Mechanisms of Persister Cell Formation in *Salmonella enterica* subsp. *enterica* serovar Typhimurium

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Declaration

I hereby certify that I have written my dissertation independently and have not used any sources or aids other than those I have specified. This dissertation was not submitted elsewhere as a final thesis.

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adenosine monophosphate	cAMP
adenosine triphosphate	ATP
Asp	aspartate
electron transport chain	ETC
extended spectrum β-lactamases	ESBL
flavin adenine dinucleotide	FAD
flavin mononucleotide	FMN
guanosine tetraphosphate	ppGpp
hibernation promoting factor	HPF
high persistence	Hip
iron–sulfur cluster	Fe-S clusters
lipopolysaccharide	LPS
lysogeny broth	LB
minimum duration for killing	MDK
minimum inhibitory concentration	MIC
multi-drug resistant	MDR
NADH:ubiquinone oxidoreductase I	NDH-1
NADH:ubiquinone oxidoreductase II	NDH-2
nicotinamide adenine dinucleotide	NADH
nicotinamide adenine dinucleotide phosphate	NADPH
outer membrane proteins	Omps
polymerase chain reaction	PCR
pyrroloquinoline	PQQ
quinolone efflux pump	Qep
quinolone resistance determining region	QRDR
reactive oxygen species	ROS
ribosome-associated inhibitor	RaiA
ribosome modulation factor	RMF
Succinate dehydrogenase	SDH/Complex-II
TCA cycle	tricarboxylic acid cycle

tRNA	transfer- ribonucleotide acid
Tyr	tyrosine

4 Summary

Drug resistance is an acknowledged and widely recognized problem, and has engaged scientists for decades. However, a new phenotypic variation of antibiotic resistance, which does not involve genetically determined resistance mechanisms, has attracted more and more attention. This phenomenon is termed persistence, and refers to the observation that despite the absence of inheritable resistant mechanisms, persister cells manage to survive lethal doses of antibiotics. Persistence is usually attributed to slow growth and metabolic inactivity of a small subpopulation of an otherwise drug sensitive bacterial population. Ironically, persister cells were reported shortly after Penicillin was commercially available in 1944, yet largely ignored until the 1980s. Since that time, persister cells were acknowledged as a noxious threat to humans, causing recalcitrant infections and even serving as a reservoir for antibiotic resistance.

The aim of this project was to obtain a deeper understanding of the role of the bacterial energy household and metabolic state of persister cells. To this end, we focused on *Salmonella enterica* subsp*. enterica* serovar Typhimurium (*S*. Typhimurium), a facultative anaerobic pathogen. To study the influence of the major energy source, adenosine triphosphate (ATP), on persister cell formation, the *atp* operon which encodes the ATP-synthase (a generator of ATP and a part of the bacterial respiration process) was deleted. In the *atp* operon deletion (Δ*atp*) strain, the intracellular ATP concentration of *Salmonella* was reduced by up to 50 %. Subsequently, the mutant and the wild type strains were challenged with ciprofloxacin while growing exponentially in lysogeny broth (LB). Surprisingly, despite a reduced ATP level, the mutant was more susceptible to antibiotic killing, and showed reduced persister cell formation. Further investigations focused on the metabolic state of the mutant and revealed increased nicotinamide adenine dinucleotide (NADH) and concomitant reactive oxygen species (ROS) formation. The Δ*atp* operon mutant relies on substrate level phosphorylation for ATP generation, whereas the wild type can also produce energy via respiration. The dependence of the mutant on glycolysis resulted in increased formation of NADH, which drives enzymatic reaction and increased respiratory activity. A side effect of this augmented activity was the formation of ROS, in particular of hydroxyl radicals, which led to additional DNA damage. However, in the absence of oxygen or when ROS quenchers were used under aerobic conditions, the difference between wild type and mutant strains disappeared. This led to the

following model: the deletion of the ATP synthase forced the mutant to generate ATP via substrate level phosphorylation/glycolysis, resulting in increased formation of NADH, which in turn enhanced the formation of ROS. Additional DNA damage caused by ROS production when treated with ciprofloxacin resulted in increased susceptibility of the mutant.

In addition to ATP, persister cell formation can be affected by many additional factors, such as endogenous phages, although the mechanisms have not yet been fully clarified. Therefore, the second aim of this project was to investigate the effect of endogenous phages on persister cell formation upon ciprofloxacin treatment. To this end, the four endogenous phages present in *S*. Typhimurium ATCC 14028, designated Gifsy-1, Gifsy-2, Gifsy-3 and ST64B, were deleted. The phage-free variant was less sensitive to ciprofloxacin, exhibiting decelerated killing and increased persister formation. Furthermore, the growth of the phage-free strain was disturbed in nutrient rich medium, indicating that phages play a role during normal growth. However, the impaired growth was not responsible for the observed increased persister cell formation. The results of these studies indicated that Gifsy-1 is strongly induced upon ciprofloxacin exposure, and that deletion of the lysis genes *S*, *R* and *Rz*/*Rz1*, which are encoded by Gifsy-1, led to the observed increased survival. However, the contribution of the other three phages was negligible.

5 Zusammenfassung

Antibiotikaresistenz ist ein anerkanntes und schwerwiegendes Problem in der heutigen Zeit, das Wissenschaftler schon seit Jahrzehnten beschäftigt. Zusätzlich dazu weckte eine neue phänotypische Form der Resistenz, bei der keine Resistenzmechanismen involviert sind, das Interesse von Forschergruppen. Dieses neue Phänomen nennt sich Persistenz und trotz fehlender vererbbarer Resistenzmechanismen überleben Persisterzellen tödliche Konzentrationen von Antibiotika, dass für gewöhnlich auf langsameres Wachstum und metabolische Inaktivität zurück zu führen ist in einer kleinen Subpopulation von sonst Antibiotika empfindlichen Bakterien. Ironischerweise wurden Persisterzellen bereits kurz nach der kommerziellen Einführung von Penicillin beschrieben, jedoch größtenteils bis zu den 1980igern ignoriert. Seitdem wurden Persisterzellen als eine Gefahr für die menschliche Gesundheit anerkannt, die wiederkehrende Infektionen auslösen und das Risiko von Resistenzbildung erhöhen.

