains were used in this work. Luria-Bertani (LB) broth was used for all cultures. Standard LB agar (Difco, Leeuwarden, The Netherlands) was used in antibiotic assays. All the antibiotics were purchased from Sigma. GlcNAc and GlcN were purchased from Sigma-Aldrich. BOCILLIN^{TM} FL Penicillin was purchased from ThermoFisher. Fru-6-P and UDP- GlcNAc were purchased from Sigma-Aldrich. HADA was synthesized following the procedure described by Kuru et al[265]. PM plate was purchased from Biolog.

3.3.2 Antibiotic Bactericidal assays

Upon reaching the exponential phase (OD_{600}=0.2), bacteria were washed and deprived of all essential nutrients by re-suspending in saline (0.85% NaCl), followed by treatment with GlcNAc (1mM), GlcN (1mM) with or without ampicillin (100μg/ml) for 24 hrs at 37°C under constant shaking (250rpm/min). Standard serial dilution and plating on LB agar was performed to determine the fraction of the test population that survived after treatment [21]. For PM assay, 1μL of cells was diluted in to 99uL LB broth and incubated for 5hrs. The cell density was determined by testing OD_{600}.

3.3.3 Electron microscopy

Cells treated with GlcNAc, GlcN, ampicillin or saline (negative control) for 24hrs were visualized by SEM. Cells were fixed in 0.4% polyoxymethylene overnight and

OSO_4 for 2hrs, then washed 3 times with PBS. The cells were dehydrated using pure ethanol, and then infiltrated and embedded in Spurr resin.

3.3.4 Analysis of intracellular accumulation products by LC/MS

The bacterial cytosolic sample was analyzed by an electrospray ionization triple quadrupole mass spectrometer (Agilent), with a 6460liquid chromatography (Agilent). Upon treatment with GlcNAc alone for 24hrs, bacteria were centrifuged at 6500×g for 10mins. The cells were then resuspended with 1mL B-PER and vortexed for 15mins to achieve cell lysis; cell debris was removed and acetone %X volume was added at to cause precipitation of macromolecules. The supernatant was transferred to a microtube and dried at 56 ℃ in a vacuum concentrator for 2hrs. The pellet was dissolved in 50μL of water before LC/MS analysis and 2μL amount were injected into an Acquity UPLC BEH Hillic column (150 by 4.6 mm; Waters) for measurement of the amount of UDP-GlcNAc, and a NH_2 column (150 by 2mm; Phenomenex) for measuring the amount of Fru-6-P, using a 45-min-gradient program set at a flow rate of 0.2ml/min. The column was subjected to 5 minutes of washing with 100% buffer A (5mM ammonium formate with 5% ammonium hydroxide for UDP- GlcNAc;20mM ammonium acetate with 5% ammonium hydroxide for Fru-6-P), followed by a linear gradient over 30mins with 40% buffer B (5mM ammonium formate with 95% acetonitrile for UDP- GlcNAc;100% acetonitrile for Fru-6-P). To quantify fru-6-P and UDP- GlcNAc concentrations in cell extracts, we performed total ion-current (TIC) in each sample and determined the area under the curve of the peak. A series of dilution of fru-6-P and UDP-GlcNAc were also analyzed by LC-MS to create a standard curve for determining the intracellular concentration of fru-6-P and UDP- GlcNAc.

3.3.5 NADH measurement

E. coli was washed and resuspended in saline until a concentration of OD_{600} of 0.2 was achieved, followed by incubation with GlcNAc for 24hrs. Cell pellets were washed and re-suspended with NADH extraction buffer, followed by determination of the concentration of NADH by using the the EnzyChrom NAD/NADH Assay Kit (BioAssay Systems).

3.3.6 ROS measurement

E. coli was washed and resuspended in saline until a concentration at OD_{600} of 0.2 was achieved, followed by incubation with GlcNAc for 24hrs. The cells were then incubated with 5mM HPF (Molecular Probes) or DCFDA (Invitrogen) for 30mins, and then washed twice in PBS to remove the excess dye. HPF is a probe highly specific for ·OH, whereas DCFDA was used to measure the total ROS level. Fluorescence was measured at an excitation wavelength of 485nm and an emission wavelength of 535nm by flow cytometry (BD Accuri C6), the cells were identified by FSC (forward scatter) and SSC (side scatter) parameters.

3.3.7 Fluorescent labelling of peptidoglycan

E. coli was washed and resuspended in saline to produce a cell suspension with an OD_{600} of 0.2, followed by incubation with GlcNAc for 24hrs. The cell suspension was then supplemented with 500μM HADA (Ex 405nm/Em 460nm) and incubated for 30mins. The cells were then fixed in 0.4% polyoxymethylene for 2hrs, washed 3 times with PBS, and then visualized by a Leica TCS SP8 MP Multiphoton Microscope; the microscopy images were analyzed by the LAS X software.

3.3.8 Mouse deep-seeded thigh infection model

For all animal experiments, 6-week-old NIH male mice (body weight, ~20g) were purchased from the Guangdong Center for experimental animals and allowed for food and water throughout the study. The NIH mice were made neutropenic by administering 150mg/kg cyclophosphamide at 3 days and 1 day before infection, respectively. An inoculum of 1×10^7 *E. coli* BW25113 were injected to the right thigh of mouse. At 24 hrs post-infection, the mice received antibacterial treatment (i.p.) every 12 hrs for 72hrs, with a dosage of 20mgCTX /kg and 1mmolGlcNAc /kg. The mice were euthanized and the infected thighs were aseptically excised, homogenized in saline and the number of *E. coli* was enumerated by serial dilution and plating on LB plate. Comparison between different treatment groups were analyzed using unpaired t-test by Graph Pad Prism. All experimental protocols followed the standard operating procedures of the approved biosafety level 2 animal facilities and were approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

3.4 Result

3.4.1Exogenous GlcNAc drastically enhances susceptibility of starvation-induced *E. coli* persister to β-lactam both *in vitro* and in vivo

Bacteria become highly tolerant to multiple antibiotics upon complete deprivation of all essential nutrients [21, 22]. We choose starvation-induced persister mode to explore the potential metabolites which revert persisters to be susceptible cells and screened for compounds that could suppress phenotypic persistence by re-sensitizing bacterial antibiotic persisters to ampicillin without supporting re-growth. Among a total of 95 carbon sources in the phenotype microarrays (PM, Biolog) tested, a

number of compounds were found to strongly re-sensitize persisters to ampicillin **(Fig 3.1)**: N-acetyl glucosamine (GlcNAc), fructose, fructose-6-phosphate, 2-deoxy adenosine, sorbitol, glucose-1-phosphate and glucuronic acid potentiate ampicillin killing activity in persisters. Among them, the effect of GlcNAc was the most pronounced. We then focused on investigating the molecular basis of the re-sensitization effect of GlcNAc.

Negative control 0.376±0.023	L-Arabinose 0.405±0.018	N-Acetyl-D-Glucosamine 0.055±0.009	D-Saccharic Acid 0.159±0.015	Succinic Acid 0.096±0.009	D-Galactose 0.104±0.012	L-Aspartic Acid 0.104±0.013	L-Proline 0.085±0.013	D-Alanine 0.071±0.014	D-Trehalose 0.075±0.016	D-Mannose 0.161±0.014	Dulcitol 0.174±0.015
D-Serine 0.072±0.013	D-Sorbitol 0.068±0.011	Glycerol 0.072±0.005	L-Fucose 0.088±0.014	D-Glucoronic Acid 0.071±0.013	D-Gluconic Acid 0.069±0.009	D,L-α-Glycerol Phosphate 0.182±0.015	D-Xylose 0.448±0.009	L-Lactic Acid 0.095±0.008	Formic Acid 0.242±0.013	D-Mannitol 0.073±0.011	L-Glutamic Acid 0.252±0.007
D-Glucose-6-Phosphate 0.071±0.014	D-Galactonic Acid-γ-Lactone 0.121±0.010	D,L-Malic Acid 0.117±0.016	D-Ribose 0.39±0.015	Tween 20 0.073±0.008	L-Rhamnose 0.14±0.015	D-Fructose 0.059±0.01	Acetic Acid 0.391±0.016	α-D-Glucose 0.077±0.011	Maltose 0.071±0.016	D-Melibiose 0.165±0.015	Thymidine 0.134±0.019
L-Asparagine 0.104±0.014	D-Aspartic Acid 0.23±0.015	D-Glucosaminic Acid 0.183±0.009	1,2-Propanediol 0.137±0.016	Tween 40 0.108±0.018	α-Keto-Glutaric Acid 0.116±0.013	α-Keto-Butyric Acid 0.119±0.009	α-Methyl-D-Galactoside 0.12±0.015	α-D-Lactose 0.123±0.014	Lactulose 0.161±0.017	Sucrose 0.126±0.012	Uridine 0.106±0.008
L-Glutamine 0.122±0.01	m-Tartaric Acid 0.214±0.009	D-Glucose-1-Phosphate 0.068±0.013	D-Fructose-6-Phosphate 0.065±0.012	Tween 80 0.118±0.011	α-Hydroxy Glutaric Acid-γ-Lactone 0.224±0.016	α-Hydroxy Butyric Acid 0.07±0.014	β-Methyl-D-Galactoside 0.127±0.018	Adonitol 0.143±0.016	Maltotriose 0.071±0.008	2-Deoxy Adenosine 0.066±0.01	Adenosine 0.137±0.015
Glycyl-L-Aspartic Acid 0.105±0.016	Citric Acid 0.101±0.013	m-Inositol 0.144±0.011	D-Threonine 0.079±0.014	Fumaric Acid 0.105±0.016	Bromo Succinic Acid 0.084±0.017	Propionic Acid 0.099±0.009	Mucic Acid 0.099±0.01	Glycolic Acid 0.24±0.018	Glyoxylic Acid 0.237±0.020	D-Cellobiose 0.113±0.014	Inosine 0.074±0.010
Glycyl-L-Glutamic Acid 0.103±0.016	Tricarballylic Acid 0.191±0.016	L-Serine 0.074±0.010	L-Threonine 0.102±0.014	L-Alanine 0.081±0.018	L-Alanyl-Glycine 0.081±0.016	Acetoacetic Acid 0.508±0.011	N-Acetyl-β-D-Mannosamine 0.082±0.013	Mono Methyl Succinate 0.114±0.014	Methyl Pyruvate 0.096±0.015	D-Malic Acid 0.202±0.020	L-Malic Acid 0.098±0.017
Glycyl-L-Proline 0.116±0.011	p-Hydroxy Phenyl Acetic Acid 0.173±0.019	m-Hydroxy Phenyl Acetic Acid 0.167±0.015	Tyramine 0.251±0.019	D-Psicose 0.15±0.012	L-Lyxose 0.553±0.011	Glucuronamide 0.109±0.009	Pyruvic Acid 0.114±0.008	L-Galactonic Acid-γ-Lactone 0.095±0.014	D-Galacturonic Acid 0.152±0.016	Phemylethyl-amine 0.21±0.014	2-Aminoethanol 0.217±0.016

Fig 3.1 The OD_{600} of the re-grown persisters that survived ampicillin treatment in the presence of the indicated nutrients in the PM plate.

Bacteria which had been starved were added to each well of the PM plate and treated with ampicillin for 4hr, followed by dilution in LB broth and allowed for re-growth at 37ºC for 5hr. Black, OD_{600} of regrown cells<0.06; dark grey, OD_{600} of regrown cells<0.07; light grey, OD_{600} of regrown cells<0.08. Error bars indicate the standard errors of the means.

We first tested whether the presence of GlcNAc affect the whole bacterial survival population under nutrient starvation. As shown in **Fig 3.2A**, the size of an *E. coli* population which had been subjected to starvation for 24 hrs did not change after supplementation of GlaNAc (black bars), indicating that GlcNAc did not change the population size of the cells cultured in saline. On the other hand, GlcNAc was found to be non-toxic, as its presence did not hamper cell growth at different growth time **(Fig 3.3A)**. We next assessed potential of GlcNAc to re-sensitize a starvation-induced tolerant population to ampicillin. Treatment with 100μg/ml ampicillin for 24hrs was only able to eradicate a very small proportion of a *E. coli* population which had been starved for 24 hrs, with ~5×10^7 CFU/ml of the original population of ~10^8 CFU/ml being able to survive the treatment, indicating persister fraction formed after starvation treatemnt **(Fig 3.2A and B)**. In the presence of GlcNAc, however, the survival rate was found to decrease drastically upon ampicillin treatment. When compared to the sample treated with ampicillin alone, the survival rate of persisters decreased ~5 logs upon treatment with ampicillin in the presence of 1mM or 10mM GlcNAc **(Fig 3.2A)**. Along with the extension of GlcNAc treatment time, cell survival decreased further, as persister population began to minor reduction in treatment 3 hours and almost complete eradication of persisters were achieved if the treatment time was extended to 33 hours, suggesting that GlcNAc was able to re-sensitize most of the persisters to ampicillin **(Fig 3.2B)**.

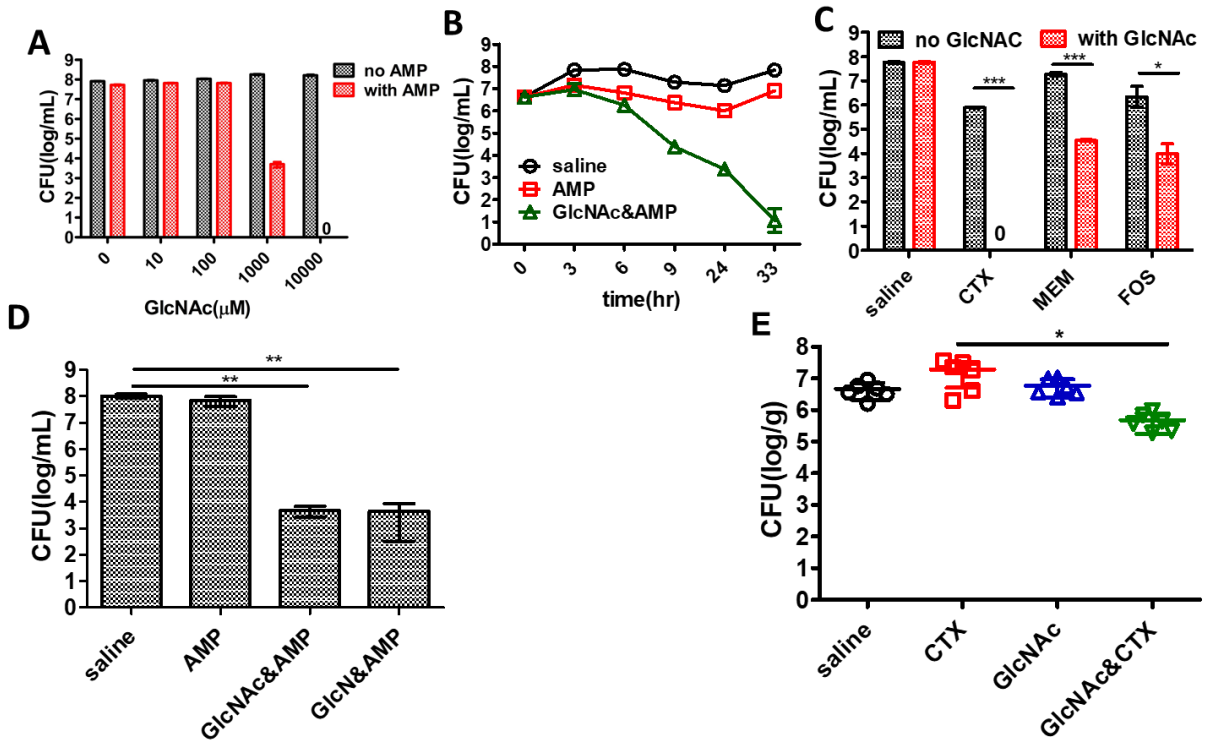

Fig 3.2 Effect of Exogenous GlcNAc or GlcN on Susceptibility of Starvation-Induced Persisters to β-lactams both *in vitro* and *in vivo*.

(A)The survival rates of starved *E. coli* incubated with or without GlcNAc and ampicillin. Starved *E. coli* were incubated with increasing concentrations of GlcNAc for 24hrs in the presence and absence of ampicillin.

(B)The time killing curve of starved *E. coli* incubated with or without GlcNAc and ampicillin. Starved *E. coli* were incubated with ampicillin (red and green lines) for up to 33 hrs in the presence or absence of GlcNAc (1mM), cells re-suspended in saline were included as negative control.

(C)The survival rates of starved *E. coli* incubated with or without GlcNAc and β-lactams. Starved *E. coli* were incubated with different β-lactams for 24hrs in the presence or absence of GlcNAc (1mM), cells re-suspended in saline were included as negative control. AMP, ampicillin; CTX, cefotaxime; MEM, meropenem; FOS, fosfomycin.

(D)The survival rates of stationary phase *E. coli* incubated with or without GlcNAc/GlcN and ampicillin. Stationary phase *E. coli* were incubated with ampicillin for 24hrs in the presence of GlcNAc (1mM) or GlcN (1mM), cells re-suspended in saline were included as negative control.

(E) The survival bacteria population after treatment with or without GlcNAc and ceftazidime in animal model. 1×10^7 cells were injected to the two thighs of each test mouse. At 24hr post-infection, the mice were subjected to the indicated dosage of antibacterial treatment (i.p.) every 12hr for 72hrs. Mice were euthanized and the thighs were aseptically excised, homogenized in saline and determination of the number of bacteria that survived (expressed in CFU).

Results are displayed as mean± SD, and the degree of significance in difference between the test and control samples (ns, no significance; *p <0.1; * *p<0.01; * * *p<0.001) was determined by Student's test. Three biological repeats were carried out.