Das Ziel dieses Projektes war es, ein tieferes Verständnis über den Energiehaushalt und metabolischen Zustand von Persisterzellen zu bekommen. Dazu fokussierte sich dieses Projekt auf *Salmonella enterica* subsp*. enterica* serovar Typhimurium (*S*. Typhimurium), ein fakultativ anaerobes Pathogen. Daher wurde das Operon, dass für die ATP-Synthase codiert, ein mikroskopischer Generator für ATP und Teil der bakteriellen Atmung, deletiert. Auf diese Weise wurde die intrazelluläre ATP-Konzentration um bis zu 50 % reduziert. Anschließend wurden die Mutante und der Wildtyp, während der exponentiellen Wachstumsphase in lysogener Brühe (LB), mit Ciprofloxacin behandelt. Überraschenderweise war die Mutante trotz reduzierter ATP-Konzentration sensibler und die Persisterzellbildung war gestört. Weitere Untersuchungen fokussierten sich auf den metabolischen Zustand, wobei sich rausstellte, dass die Mutante erhöhte Mengen an Nicotinamidadenindinukleotid (NADH) und reaktiven Sauerstoffradikalen (ROS) produzierte. Die *atp* Operon-Mutante benötigt Substrat-Levelphsophorylierung, um ATP zu generieren, während der Wildtyp auch zusätzlich die Zellatmung zur Energieproduktion nutzen kann. Die Abhängigkeit der Mutante von der Glykolyse resultierte in der erhöhten Bildung von NADH, das wiederum enzymatische Reaktionen, sowie die respiratorische Zellatmung, erhöht. Als Nebeneffekt der erhöhten Aktivität werden gehäuft reaktive Sauerstoffradikale gebildet, insbesondere Hydroxylradikale, die zusätzliche DNA-Schäden verursachen. Bei Sauerstoffentzug verschwindet jedoch der

Unterschied zwischen Wildtyp und Mutante, ähnlich wie unter aeroben Bedingungen mit ROS-Quenchers. Das führt zum folgenden Model: die Deletion der ATP-Synthase zwingt die Mutante, via Glykolyse ATP zu generieren, dass zur erhöhter Bildung von NADH und metabolische Aktivität sowie der Bildung von reaktiven Sauerstoffradikalen führt. Die zusätzlichen DNA-Schäden, die durch Sauerstoffradikale und Ciprofloxacin verursacht werden, führen schließlich zur einem effizienteren Absterben der Mutante.

Neben ATP, wird die Persisterzellbildung durch viele weitere Faktoren beeinflusst, unter anderem durch endogene Phagen, wie wurde jedoch noch nicht gänzlich geklärt. Daher beschäftigt sich das zweite Ziel dieses Projektes mit der Untersuchung von endogenen Phagen und dem Einfluss auf die Persisterzellbildung nach Ciprofloxacinzugabe. Dazu wurden vier Phagen, die als Gifsy-1, Gifsy-2, Gifsy-3 und ST64B bezeichnet werden, in *Salmonella* deletiert. Die phagenfreie Variante war weniger empfindlich gegen Ciprofloxacin, was sich durch langsameres Absterben und mehr Persisterzellen wiederspiegelte. Außerdem war das Wachstum des phagenfreien Stammes im nährstoffreichem Medium gestört, was darauf hindeutete, dass diese Phagen eine wichtige Rolle beim Wachstum spielten. Allerdings war das langsamere Wachstum nicht für die erhöhte Bildung von Persisterzellen verantwortlich. Es stellte sich heraus, dass Gifsy-1 bei Ciprofloxacinzugabe besonders stark induziert wurde und das die Deletion der Lysisgene *S*, *R* und *Rz*, die von Gifsy-1 codiert werden, das Überleben erhöhten. Der Beitrag der übrigen Phagen war allerdings vernachlässigbar.

6 General introduction

6.1 *Salmonella enterica* subsp. *enterica* serovar Typhimurium

Salmonella is a rod-shaped Gram-negative facultative anaerobic bacterium, belonging to the family of *Enterobacteriaceae* [1] (Fig. 1). *Salmonella* is classified into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into 6 subspecies and into over 2500 different serovars in which *Salmonella enterica* subspecies *enterica* is the most important clinically relevant subspecies accounting for 99 % of the infections in humans and animals by causing gastroenteritis, enteric fever, bacteraemia and a chronic carrier state [1-4]. Among the subspecies *enterica*, the serovar Typhimurium is the second most common isolated serovar, accounting for approximately 12 % of the infections worldwide [1].

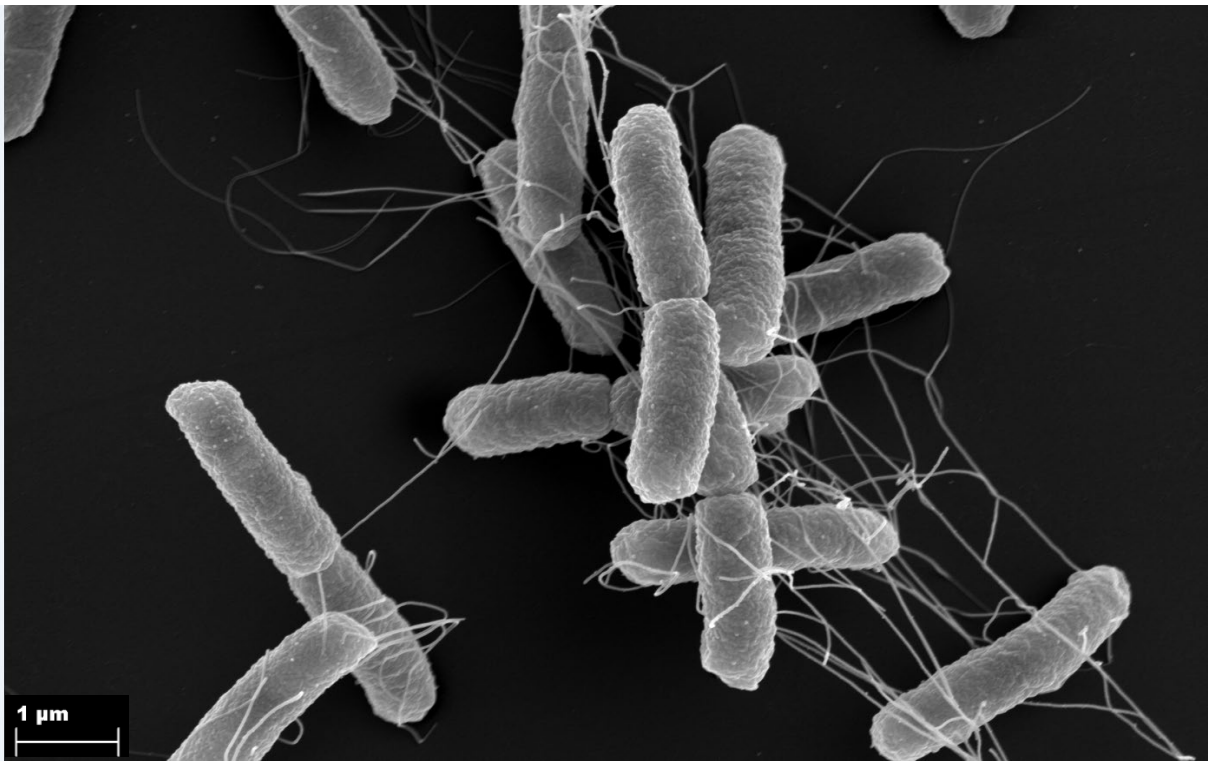

Fig. 1: Electron-microscopical image of *Salmonella enterica* serovar *enterica* Typhimurium. The bacteria were incubated to the exponential phase while shaking in nutrient rich medium. Subsequently, *Salmonella* was centrifuged and fixed with glutaraldehyde and formaldehyde before visualization using an electron microscope. Magnitude = 20000 x; Voltage = 5.00 kV; Working Distance = 2.0 mm. Pictures were kindly provided by Dr. Manfred Rohde.