Apart from ampicillin, GlcNAc was also found to re-sensitize starvation-induced persistent cells to other β-lactams or fosfomycin, which target on peptidoglycan synthesis. β-lactam antibiotics target and inhibit transpeptidase, commonly known as penicillin-binding proteins (PBPs), which mediates the final assembly of peptidoglycan by catalyzing cross-linkage between the neighboring stem peptides via formation of pentaglycine bridges. Fosfomycin prevents the formation of UDP-GlcNAc-3-O-enoplyruvate from UDP-GlcNAc, the first step in peptidoglycan biosynthesis [266]. In the presence of GlcNAc, the susceptibility of persisters to cefotaxime sharply increased and cell survival decreased to 0 **(Fig 3.2C)**. Cell survival in the presence of meropenem or fosfomycin plus GlcNAc was 3 or 2 logs lower than that recorded upon treatment with meropenem or fosfomycin alone **(Fig**

3.2C). Apart from *E. coli* BW25113, cell survival was also found to decrease ~4 logs in *E. coli* strain BW3110 upon treatment with the GlcNAc and ampicillin combination; likewise, survival also decreased ~4 logs in J53 (*E. coli 2*) when treated with GlcNAc and meropenem **(Fig 3.3A and C)**.

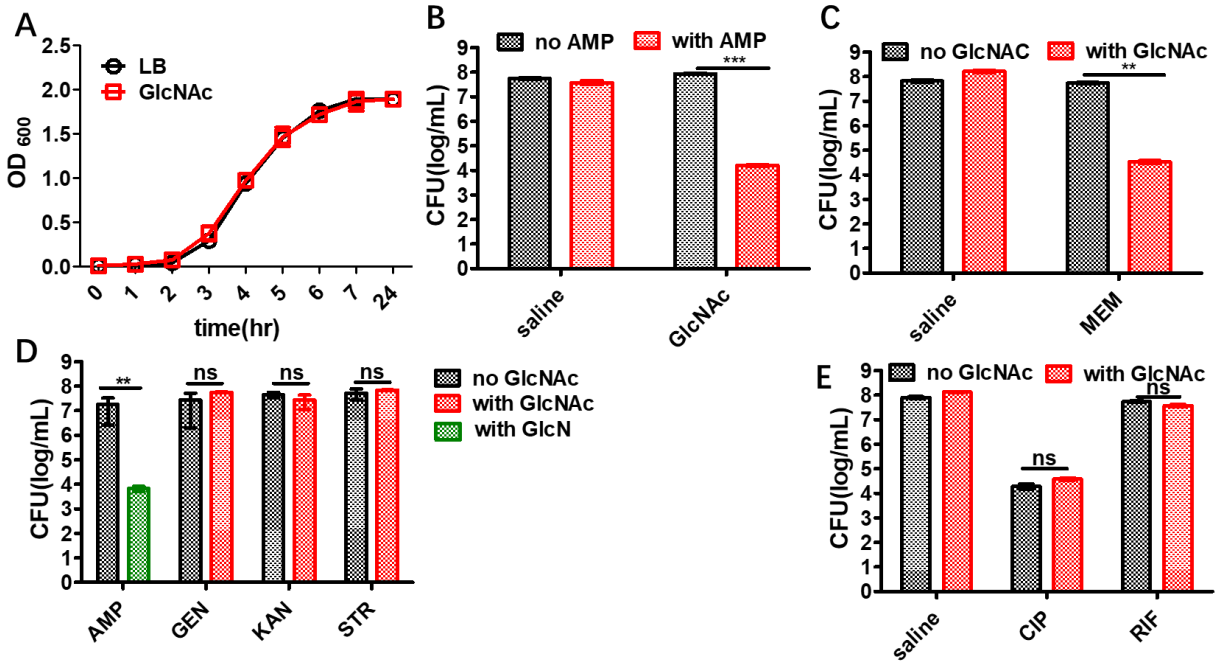

Fig 3.3 Effect of Exogenous GlcNAc or GlcN on different strains or different antibiotics.

(A)Growth rate of *E. coli* with or without GlcNAc (1mM), indicating that GlcNAc is non-toxic and does not inhibit cell growth.

(B)The survival rates of starved *E. coli* BW3110 incubated with or without GlcNAc and ampicillin. Starved *E. coli* BW3110 were incubated with ampicillin (red bar) for 24hrs in the presence or absence of GlcNAc (1mM), cells resuspended in saline was included as negative control.

(C)The survival rates of starved *E. coli* 2 incubated with or without GlcNAc/GlcN and meropenem. Starved *E. coli* 2 were incubated with meropenem (red bar) for 24hrs in the presence or absence of GlcNAc (1mM), cells resuspended in saline were included as negative control.

(D)The survival rates of starved *E. coli* 2 incubated with or without GlcNAc/GlcN and antibiotics. Starved *E. coli* were incubated with different antibiotics for 24hrs in the presence or absence of GlcNAc (1mM) or GlcN (1mM), cells resuspended in saline were included as negative control. AMP, ampicillin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin.

(E)The survival rates of starved *E. coli* 2 incubated with or without GlcNAc/GlcN and antibiotics. Starved *E. coli* were incubated with different antibiotics for 24hrs in the presence or absence of GlcNAc (1mM), cells resuspended in saline were included as negative control. CIP, ciprofloxacin; RIF, rifampin.

Results are displayed as mean± SD, and the degree of significance in difference between the test and control samples (ns, no significance; *p <0.1; * *p<0.01; * * *p<0.001) was determined by Student's test. Three biological repeats were carried out.

Transportation of GlcNAc into the bacterial cell was accompanied by phosphorylation and deacetylation, producing glucosamine 6-phosphate (GlcN6P). Likewise, Glucosamine (GlcN) is also converted to GlcN6P upon entry into the bacterial cell (NagA; EC 3.5.1.25)[267]. GlcN possesses similar physiological function with GlcNAc and we found that GlcN exhibited a persister-re-sensitizing effect resembling that of GlcNAc **(Fig 3.3D)**. Apart from starvation-induced persisters, GlcNAc and GlcN was also able to stationary phase persisters, with both

being able to cause 5 logs reduction in the size of the persister population upon ampicillin treatment, when compared to treatment with ampicillin alone **(Fig 3.2D)**.

The efficacy of the GlcNAc and β-lactam combination to eradicate bacterial persisters was further investigated in a mouse infection model. Ceftazidime (CTX), a commonly used antibiotic in clinical treatment of bacterial infection, was used as the test agent. A deep-seeded thigh persister model using *E. coli* BW25113 was established; the results showed that the size of the surviving bacterial population recorded after treatment with the GlcNAc and CTX combination was 4.8×10^5 CFU/g, which was significantly lower than that recorded after treatment with CTX only (1.9×10^7 CFU/g, *P*=0.02) **(Fig 3.2E)**.

Interestingly, GlcNAc did not alter cell susceptibility to aminoglycosides, quinolones and rifampin under starvation as cell survival did not change when GlcNAc was included in treatment with gentamicin, kanamycin or streptomycin, suggesting that the underlying mechanism of GlcNAc-mediated sensitization was confined to the -lactam-sensitive peptidoglycan synthesis, and is different from that mediated by glucose and fructose, which rendered persisters susceptible to aminoglycosides as the catabolism of these carbohydrates provided proton motive force (PMF) to drive aminoglycosides into cells, reported by Allison *et al* **(Fig 3.3D)**. Phenotypic persistence was not suppressed when GlcNAc was used in combination with ciprofloxacin or rifampin **(Fig 3.3E)**; this observation was consistent with Allison *et al* report that carbohydrates could only re-sensitize persisters to aminoglycosides [200].

3.4.2 Morphology of ampicillin treated cells in the presence of GlcNAc or GlcN

SEM assay was performed on *E. coli* that had been treated with GlcNAc or GlcN in the presence or absence of ampicillin. Compared to the untreated cells which had integrated and smooth membrane and cell wall, cells treated with GlcNAc and ampicillin exhibited signs of complete membrane and cell wall damage including cell lysis **(Fig 3.4 arrow 1)**. The morphology of bacteria subjected to GlcNAc treatment only was identical to that of the untreated control, with integrated and smooth membrane and cell wall, confirming that GlcNAc itself did not exert any cellular damages. The cell structure and cell wall integrity were not altered if only ampicillin was involved in treatment, indicating that the bacterial cells were tolerant to ampicillin under starvation stress **(Fig 3.4)**. Likewise, cell wall was completely damaged and cell structure ruin was observed upon treatment with the GlcN and ampicillin combination, but not GlcN alone **(Fig 3.5)**. The SEM observations are consistent with the results of phenotypic assay results, which showed that GlcNAc or GlcN could effectively re-sensitize persister cells to β-lactams.

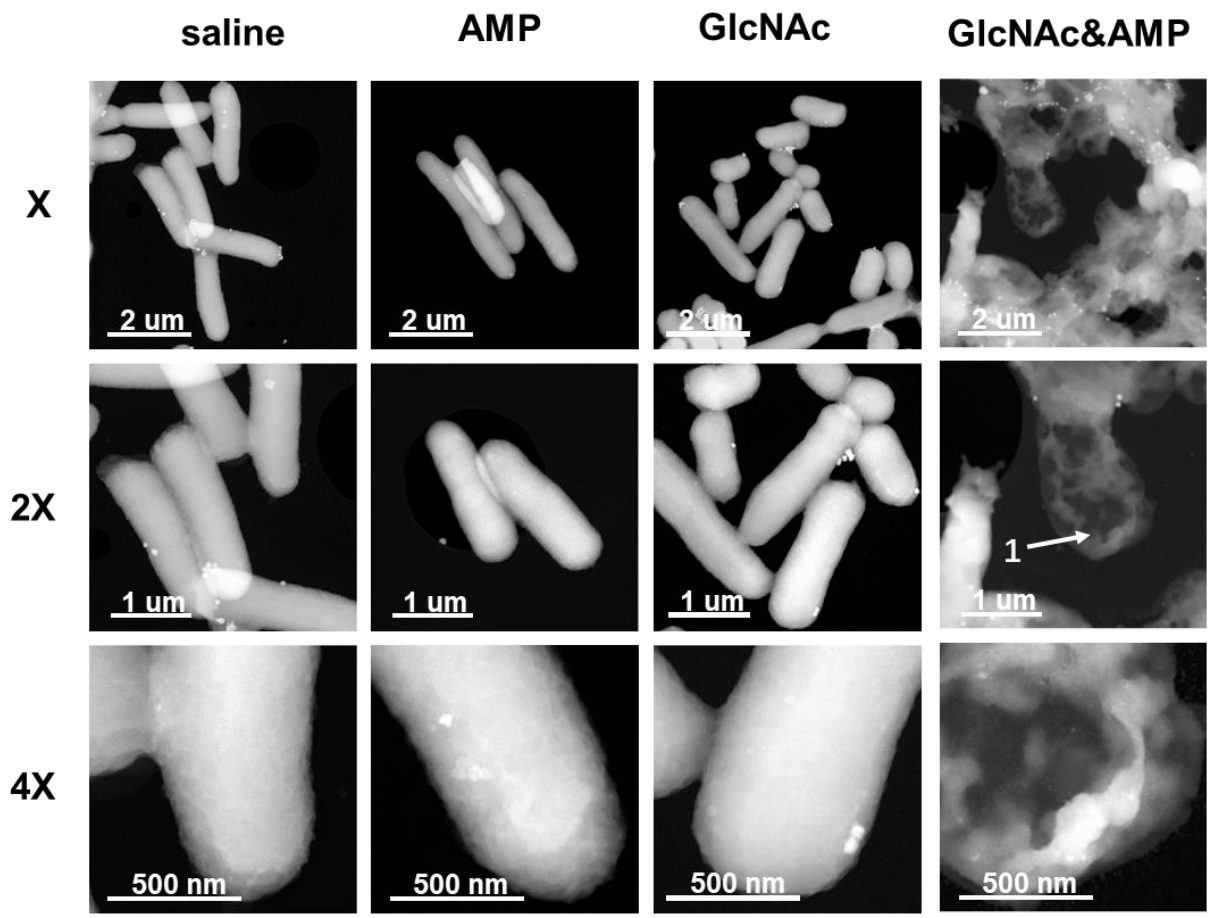

Fig 3.4 SEM image of *E. coli* cells under GlcNAc treatment.

Starved cells were incubated with ampicillin (100μg/ml), GlcNAc (1mM) or GlcNAc plus ampicillin (saline as negative control) for 24hrs before SEM image.

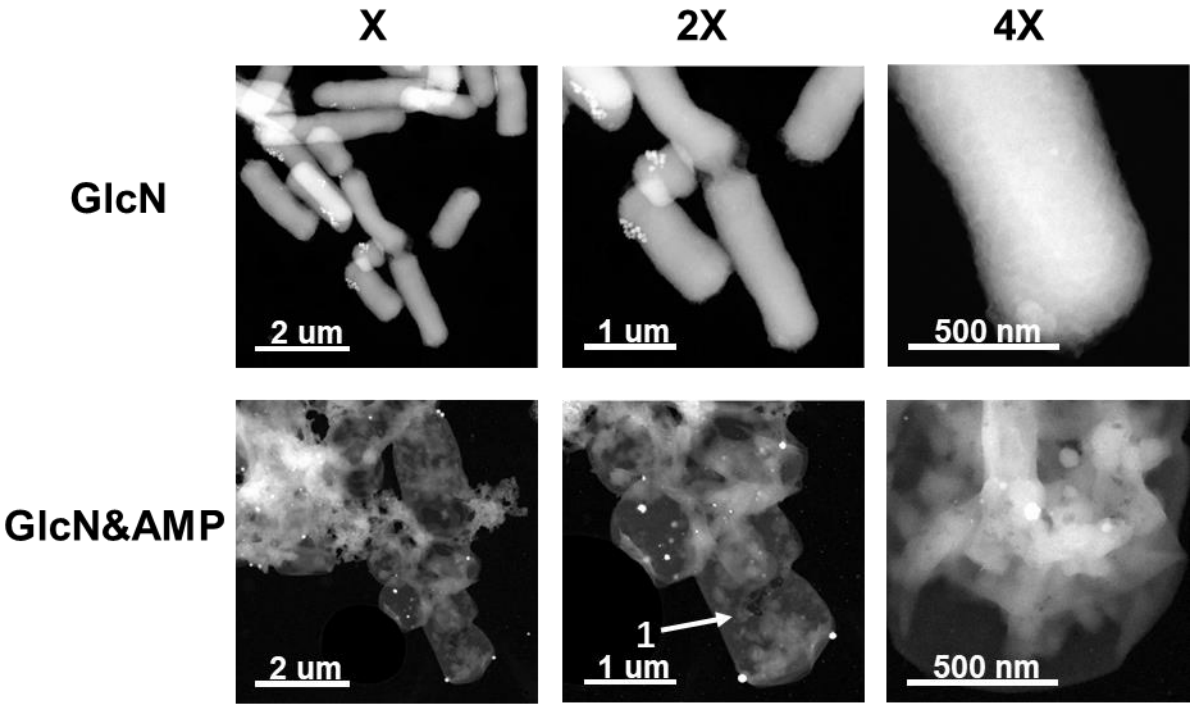

Fig 3.5 SEM image of *E. coli* cells under GlcN treatment.

Starved cells were incubated with GlcN (1mM) or GlcN plus amipicllin for 24hrs before SEM image.

3.4.3 GlcNAc Stimulates peptidoglycan synthesis

Upon internalization, GlcN or GlcNAc was phosphorylated and converted to GlcN-6-P, which then enters the GlcNAc metabolic pathway. In this work, we chose GlcNAc as a test compound and investigated the mechanism that underlies the strong GlcNAc re-sensitization effect. First, we checked if the persister re-sensitization effect exhibited by GlcNAc was due to its ability to activate the GlcNAc metabolic pathway; we investigated if knockout of the *nagA* gene (Δ*nagA*), which encodes GlcNAc-6-P deacetylase and plays a role in coverting GlcNAc-6-P to GlcN-6-P (NagA; EC 3.5.1.25), could diminish the strength of persister re-sensitization. Our data showed that the cell survival rate in the *E. coli*::Δ*nagA* strain treated with ampicillin did not alter when supplemented with GlcNAc, indicating that activation

of the GlcNAc metabolic pathway is sufficient to convert persister cells back to the ampicillin-susceptible mode **(Fig 3.6)**.

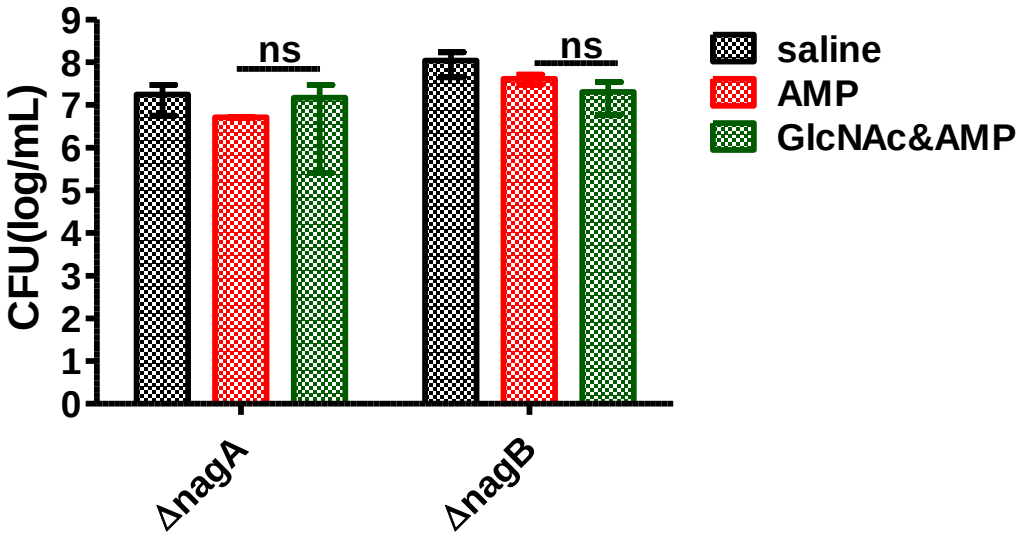

Fig 3.6 The failure of GlcNAc resensitization after inhibition of GlcNAc metabolism. Starved strains were incubated with ampicillin for 24hrs in the presence or absence of GlcNAc (1mM), cell re-suspended in saline were included as negative control. Results are displayed as mean± SD, the degree of significance in difference between the test and control samples (ns, no significance; *p <0.1; * *p<0.01; * * *p<0.001) was determined by Student's test. Three biological repeats were carried out.