Salmonella is frequently found in uncooked poultry, eggs or dairy products, but also in fresh fruits and vegetables [5-7]. Gastroenteritis, caused by consumption of contaminated food, is

characterized by nausea, vomiting, diarrhea as well as fever, and is among the most common gastrointestinal infections in Europe [8]. The symptoms are usually self-limiting, but can also be life-threating for infants, the elderly, and immunocompromised patients [1]. In rare cases, infection with *Salmonella* can also lead to hepatomegaly and splenomegaly, cholecystitis, pancreatitis as well as appendicitis [1, 9]. Antibiotic treatment is only recommended during severe infectious diseases because the usage of antibiotics during uncomplicated gastroenteritis increases the risk of relapsing infections with *Salmonella*, presumably due to the killing of protective bacteria of the intestinal flora [9, 10]. Moreover, the emergence of multi-drug resistant (MDR) *Salmonella* against chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole exacerbates the treatment of acute salmonellosis with antibiotics, limiting the choice of antibiotics for treatment. Therefore, quinolones, such as ciprofloxacin, have become the first choice to treat infections with MDR *Salmonella* [11, 12]. However, a number of quinolone resistant *Salmonella spp.* have already been reported in food-producing animals [13]. High resistance to the quinolones nalidixic acid and ciprofloxacin has been frequently observed among broilers with an occurrence of 51.8 % for nalidixic acid-resistant strains and 48.8 % for ciprofloxacin-resistant strains in livestock within the European Union. In turkeys 42.7 % of the isolates were nalidixic acid-resistant while 33.7 % were ciprofloxacin-resistant [13]. Nevertheless, treatment of fluoroquinolone-sensitive *Salmonella* does not automatically result in the eradication of all bacteria. Instead, a small fraction of bacteria are able to persist while exposed to ciprofloxacin [14]. These bacterial survivors are designated as persister cells, a small subpopulation of drug tolerant bacteria, which are genetically identical to the drug sensitive bulk of bacteria but nevertheless manage to survive antibiotic exposure. These surviving persister cells can re-establish a new infection, through persistent *S*. Typhimurium residing in mesenteric lymph nodes within macrophages, and can further promote bacterial dissemination by fecal shedding [15, 16]. Interestingly, internalization of *S*. Typhimurium into macrophages increases the drug tolerance due to the activation of stress responses triggered by starvation and vacuole acidification, resulting in reduced cell division and dormancy [17]. Furthermore, these persister cells have the potential to further spread or develop resistances to diverse antibiotics [18-20]. For example, it was demonstrated that treatment with ciprofloxacin resulted in survival of a small fraction of bacteria in the host tissues of mice, which were able to migrate back into the gut lumen after cessation of drug treatment, in order to transfer extended-spectrum β-lactamase encoding plasmids to non-related bacteria [18].

Another important factor for drug-persistence is the involvement of bacterial DNA repair mechanisms, which are activated during the so-called SOS response, because it allows a small subpopulation of bacteria to survive the treatment with DNA damaging agents and thus enhances the risk of drug-resistance [19].

7 Antibiotic resistance

7.1 A global threat

The first official antibiotic was named Penicillin because it was extracted from the mould *Penicillium* by Alexander Flemming after observing its inhibitory effect on *Staphylococcus aureus* [40]. As soon as they were commercially available, antibacterial drugs were the first choice to counteract bacterial infections. Penicillin was already broadly administered during World War II shortly after its introduction in 1943 [41]. Undoubtedly, the usage of antibiotics has saved millions of lives up until the present day and has extended life spans as well as health-related quality of life. However, the increasing emergence of antibiotic resistant bacteria have become a threat for humans as a result of overuse and lack of new drug development [42]. Already in the 1940s, Penicillin resistant Staphylococci strains were discovered after isolation from patients suffering from infectious lesions [43]. Therefore, the following decades brought out new antibacterial drugs to keep pace with the evolving pathogens, which is however concomitant with new drug resistance developments. Contributing to this situation is the poor control of antibiotic use in some countries, where many antibiotics can be obtained without prescription or regulation [44]. Furthermore, the usually low costs and copious availability of standard antibiotics promotes overuse as well, resulting in thoughtless administration of antibiotics in livestock and in the medical sector. For example, a study in Germany revealed that in many cases prescribed antibiotics were unnecessary, and were prescribed without respecting the indications or national guidelines [45]. It should also be considered that each treatment with antibiotics raises the risk of resistance development due to an increased drug-induced mutation rate [19]. Unfortunately, bacteria are constantly exposed to high and low amounts of antibiotics, and the usage of antibiotics as food supplements in livestock to prevent diseases and to preserve food, results in residual amounts of antibiotics accumulating in meat, which are consumed and digested by humans [46-48]. Furthermore, it has been reported that sub-lethal concentrations of antibiotics induce the formation of ROS, which in turn increase the mutagenesis, presumably

via the SOS response, and minimum inhibitory concentration (MIC) of antibiotics [49]. For example, constant exposure to low amounts of β-lactam antibiotics resulted in an increased MIC towards norfloxacin [49], a structurally related fluoroquinolone of ciprofloxacin. This could be problematic because ciprofloxacin is one of the most prescribed antibiotics in Germany and, as mentioned above, is also used to treat salmonellosis [45, 50]. Furthermore, it was reported that increased resistance to ciprofloxacin (marked by an increased MIC when compared to a susceptible strain) also resulted in more persister cells when treated with four-fold the MIC of ciprofloxacin, demonstrating a positive correlation between resistance and persistence [51]. This might be relevant under clinical conditions when incomplete eradication of persister cells further increases drug tolerance or resistance, in particular when taking into account that fluoroquinolones, such as ciprofloxacin, have high mutagenic activity [52-54].

8 Persistence, tolerance and heteroresistance - a phenotype of classical resistance

8.1 Drug persistence

8.1.1 General definition

Exposing bacteria to a lethal concentration of antibiotics will rapidly kill all drug susceptible bacteria until only a few bacterial survivors remain, which are genetically identical to the previously killed clones. These remaining persistent bacteria and are generally regarded as slow growing or dormant cells with a low metabolic activity [20]. This phenomenon has been described for a wide range of bacterial species [55-57], for eukaryotic pathogens [58] and even for multicellular protozoa [59]. Persister cells were originally described shortly after the discovery of penicillin by the Irish physician Jospeh Bigger in 1944, who observed that a culture of *S. aureus* was not fully sterilized by penicillin [57]. The survival was not attributed to the emergence of classical resistance because re-cultivation of the bacterial survivors and subsequent treatment with penicillin resulted in the same killing kinetics as for the original population. Therefore, the phenomenon of persistence is non-inheritable. Rather, survival and persister cell formation upon drug exposure is believed to be conferred by a slow- or non-growing subpopulation [60, 61]. Furthermore, persistence is relevant under clinical conditions because persister cells have been linked to treatment failure and for promoting resistance mutations [23, 62].