In the GlcNAc hexosamine pathway, GlcNAc is eventually converted to UDP-GlcNAc, which then enters the peptidoglycan biosynthesis pathway. It is widely accepted that dormancy is a key mechanism underlying bacterial antibiotic persistence, as the targets of antibiotics are inactive in dormant state [39, 123]. If a specific antimicrobial target can be converted to the active state by metabolite stimulation, persister cells may become sensitive to antibiotics again. To examine whether supplementation of GlcNAc can activate peptidoglycan synthesis in

persisters, we quantified the amount of intracellular UDP-GlcNAc after treating the persister cells with GlcNAc for 24 hours. UDP-GlcNAc identification was based on the retention time of the standard subjected to high-performance liquid chromatography (HPLC) and confirmed according to the multiple reaction monitoring (MRM) transition mode (retention time on the HPLC column of 7min, m/z^{-1} of 606.2 to 385 collision energy 25V). The concentration of UDP-GlcNAc in cells after starvation for 24 hours was found to be 0.06μM, but increased ~80 folds upon treatment with GlcNAc (4.95μM) **(Fig 3.7 and 3.8)**.

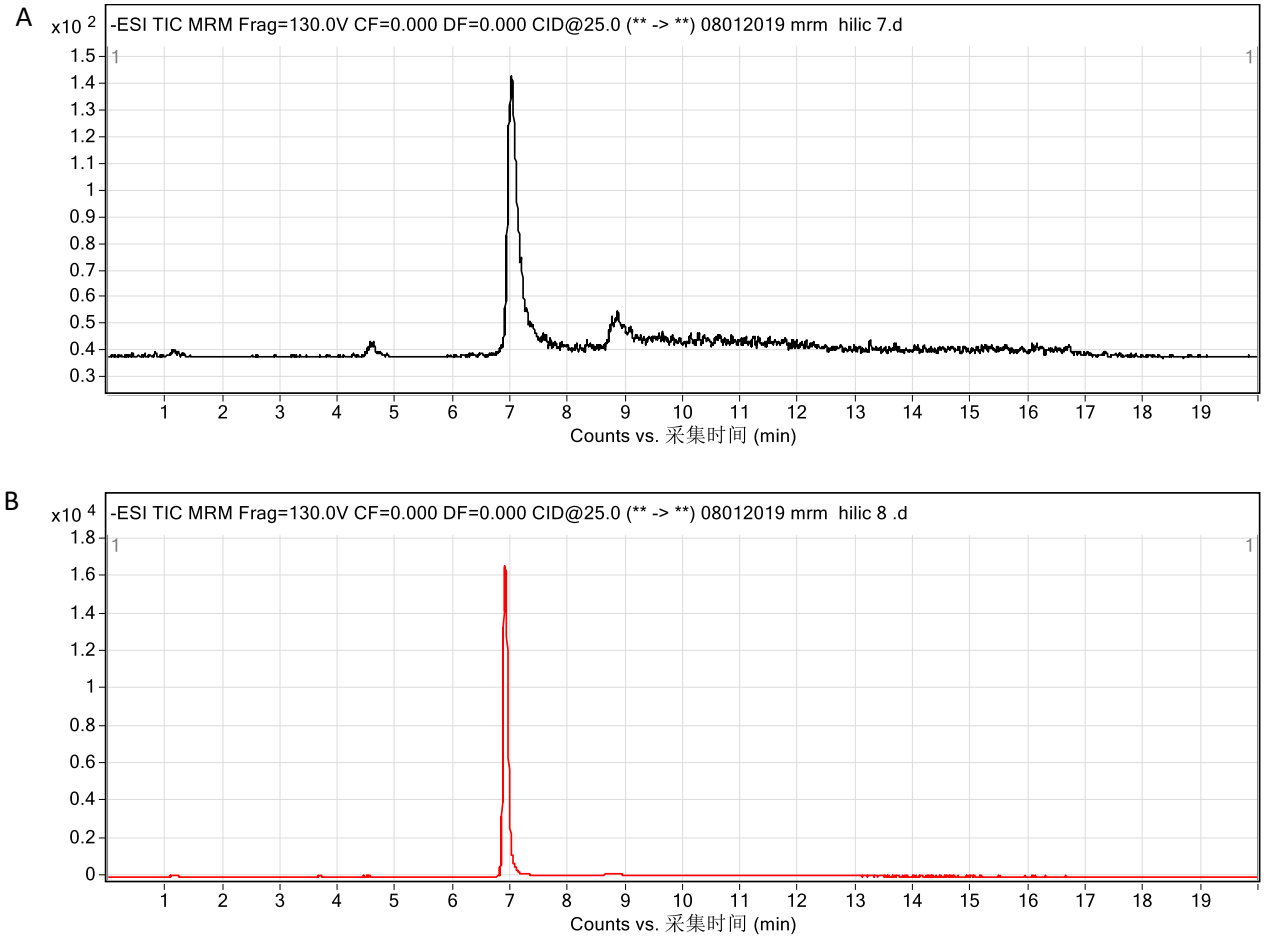

Fig 3.7 Intracellular amount of UDP-GlcNAc in the absence (A) or presence (B) of GlcNAc.

Mass spectra of UDP-GlcNAc in the samples are presented with the total-ion chromatograms (TIC) (m/z^{-1} of 606.2 to 385 collision energy 25V and retention time on the HPLC column of 7min)

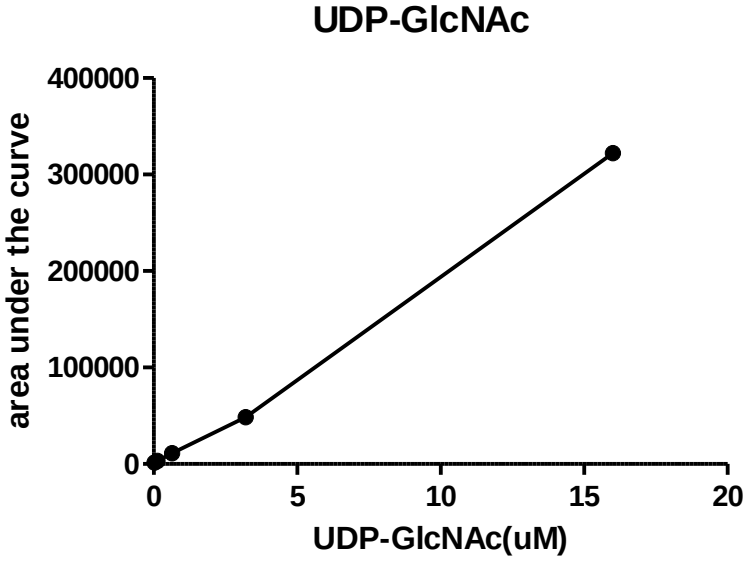

Fig 3.8 Dilution of UDP-GlcNAc standards measured by LC-MS.
2μL of gradient diluted standard with concentrations from 25.6nM to 16μM were analyzed by HPLC-MS operated in negative ion mode. The area under the curve of each sample was determined with GraphPad Prism.

The sharply increased amount of UDP-GlcNAc detectable in persister cells upon supplemented with GlcNAc suggests that dormant persister cells had resumed GlcNAc metabolism and peptidoglycan biosynthesis. To further confirm that peptidoglycan was synthesized in persisters, we tested whether fluorescent D-amino acid HADA (7-hydroxycoumarin-3-carboxylic acid-3-amino-D-alanine), which specifically binds to and stains newly synthetized peptidoglycan, could stain the cell wall of persisters in the presence of GlcNAc [268]. Our data showed that none of the

persister cells under starvation could be stained by HADA, indicating that peptidoglycan biosynthesis has grounded to a halt. However, most of the persister cells tested were stained by HADA after supplementation with GlcNAc 24hr, indicating that peptidoglycan biosynthesis had resumed when GlcNAc was available **(Fig 3.9)**. These data indicate that an active peptidoglycan biosynthesis was regained after GlcNAc supplementation.