8.1.2 Clinical relevance of persister cells

As noted above, bacterial persister cells have been attributed to treatment failure and recurrent chronic infections [15]. This phenomenon has been long overlooked because routine clinical susceptibility assays concentrate on the bulk of bacterial populations, whereas the smaller and drug-tolerant persister cells are ignored. In 2006, using mathematical models and computer simulation, it had been foreshadowed that non-inherited antibiotic resistance would prolong antibiotic therapy, causing treatment failure [63]. A prominent example for a chronic infection caused by drug tolerant persister cells are infections with *Pseudomonas aeruginosa* in cystic fibrosis patients. Although cystic fibrosis leads to severe malfunction of the respiratory organs, owing to an imbalance in chloride and sodium levels of the corresponding epithelial cells [64], most of the patients eventually die from persistent infection with *P. aeruginosa* due to incomplete eradication after antibiotic therapy [65]. Interestingly, treatment failure due to persistent pathogens are not restricted to bacterial infections, hard-to-treat infections were also described for eukaryotic pathogens, such as *Candida albicans* [15]. Treatment of *C. albicans* with chlorhexidine failed to eradicate oral *C. albicans* in cancer patients, even leading to increased drug persistence after intermittent treatment [66]. Usually, antibiotic persistent bacteria, which have escaped killing by antibacterial agents, are eliminated by the immune system. However, pathogens have also evolved mechanisms to circumvent the problem by modulating the host immune system and to avoid an inflammatory response. For example, *Mycobacterium tuberculosis* interferes with interferon-γ response by inducing the formation of interleukin-10 in macrophages [67]. Furthermore, *S.* Typhimurium, once internalized by macrophages, evades its digestion in the phagosomes and reduces its growth due to intracellular vacuole acidification and nutrient deprivation, leading to increased drug tolerance [17]. This could also explain the recalcitrant nature of non- typhoidal infections in humans and animals [68].

8.1.3 Persister cells – resistance development and eradication

Persister cells are not only responsible for recalcitrant infections, but can also serve as a source of antibiotic resistance and genetic diversity [23, 69, 70]. Persister cells support the evolution of resistance by increasing the likelihood of survival and fixation of beneficial mutations in a bacterial population. For example, DNA damage, induced by the fluoroquinolone ofloxacin, resulted in the activation of the SOS response and an increased mutation rate in the remaining persister cells, which in turn gave rise to antibiotic resistant mutants [19]. Indeed, it is more

likely that increased tolerance precedes resistance as a second mutational event when drug tolerance formation is already established [23]. Therefore, it is important to eradicate all bacteria during antibiotic therapy to minimize the emergence of resistant pathogens. One way to achieve this goal is by inhibition of the SOS response to decrease the mutation rate, for example by using N-acetylcysteine to quench the formation of ROS, which otherwise would damage the DNA [71]. Another possibility is the complete eradication of persister cells by providing additional carbon sources to stimulate the metabolism of pathogens [72, 73]. Supplementation with glucose augments killing efficiency of *S. aureus* with daptomycin and *E. coli* with aminoglycosides because glucose triggers the metabolism, thereby increasing bacterial activity. Furthermore, glucose usage increases the membrane potential and facilitates the uptake of aminoglycosides, demonstrating the importance of the aerobic respiration in the context of persister cell formation [73], which is why the aerobic respiration will be discussed in more detail in the next section. However, supplementation with metabolites, which can only be utilized by bacteria, may not be sufficient for effective killing because providing oxygen for respiratory activity is as important as the carbon source itself [74]. This makes the aerobic respiration of bacteria a promising target to increase antibiotic efficiency, which in turn could decrease persister cell formation. Therefore, it is crucial to understand the mechanism of respiration, which is also known as electron transport chain.

8.1.4 Aerobic respiration and ATP synthesis

The enzymes involved in aerobic respiration are located in the inner membrane of bacteria and comprise dehydrogenases, reductases and oxidases, which are connected by quinones [75]. The alternative name of the aerobic respiration is the electron transport chain (ETC) because electrons are transferred from one complex to the next while protons (H^+) are pumped out into the periplasm, generating a membrane potential (Fig. 2). Eventually, this proton gradient is used by the ATP synthase to convert adenine dinucleotide phosphate (ADP) into ATP [76]. NADH dehydrogenase I, encoded by *nuo* genes (also NADH:ubiquinone oxidoreductase I (NDH-1)), is the first enzyme complex of the ETC, consisting of 14 subunits with diverse prosthetic cofactors, such as molybdenum, nickel, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), iron–sulfur cluster (Fe-S clusters), pyrroloquinoline (PQQ) and heme b, c, d or o. Alternatively, NDH-1 can be replaced by NDH-2 (NADH:ubiquinone oxidoreductase II), which is encoded by the *ndh* gene. NDH-1 oxidizes NADH to NAD^+ by transferring two electrons via FMN and the Fe-S cluster on ubiquinone while

extruding four protons into the periplasm [77, 78]. Subsequently, NAD^+ is regenerated either in the tricarboxylic acid cycle (TCA cycle) or during glycolysis. In contrast to NDH-1, the alternative dehydrogenase NDH-2 has no proton pumping function. The reduced ubiquinone transmits both electrons on either cytochrome *bd* or cytochrome *bo* while releasing two protons into the periplasm. The composition of the ECT varies under different circumstances, so bacteria can adapt their respiratory activity to various environmental conditions and oxygen availability.