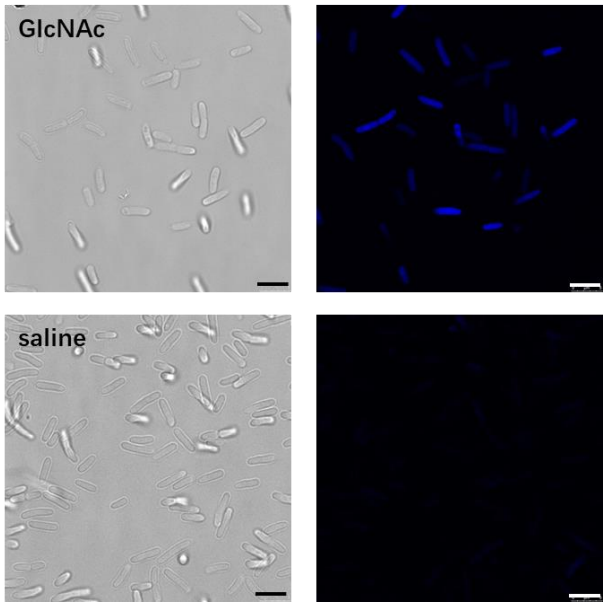

Fig 3.9 Peptidoglycan biosynthesis was reactivated after GlcNAc supplementation. Confocal microscopy images of HADA labelling newly synthesized peptidoglycan in *E. coli* subjected to 24hrs starvation in the presence or absence of GlcNAc. The left and right panels are the bright field and fluorescence images respectively (scale bar:5μm).

3.4.4 Exogenous GlcNAc Promote Glycolysis

Apart from being a precursor of peptidoglycan biosynthesis, GlcNAc could also be catabolized to Fru-6-P by deaminase, which is encoded by the *nagB* gene [269]. Fru-6-P is a substrate of glycolysis. Previous studies reported that glucose, fructose and mannitol re-sensitized persister cells to aminoglycosides via activating glycolysis or

TCA cycle [200, 228]. Both Fru and Fru-6-P were shown to exhibit ampicillin resensitization effect in the PM assay in this work **(Fig 3.1)**. To determine whether supplementation of GlcNAc caused an increase in the amount of Fru-6-P and subsequently activated glycolysis and initiation of the TCA cycle in persisters, the amount of Fru-6-P in persister cells was quantified by HPLC. Upon starvation for 24 hours, the amount of Fru-6-P in the persister cells was 0.02uM; however, the level was found to increase ~20 folds in the presence of GlcNAc (0.42uM) **(Fig 3.10 and 3.11)**. This sharp increase in the amount of Fru-6-P indicated supplementation of exogenous GlcNAc stimulated its catabolism in persisters.

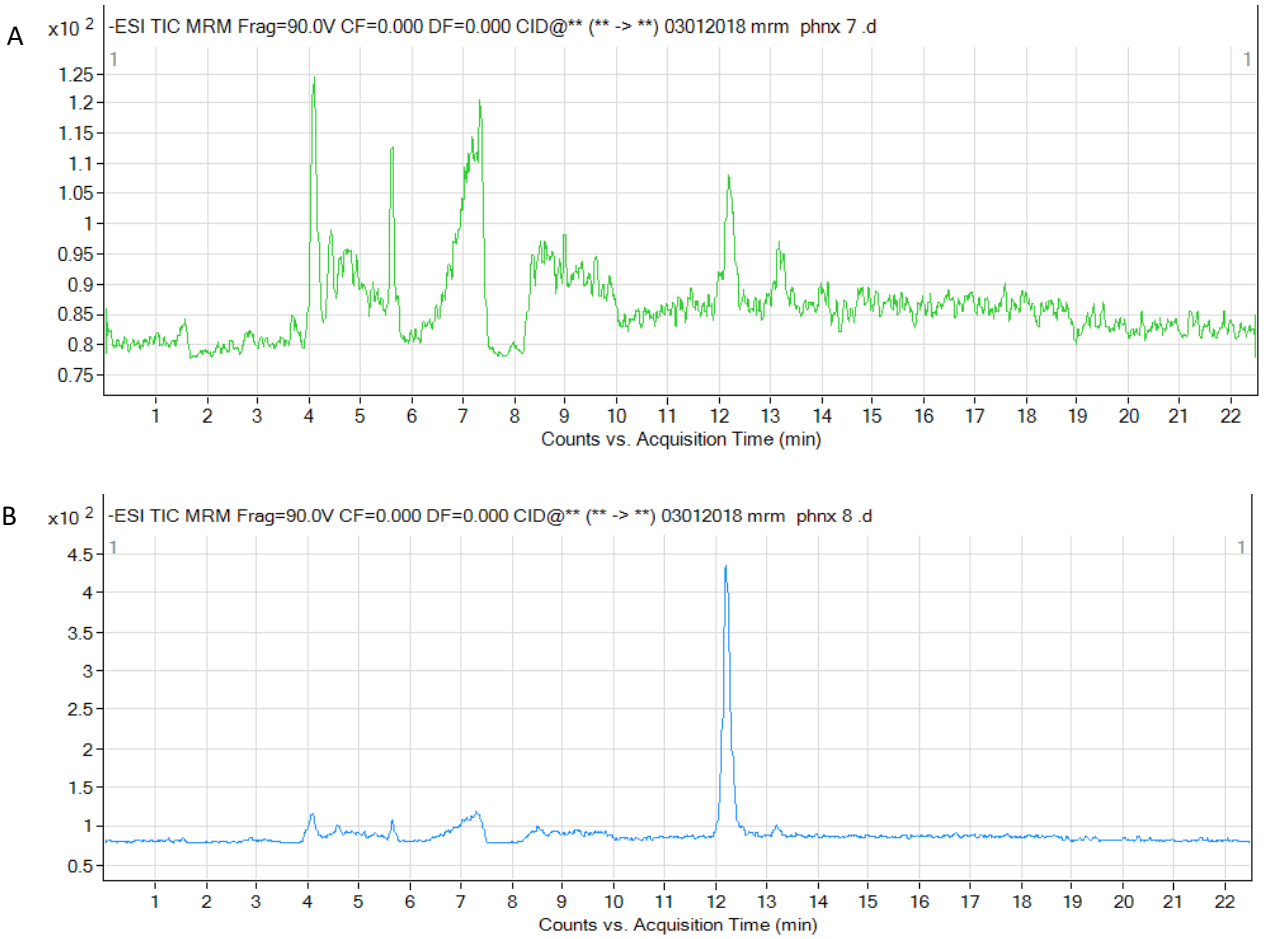

Fig 3.10 Intracellular amount of Fru-6-P in the absence (A) or presence (B) of GlcNAc.

Mass spectra of Fru-6-P in the samples are presented with the total-ion chromatograms (TIC) (m/z^{-1} of 259 to 79 collision energy 25V and retention time on the HPLC column of 12.2min)

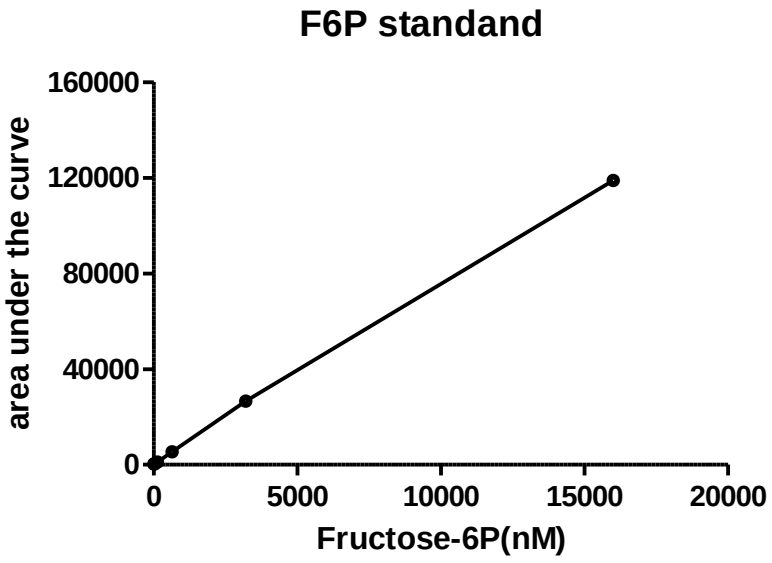

Fig 3.11 Dilution of Fru-6-P standards measured by LC-MS.
2μL of gradient diluted standard with concentrations from 5.12nM to 16μM were analyzed by HPLC-MS operated in negative ion mode. The area under the curve of each sample was determined with GraphPad Prism.

To further explore the relationship between GlcNAc catabolization and persister re-sensitization, we tested the effect of GlcNAc in the Δ*nagB* mutant, in which the gene encoding GlcN-6-P deaminase, the enzyme responsible for transferring GlcN-6-P into Fru-6-P (NagB; EC3.5.99.6), was deleted. Unlike the wild type cell, the survival rate of persisters of the Δ*nagB* mutant did not decrease upon treatment with the GlcNAc and ampicillin combination, indicating that persisters remained tolerant to ampicillin if GlcNAc catabolism was disturbed **(Fig 3.6)**. Although GlcNAc

catabolism is essential for its ability to re-sensitize persisters to ampicillin, the state of glycolysis or TCA cycle upon supplementation of GlcNAc and whether these processes are involved in the re-sensitization effect of GlcNAc remain unknown. NADH is the major product of glycolysis and TCA cycle and is a reducing agent directly involved in energy production through donating electrons in the electron transport chain to produce ATP. An elevated NADH level was found to be involved in glucose-mediated re-sensitization of persisters to aminoglycosides [228]. We tested the amount of NADH in persisters in the presence of GlcNAc and found that the level of NADH was about three times higher than that recorded in the absence of GlcNAc **(Fig 3.12A)**. The increasing amount of NADH in persisters indicated that glycolysis and TCA cycle were activated by GlcNAc. We then tested whether deletion of either one or both genes that encode NADH dehydrogenase (NADH dehydrogenase I (Δ*nuoI*), NADH dehydrogenase II (Δ*ndh*) and both NADH dehydrogenases (Δ*ndhnuoI*)), which play an essential role in energy generation, affected the size of the persister sub-population. We found that persisters could not be eradicated by the GlcNAc and ampicillin combination in the Δ*ndhnuoI* mutant, with the size of the tolerant population being similar to that of the ampicillin treatment (~10^8 CFU/ml). However, the re-sensitization effect of GlcNAc could still be observed upon deletion of a single NADH dehydrogenase gene **(Fig 3.12B)**, suggesting that activation of glycolysis and TCA cycle via enhancement of NADH production and dehydrogenation was an essential step in the GlcNAc re-sensitization process.