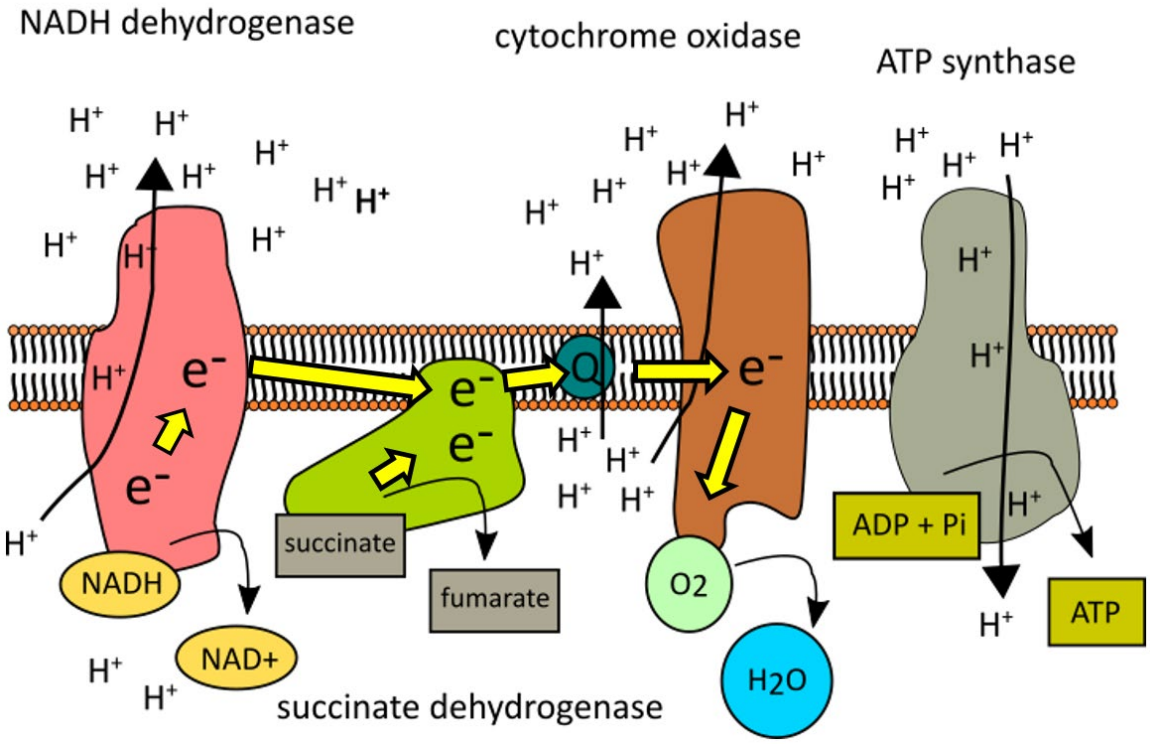

Fig. 2: Membrane located electron transport chain of bacteria. Bacterial respiration, also known as electron transport chain, is used for ATP synthesis by exploiting the proton gradient between cytosol and periplasm. NADH transfers one electron (e^-) on the first complex (NADH dehydrogenase) of the electron transport chain. This electron is further transferred to succinate dehydrogenase and via ubiquinone (Q) and the terminal cytochrome oxidase on oxygen to form water. During the process of electron transfer, protons are pumped out of the cytosol into the periplasm to build up a membrane potential, which is used by the ATP synthase to generate ATP. Succinate dehydrogenase uses succinate as electron donor instead of NADH and is not involved in proton expulsion. The yellow arrows show the trajectory of the electrons. Adapted from Szabo et al. (2020) [79].

For example, cytochrome *bd* is preferentially expressed under oxygen-limiting conditions while cytochrome *bo* is primarily used under aerobic conditions [80, 81]. Cytochrome *bo*

contributes to the membrane potential by pumping out two protons while cytochrome *bd* has no such function. Under aerobic conditions, the electrons are transferred on oxygen as terminal electron acceptor to form water. The generated membrane potential is ultimately used by the ATP synthase to synthesize ATP in which approximately three protons are necessary to obtain one ATP [82]. Succinate dehydrogenase (SDH/Complex-II) is also a part of the ECT as well as part of the TCA cycle; however, the electron donor is not NADH, as in the case of NDH-1 or NDH-2, but succinate. Electrons are imported into the ubiquinone pool by oxidizing succinate to fumarate, which are transferred via cytochrome to oxygen, thus contributing to the membrane potential as well [83]. Despite efficient ATP generation by respiration, the formation of cell damaging ROS is possible when electrons are accidentally transferred to oxygen during respiration, resulting in either superoxide or hydrogen peroxide [84, 85]. Therefore, bacteria have evolved counter measures to degrade ROS, including catalases/peroxidases and superoxidedismutases [86]. Nevertheless, in rare cases, extremely reactive hydroxyl radicals are formed via the Fenton reaction, with a high potential to damage the DNA [87]. As a consequence, cellular respiration as well as glycolysis are important targets to reduce persister cell formation because stimulating the respiratory/glycolytic activity with additional carbon sources, or supplementation with terminal electron acceptors, promotes oxidative stress and killing of bacteria upon antibiotic exposure [73, 74, 88-90]. In contrast, interfering with the TCA cycle or glycolysis, which supplies the ETC with NADH and nicotinamide adenine dinucleotide phosphate (NADPH), increases survival by affecting the respiration, bacterial growth, membrane potential and ATP concentration [91-94]. Low ATP levels were suggested to be particularly important because a reduced ATP concentration leads to low target activity of fluoroquinolones, aminoglycosides and β-lactam antibiotics with concomitant increased persister cell formation, while a high ATP level has the opposite effect of promoting antibiotic susceptibility [95-97]. However, during these studies, the ATP concentration was artificially reduced in *E. coli* and in *S. aureus*, using sodium arsenate, which binds ADP and interrupts the regeneration of ADP to ATP [95, 96, 98]. In a more direct approach, when the operon of the ATP-synthase was deleted in *S.* Typhimurium, the resulting reduced ATP pools did not increase persister cell formation upon fluoroquinolone exposure [99, 100]. In contrast, mutation of the ATP synthase, which interferes with ATP synthesis, resulted in disturbed persister cell formation in *E. coli* as well as *Salmonella*, presumably due to increased ROS formation [88, 99-101]. This renders the energy household of bacteria an

attractive target for future drug development. However, pathogens have also evolved countermeasures, so-called toxin-antitoxin modules, to interfere with metabolic and respiratory activity to increase survival during stress conditions, for example during exposure to antibiotics [102] (see Section **8.1.7**). Nevertheless, when induction of toxin-antitoxin modules is insufficient to avoid DNA damage, bacteria possess the SOS stress response to repair drug-induced DNA lesions.

8.1.5 SOS response induced persister cell formation

The SOS repair system is a bacterial stress response to adverse environmental conditions triggered by DNA damage and generates genetic variability as a result [21]. This genetic variability may increase drug tolerance as well as resistance against diverse classes of antibiotics [19, 22, 23]. *S*. Typhimurium also possess an SOS response, which is activated upon DNA damage, leading to the induction of at least 48 loci [24]. The DNA damaging agent ciprofloxacin, as well as other fluoroquinolones, is a potent inducer of DNA repair mechanisms [25]. The trajectory of the SOS response is well described for *Escherichia coli* and there are high similarities between *S*. Typhimurium and *E. coli* [26-28]. Upon DNA damage, the expression of several genes is induced, which are involved in homologous recombination, nucleotide excision repair or translesion synthesis [28]. Treatment with DNA damaging agents cause DNA double strand breaks, generating blunt ends [132], which are degraded into single stranded DNA by exonuclease RecBCD, enabling the binding of RecA, the activator of the SOS response, to the DNA [133]. The accumulation of RecA at the DNA results in the proteolytic self-cleavage of the SOS-response repressor LexA, leading to derepression of genes with a LexA-binding box [29, 30]. Furthermore, the recruitment of RecA to the DNA initiates homologous recombination between two chromosomes with identical DNA sequences, therefore allowing mutation-free repair. During this process, the so-called Holliday junction is formed, an important intermediate step of homolog recombination, which is catalysed by the enzymes RuvABC and RecG, ultimately resulting in two recombinant DNA strands [31]. In addition, among the derepressed genes is the *tisB* gene, encoding a toxin which has been suggested to reduce the expression of SOS genes and which is also connected to drug tolerance [32, 33]. Alternatively to homolog recombination, the nucleotide excision repair pathway detects nicks in the DNA preferentially caused by ultra violet radiation, which is repaired by the endonuclease UvrABC [34]. UvrABC recognizes lesions in the DNA strand and removes the damaged DNA patch via its endonuclease and helicase activities [35].

Subsequently, DNA polymerase I adds the complementary DNA bases [28]. The aforementioned repair strategies usually do not result in mutations. However, if the SOS response lingers due to persistent DNA damage, additional, alternative error-prone polymerases are activated, therefore occasionally introducing the wrong base. Polymerase V, encoded by *umuDC*, is such an alternative polymerase, which increases the mutation rate in response to persistent SOS response [36]. SOS response regulated DNA polymerases are able to synthesise DNA, despite DNA lesions, because of the missing proof-reading capability, whereas the highly reliable polymerase III, which is active under normal conditions, is incapable of replicating a DNA strand when encountering DNA lesions or gaps due to its proof-reading function [37]. Therefore, the SOS response increases the mutation rate and the evolution of drug resistance [19, 38, 39]. The DNA repair mechanism is also indispensable for survival upon fluoroquinolone exposure and for persister cell formation, either in biofilms or planktonic cultures [38, 39, 129]. In fact, not all genes regulated by RecA and LexA contribute equally to the formation of persister cells. The most important genes in terms of persister cell formation are *uvrD*, *dinG* and *ruvAB* [38]. UvrD possess a helicase activity and is important for dismantling RecA from the DNA, allowing proper repair of the damaged DNA [38, 134-136]. DinG enables replication re-start by removing R-loops [137] and RuvAB catalyzes the final step of homolog recombination by resolving the Holliday junction [28, 138]. It has been demonstrated that DNA damage in both persister cells and drug susceptible bacteria are comparable and that the timing of repair after the removal of fluoroquinolones was crucial, otherwise cell division before DNA repair is accomplished would result in cell death [139, 140]. In addition to the induction of the DNA repair genes, the toxin gene *tisB*, which encodes for a small membrane protein, is expressed after derepression of LexA, promoting persister cell formation by corrupting ATP synthesis [33, 122, 141]. TisB has an exceptional position among toxins because it is not only involved in persister cell formation, but also in the regulation of gene expression of the SOS response [32]. Once activated, TisB forms small selective pores in the inner bacterial membrane, causing proton leakage and interferes with ATP formation by the ATP synthase [142]. Low ATP results in reduced target activity of the antibiotics, leading to more persister cells as well as inhibits an over-initiation of the SOS response [32, 95, 96]. Furthermore, overexpression of TisB interfered with the induction of the phage λ, implying that TisB may control phage induction as well [32]. Interestingly, a homolog of *tisB* is also located in the chromosome of all *Salmonella* species [104].

8.1.6 Persistence and prophages

Salmonella harbours a number of endogenous prophages integrated into its chromosome, which also significantly contribute to its pathogenicity [143]. However, the contribution of phages regarding drug persistence in *Salmonella* is so far completely unknown. *Salmonella* Typhimurium serovars can potentially harbour six or more different phages in its chromosome, which are designated as Fels-1, Fels-2, Gifsy-1, Gifsy-2, Gifsy-3, and ST64B [144-146]. The wild type strain ATCC 14028s, which was used in this study, contains at least four inducible phages in its chromosome, namely Gifsy-1, Gifsy-2, Gifsy-3 and ST64B [144-146]. Normally, these phages remain dormant as lysogens in the bacterial chromosomes; however, exposing *S*. Typhimurium to Mitomycin C, a DNA damaging agent, causes the switch from the lysogenic cycle to the host killing lytic cycle [147, 148].

8.1.6.1 The lysogenic/lytic cycle

There are two general pathways which allow phages to multiply within their hosts, either by integrating the viral DNA into the host`s chromosome or by exploiting the hosts metabolism to generate new viral progenies with subsequent lysis of the host shortly after infection [149]. The latter cycle is designated as the lytic cycle, whereas the first pathway is referred to as the lysogenic cycle, which allows phages to remain over multiple generations within their host without causing lysis (Fig. 4). Phages are termed as temperate phages if phages remain integrated within their host`s chromosome. The four phages of ATCC 14028s (Gifsy-1, Gifsy-2, Gifsy-3 and ST64B) belong to the group of temperate phages, ergo they are part of the bacterial chromosome as long as they are not triggered by environmental stresses, such as treatment with mitomycin C or hydrogen peroxide [148, 150, 151]. Due to the nature of the inducing signal, it is clear that Gifsy-1, Gifsy-2, Gifsy-3 and ST64B are activated upon DNA damage. For the Gifsy phages, it was demonstrated that induction of the SOS response and the concomitant cleavage of LexA allows the expression of anti-repressors [148]. These anti-repressors bind the actual phage repressor, whereby the binding forces the repressor to dissociate from the DNA, thus allowing the expression of the phage genes. The SOS response contributes also to the excision of ST64B, but the exact mechanism is still elusive [147]. A crucial moment of phage activation is the lysis of the host and the dissemination of the new viral particles. To initiate the lysis of the host, the phages have to encode for proteins, which can degrade the bacterial cell wall [152]. The responsible proteins include the pore-forming protein S, an endolysin designated as R, and two auxiliary proteins, named as Rz and Rz1. So

far, all known lambdoid phages have their lysis genes arranged as in the phage λ, namely *SRRzRz1* [152]. The protein S accumulates in the inner bacterial membrane, in which pore-formation depends on the membrane potential, i.e. an energized membrane favours the formation of pores by S, whereas low potential decelerates pore-formation [153]. As soon as the pores congregate, the endolysin, encoded by *R*, diffuses through the channel into the periplasm, where it degrades the cell wall. Endolysins comprise diverse classes of enzymes, including lysozymes, transglycosylases or amidases, which target either glycosidic or peptide bonds to disrupt the peptidoglycan layer [154]. The last act of host lysis of Gram-negative bacteria involves the disintegration of the outer membrane by the auxiliary proteins Rz/Rz1, also termed as spanins [155]. The spanins fuse the inner with the outer membrane, thereby causing topological changes, which ultimately lead to the disruption of the membrane and the release of the phages.

Considering the fact that phages are induced as a result of DNA damage, it is an interesting question to ask what impact endogenous phages have on persister cell formation and survival upon antibiotic treatment.