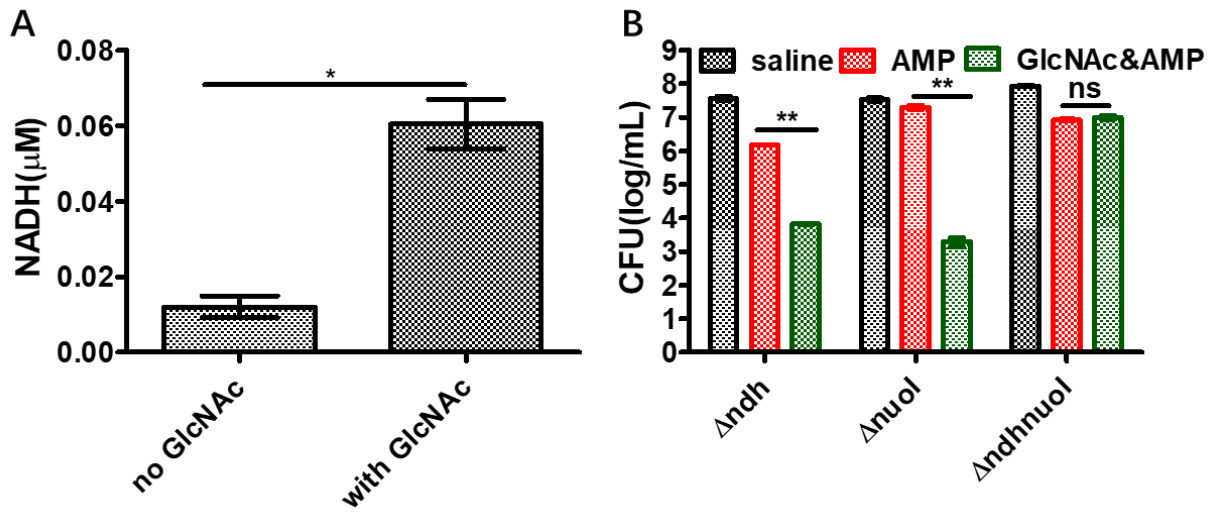

Fig 3.12 The effect of glycometabolism on GlcNAc resensitization.

(A) NADH level in the presence or absence of GlcNAc.

(B) Starved strains were incubated with ampicillin for 24hrs in the presence or absence of GlcNAc, strains treated with saline only were included as negative control.

Results are displayed as mean± SD. The degree of significance in difference between results of the test samples were determined by Student's T test (ns, no significance; *p <0.1; * *p<0.01; * * *p<0.001).

3.4.5 cAMP regulation and ROS was not involved in GlcNAc re-sensitization

It was reported that GlcNAc elicited onset of the cAMP signaling pathway, which in turn induced morphological changes, ROS generation and eventually triggered rapid cell death in *C.albicans* [270, 271]. A recent study showed that cAMP played a role in regulating the level of *E. coli* persistence to β-lactams via mediating oxidative stress response and SOS-dependent DNA repair[113]. The cAMP receptor protein (CRP) is a global regulator of genes involved in carbon catabolite repression [272]. Based on these previous findings, we hypothesized that the cAMP signal-

dependent regulation and ROS production were also involved in GlcNAc re-sensitization and tested the cell survival rate of persisters of the Δ*crp*, Δ*cyaA* and Δ*cpdA* gene knockout mutant upon treatment with the GlcNAc and ampicillin combination. The *cyaA* encodes the cAMP synthase; the *cpdA* gene product is a cAMP-specific phosphodiesterase which hydrolyzes cAMP; the *crp* gene encodes the cAMP receptor protein [273, 274]. However, our results showed that the synergistic antimicrobial effect of the GlcNAc and ampicillin combination could still be exerted on persisters of the Δ*crp*, Δ*cyaA* and Δ*cpdA* mutants **(Fig 3.13)**, indicating that cAMP mediated regulations were not involved in the GlcNAc re-sensitization process. To identify whether ROS was involved in the killing of persisters by the ampicillin in the presence of GlcNAc, we used 3'-p-hydroxyphenyl fluorescein (HPF) to measure the level of hydroxyl radicals (·OH) before and after treatment with the GlcNAc and ampicillin combination and found the amount of hydroxyl radicals remained unchanged upon treatment with GlcNAc and ampicillin **(Fig 3.14A)**. We also used 2',7'-Dichlorofluorescein diacetate (DCFDA) staining to detect total cellular ROS level and found that GlcNAc-treated cells exhibited the same level of ROS with the untreated persisters **(Fig 3.14B)**. These findings confirmed that GlcNAc did not induce ROS production and that ROS was not involved in persister killing due to GlcNAc-mediated persister re-sensitization to ampicillin.

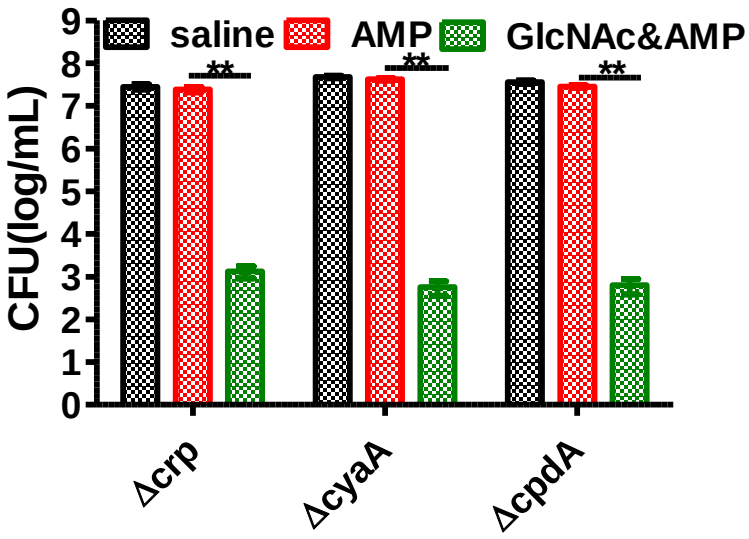

Fig 3.13 GlcNAc resensitization effect was not regulated by cAMP.

Survival rate of three gene knockout strains Δ*crp*, Δ*cyaA* and Δ*cpdA* subjected to starvation by washing and re-suspending in saline and then treatment with ampicillin for 24hrs in the presence or absence of GlcNAc. Strains treated with saline only were included as negative control. Results are displayed as mean± SD. The degree of significance in difference between results of the test samples were determined by

student's T test (ns, no significance; *p <0.1; * *p<0.01; * * *p<0.001).

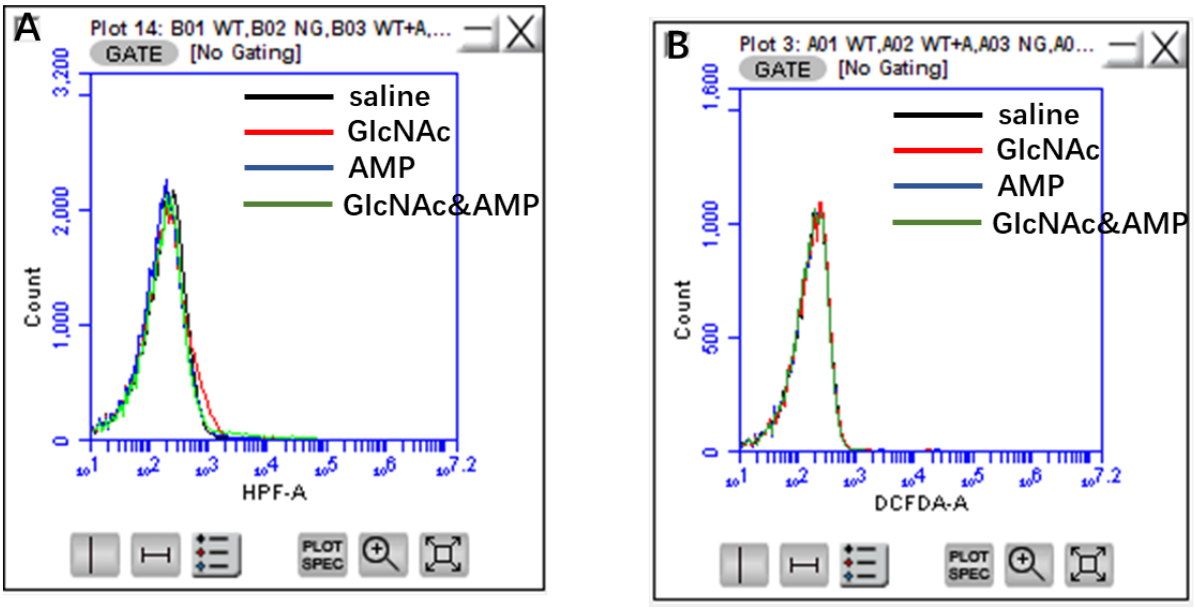

Fig 3.14 ROS level had no change in the presence of GlcNAc.

The level of intracellular oxidative stress was measured by flow cytometry using two different fluorescence probes of different affinities to free radicals. Starved *E. coli* were treated with ampicillin, GlcNAc or a combination of ampicillin and GlcNAc for 24hrs before staining. (A) Staining by HPF to quantify hydroxyl radical (·OH), (B) Staining by DCFDA to quantify total cellular ROS.

3.5 Discussion

Bacterial antibiotic tolerant persisters, which are the main culprit of chronic and recurrent infections, have been identified in almost every bacterial species. Currently, research reports on persisters mostly focus on identification of genes and metabolic pathways involved in persister formation [39] without providing sufficient knowledge that allows design of an effective strategy for eradication of bacterial persisters. In this work, we aimed to identify non-toxic compounds that can alter the

metabolome of physiologically dormant persister cell so that they become awaken and reverted to the antibiotic-susceptible status.

We first used the PM assay to perform high-throughput screening of known nutrients that could suppress phenotypic persistence without supporting bacterial growth and found that GlcNAc exhibited the most pronounced effect in converting persister cells back to the susceptibility status. Based on results of the HADA and BOCILLIN FL labeling experiments, we propose a re-sensitization mechanism: uptake of GlcNAc or GlcN by persister cells allows them to switch from dormancy to a metabolically active state, in which peptidoglycan biosynthesis resumes and antibiotic is increasingly being accumulated in the cell cytosol. Allison *et al* previously reported that carbon metabolites, such as glucose, fructose and mannitol, could significantly enhance the susceptibility of persisters to aminoglycosidea via activation of glycolysis, generation of PMF and subsequently enhancement of aminoglycoside uptake [200]. Trehalose-catalytic shift was discovered in *M. tuberculosis* persister as these cells utilized trehalose as an internal carbon to biosynthesize central carbon metabolism intermediates involved in glycolysis and pentose-phosphate pathway, thus maintaining levels of ATP and NADPH. Inhibitor of trehalose-catalytic shift process could be used as an adjuvant as it potentiated antibiotic efficacy by interfering with these adaptive strategies[275]. As a carbon-based metabolite, GlcNAc also activates glycolysis or TCA cycle as the level of Fru-6-P and NADH level were found to have increased. The finding that GlcNAc lost the re-sensitization effect if the NADH dehydrogenase gene was deleted indicates that glycolysis and dehydrogenation of NADH by the electron transport chain are essential steps in the GlcNAc re-sensitization process. To conclude, our data showed that the underlying mechanisms by which GlcNAc can re-sensitize bacterial persisters to ampicillin

involve (i) eliciting glycolysis and (ii) allowing peptidoglycan biosynthesis to resume.

GlcNAc is known to induce cell death in *C. albicans* by inducing alteration in mitochondrial metabolism and activating the cAMP-regulated pathways, which in turn leads to accumulation of ROS and cell death[259]. Our data showed that the underlying mechanism of GlcNAc-mediated persister killing in *E. coli* differs from that in *C. albicans*, as ROS level did not change in the presence of GlcNAc. It should also be noted that GlcNAc alone can induce cell death in *C. albicans* due to its ability to cause ROS accumulation, whereas GlcNAc alone in *E. coli* cannot. GlcNAc can also trigger an increase in cAMP level in *C.albicans* [270]. The signaling molecule cAMP plays an important role in regulation of the *nag* regulon, which contains genes whose products play a role in GlcNAc and GlcN metabolism in *E. coli*, and was reported to be involved in persister formation.[113, 270, 276]. However, we found that the GlcNAc re-sensitization effect was not affected by deletion of the *crp*, *cyaA* and *cpdA* genes, suggesting that cAMP signaling is not involved in GlcNAc-re-sensitization of persisters to ampicillin, and that the bactericidal / re-sensitization effect of GlcNAc on bacteria and fungus involve activation of different cellular mechanisms.

GlcNAc and GlcN are known to exhibit beneficial pharmacological effects in treatment of osteoarthritis [277]. The therapeutic potential of GlcNAc is mainly attributed to its anti-inflammatory and chondro-protective effects[278]. GlcNAc also shows promising therapeutic efficacy in treatment chronic inflammatory bowel disease by restoring the intestinal matrix and improving the epithelial

morphology[279].. As GlcNAc is non-toxic, we believe that our approach can be applied clinically to reduce the chance of chronic and recurrent infections in immuno-compromised patients caused by members of enterobacteriaceae and other Gram-negative bacterial pathogens.

The experimental system reported here demonstrates a novel approach to restore the antibiotic susceptibility of bacterial persisters by using GlcNAc as a β-lactam adjuvant. Findings in this work broaden the approach for eradicating persisters which is based on metabolism by demonstrating that activating specific metabolic pathways of persisters can significantly enhance the susceptibility of persisters to specific antibiotic.

Chapter Four: Conclusion and Summary

Persisters widely exist and have been identified in almost all the bacterial species. It is commonly recognized that persistence is a culprit of failure in treatment of infections caused by major bacterial pathogens such as *E. coli* [25], *S. aureus* [280],*P. aeruginosa* [232],and *M. tuberculosis* [281]. In particular, chronic infections caused by persisters are difficult to treat in immunocompromised patients undergoing cancer chemotherapy, or those infected by HIV. As persisters constitute a subpopulation of bacteria which remains viable after treatment with a lethal dose of antibiotic, they are often regarded as a nature reservoir of resistant mutants. The threat and hazard that persisters bring about are huge and it is urgent to understand mechanisms underlying persistence formation and develop efficient therapeutic strategies for persister eradication.

In this thesis, we investigated the active cellular responses involved in persistence formation and devise an efficient way to treat persister-mediated infections. Active physiological adjustment mechanisms are essential for persistence formation and maintenance, as mere dormancy is not enough to maintain a persistence phenotype for a prolonged period. We describe in chapter two that many classes of genes were upregulated under starvation, according to our RNASeq results, among which expression of genes in the *psp* family was all found to be significantly upregulated. We then demonstrated that the up-regulated *psp* genes were involved in maintaining a basic level of PMF, which was required for long term persistence as treatment with compounds which caused dissipation of PMF, such as CCCP or sodium azide, resulted in a reduced persister population. Inhibition of the PMF generation process by deletion of the NADH dehydrogenase genes also caused reduction in the size of

the persistence fraction. Importantly, Econazole, an FDA-approved anti-fungus drug, could cause dissipation of bacteria PMF and eradicate persister population in a non-toxic manner when used in combination with β-lactams both *in vitro* and *in vivo*. We found that efflux activity decreased upon PMF dissipation, this finding may partially explain why PMF maintenance was essential for long term antibiotic tolerance.

In chapter three, we aimed to screen for non-toxic compounds which exhibited potential antibiotic bacterial effect and were also able to eradicate kill persister sub-populations. We focused on nutrients that suppressed starvation-mediated tolerance response and utilized a phenotype array in screening assays. GlcNAc and GlcN were found to exhibit β-lactam potentiation effect upon persisters. Supplementation of these compounds was found to reduce the persister population by five orders of magnitude in the presence of ampicillin. Hence these compounds could be developed as β-lactam adjuvants for anti-persister therapy. Peptidoglycan biosynthesis was found to be activated in the presence of GlcNAc; sugar catabolism was also activated, with an increasing level of NADH being detected. We showed that the GlcNAc re-sensitization effect was mediated by NADH-related respiration as deletion of the gene encoding NADH dehydrogenase could abolish such effect.

In summary, persistence and chronic infections caused by antibiotic persisters become a worldwide challenge which are urgent to solve. The underlying mechanisms of bacterial persistence remain undefined, preventing researchers to devise effective persister eradication strategy. In this work, we found that PMF maintenance was required for long term persistence formation, we identified an FDA-approved drug, econazole, which could cause dissipation PMF and may be

further developed as an anti-persister drug. We also found that the nutrients GlcNAc and GlcN could effectively re-sensitize persisters to β-lactam antibiotics via activating the peptidoglycan synthesis and sugar catabolism pathways in persister cells. Our discoveries broaden the knowledge on the molecular basis of persistence formation and maintenance and provide the basis for the design of effective strategies for persister eradication.

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