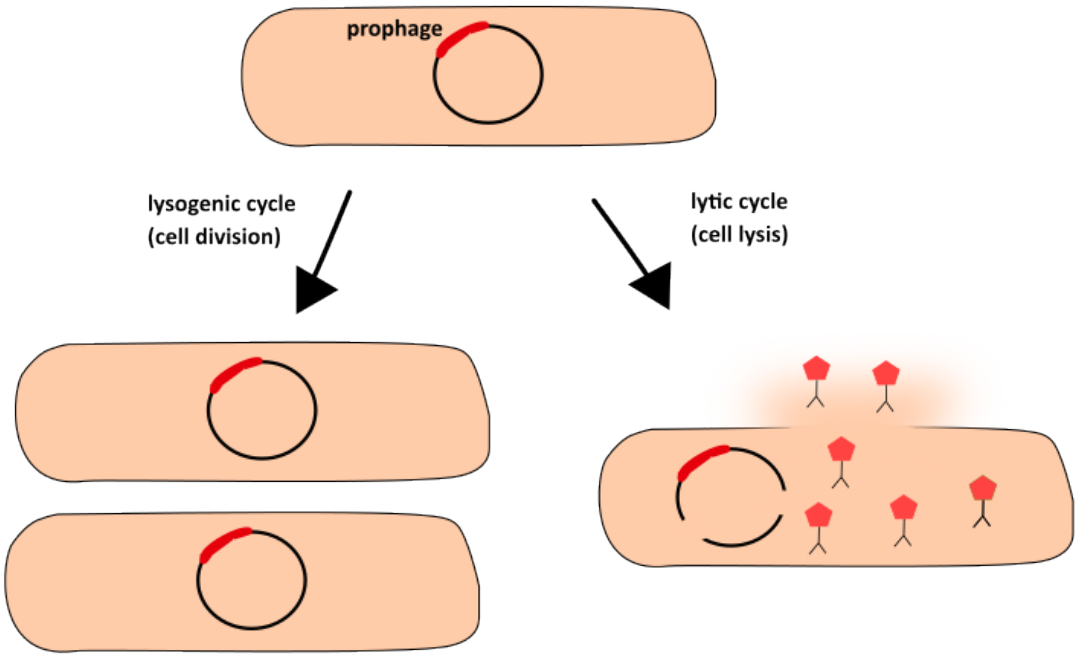

Fig. 3: Simplified model of the lysogenic and lytic cycle. Prophages are integrated into the bacterial chromosome where they remain dormant and are propagated to the next generation after cell division (lysogenic cycle). However, when bacteria encounter harsh environmental stresses, which cause the damage of the DNA, then prophages can switch from the lysogenic

cycle to the lytic cycle to escape from their dying host. To this end, prophages encode for cell wall degrading enzymes. This figure has been adapted from Zhang et al. (2022) [156].

8.1.6.2 The impact of prophages on drug persistence

For a long time, the role of phages with regard to persister cell formation has been largely overlooked, although it has been acknowledged that phages play a crucial role in bacterial evolution by shuffling genetic material between bacteria [157]. However, the correlation between endogenous phages and antibiotic tolerance has never been thoroughly investigated. So far, it has been reported that cryptic phages significantly contribute to stress tolerance in *E. coli* [158]. Cryptic phages remain in the bacterial chromosome despite induction, because they have lost their ability to induce the lytic pathway (excision and cell lysis of the host). Nevertheless, genes of these cryptic phages are expressed, leading to increased fitness of their host under certain conditions [158]. In *E. coli*, deletion of 10 cryptic phages had a great impact on the bacterial physiology by impairing the growth, the resistance against oxidative and osmotic stress, as well as decreasing persister cell formation upon exposure to β-lactam and quinolone antibiotics. In particular, the phages Rac and Qin were involved in drug tolerance, hence deletion of these phages reduced persister cell formation.

Decades ago, an apparent paradox regarding antibiotic killing of bacteria had been observed, where treatment of *E. coli* with high antibiotic concentrations yielded higher bacterial survival compared to moderate concentrations [159-162]. It was demonstrated that RNA and DNA synthesis is inhibited at high antibiotic concentrations, while moderate concentration allows metabolic activity. Hence, it is conceivable that low metabolic activity also results in low or zero induction of phages because transcription and translation are essential for lytic gene expression [154, 163]. This assumption is in agreement with the heat mediated induction of λ in *E. coli*, resulting in the lysis of the heat-sensitive bacteria, while in the smaller persistent subpopulation, which have survived heat exposure, no induction was observed [164]. Under these conditions, the survival was further increased by overexpressing the toxin HipA, which in turn significantly diminished the lysis of *E. coli* by phage λ, as a result of reduced translation. Furthermore, the antibiotic paradox was circumvented by using a derivative of an *E. coli* strain, which lacks nine cryptic phages [108]. To this end, *E. coli* and its corresponding phage-free variant were treated with various concentrations of ciprofloxacin in which survival of the *E.*

coli strain, which still carried the endogenous phages, was significantly increased at higher drug concentration, while deletion of all phages abolished this phenomenon, resulting in the same killing at low and high ciprofloxacin concentration [108]. The same effect was demonstrated for *S. aureus* in which paradoxically 1 µg/ml ciprofloxacin was more efficient than 50 µg/ml [160]. However, using tetracycline to inhibit the induction of phages at 1 µg/ml, and to prevent host lysis, resulted in comparable survival rates at low ciprofloxacin concentration and high drug concentration [160].

As described above, *S*. Typhimurium (ATTC 14028s) contains at least four phages in its chromosome, which are all inducible [144, 146, 150]. All four phages are either under the control of RecA or LexA, which are both involved in the regulation of the SOS response system [147, 148]. The contribution of these phages to antibiotic tolerance and persister cell formation is unknown so far. It was only reported that all phages carry virulence genes, which selectively play an important role during infection in mice [146, 165]. Thus, one major aim of this study was to investigate the impact of the phages during antibiotic treatment in our *Salmonella* strain, which will be further discussed in the "Discussion" section.

8.1.7 Toxin-antitoxin module

One crucial factor is the involvement of toxin-antitoxin modules, which has always been connected to persister cell formation. These modules consist of a toxin and its respective inhibiting antitoxin (Fig. 3). Upon stress, the antitoxin is degraded and the toxin is released into the cytosol where it can inhibit important cellular functions, such as interfering with translation [103]. Apparently, there is a rough correlation between pathogenicity and the amount of toxin-antitoxin modules, for example *S.* Typhimurium contains at least 28 toxin-antitoxin modules, whereas non-pathogenic *S. bongori* has only 10 [104]. The same phenomenon is observed for *M. tuberculosis*, *Yersinia pestis*, *Streptococcus pneumonia* and other pathogens compared to their non-pathogenic counterparts [105]. Toxins are usually deactivated as long as bacteria are growing under favourable conditions, whereas adverse situations induce the degradation of the respective antitoxins, for example during starvation. The following model has been suggested for *E. coli*, which leads supposedly to the degradation of the antitoxins during nutrient limitation: starvation causes the accumulation of the alarmone guanosine tetraphosphate (ppGpp), synthesized by SpoT or RelA, which leads to an increased intracellular polyphosphate concentration [106].

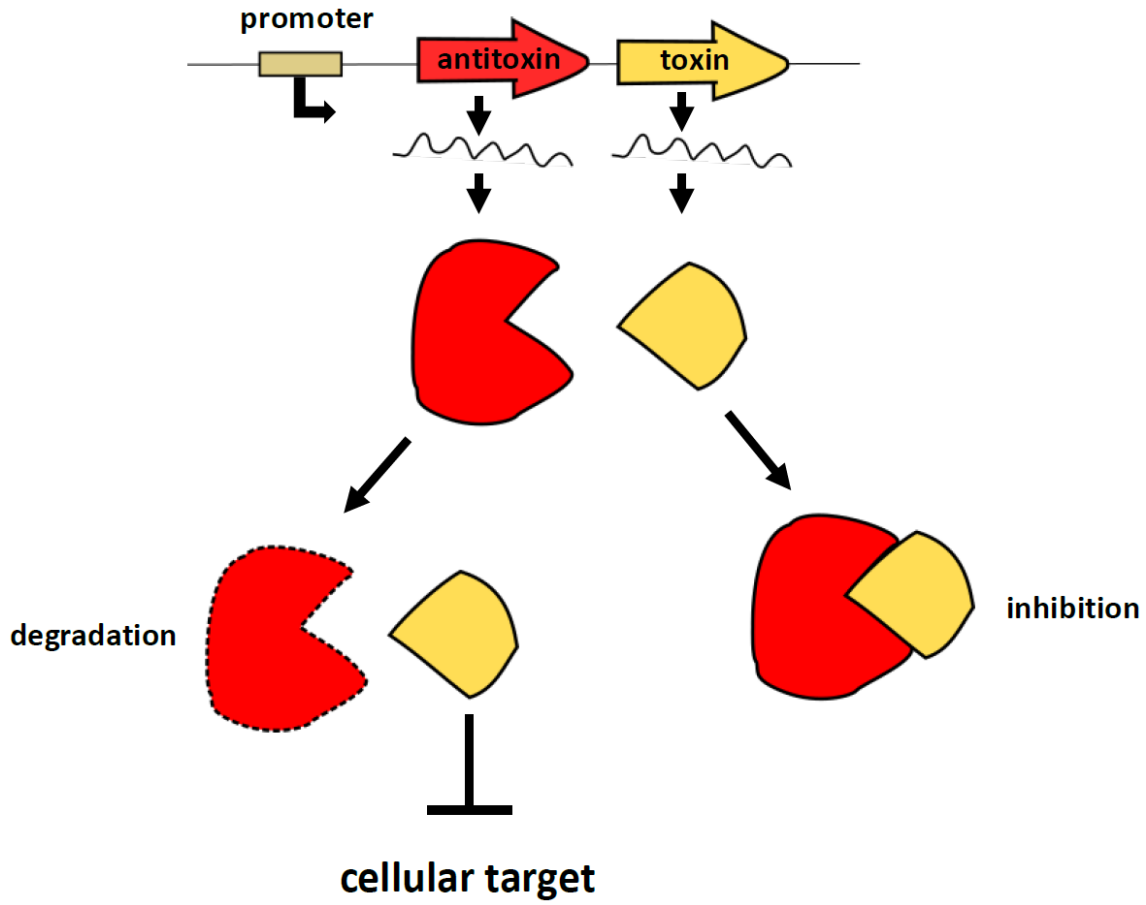

Fig. 4: Simplified illustration of an antitoxin-toxin module. An antitoxin-toxin module consists of an unstable antitoxin and a comparatively stable toxin. Usually, both are co-transcribed whereby under normal conditions, the antitoxin inhibits its toxin as long as it is not degraded by stress induced proteases [127]. However, when the toxin is released from its antitoxin, it can inhibit important cellular functions, such as replication, translation or ATP synthesis. Adapted from Wang and Wood (2011) [128].

Polyphosphate stimulates the degradation of the antitoxin by the protease Lon, releasing the toxin (endoribonuclease) into the cytoplasm [106]. However, this model was later retracted because one substrate of Lon is SulA, a cell division inhibitor [107]. Therefore, deletion of only *lon* resulted in decreased persister cell formation because cell division is inhibited by SulA, leading to less colonies on plates, whereas deletion of both genes restored the wild type phenotype [38]. Furthermore, deletion of all endoribonuclease toxins in *E. coli*, which were, according to Maisonneuve and colleagues, responsible for persister cell formation upon induction [106], did not bias persister cell formation, in the case where prophages were additionally deleted [108]. Nevertheless, toxin-antitoxin modules still play an important role in persister cell formation by modifying the cell physiology. The first toxin which was

associated with increased persister cell formation in *E. coli*, was the toxin HipA (high persistence) [109]. HipA phosphorylates a serine residue near the centre of glutamyl-tRNA synthetase (GltX), inhibiting aminoacylation and efficient protein synthesis [110]. Uncharged tRNAs trigger the formation of (p)ppGp via SpoT and RelA, leading to the activation of the transcription regulator DksA, which inhibits the RNA-polymerase activity and DNA supercoiling, ultimately resulting in growth arrest and increased persistence [111, 112]. The artificial inhibition of translation promotes persister cell formation upon ciprofloxacin and ampicillin treatment as well [113]. The final effect of HipA activity is the degradation of rRNAs and tRNAs, leaving only a small fraction of ribosomes intact [114]. The reduced translation, due to low active ribosomes, would ultimately result in a decreased glycolytic as well as respiratory activity, which is beneficial for persister cell formation during antibiotic treatment, as described above. There are a variety of toxin-antitoxin modules, which interfere with translation, depolarization of the membrane and ATP synthesis, ribosome assembly, regulation of outer membrane proteins, indole formation, membrane integrity and inhibition of programmed cell death [33, 115-123]. It has been suggested that toxins confer persistence when toxins exceed a certain threshold and that the duration of dormancy depends on how high this threshold is exceeded by the toxins [124]. Therefore, another basic factor for persister cell formation is heterogeneity because even in an isogenic bacterial population, gene expression can vary extensively [125]. With regard to antitoxin-toxin modules, this implies that expression of antitoxins and toxins can differ enormously between genetically identical strains, whereby only a few bacteria reach the optimum amount of toxins to become dormant and persistent. This phenotypic diversity of bacteria ensures the survival of at least a small subpopulation in suddenly changing conditions. This mechanism is designated as bet-hedging and is the result of stochastic expression of genes [126]. However, the heterogeneous induction alone of antitoxin-toxin modules are not sufficient for survival, which is why further stress responses are of significant importance to survive antibiotic exposure, such as the bacterial SOS response [38, 39].

8.1.8 Resuscitation of persister cells

Dormancy contributes to persister cell survival from antibiotic mediated death. Nevertheless, persister cells have to resuscitate in order to establish a new infection. How persister cells re-awake was and is still a matter of debate. Toxin-antitoxin modules were discussed to be responsible for increased drug tolerance, so it has been proposed that the inhibition of the

toxin allows resumption of metabolic activity after antibiotic treatment is ceased [119, 166]. *S.* Typhimurium contains the toxin TacT, an acetyltransferase, which acetylates tRNAs to block translation [119, 167]. By detoxifying TacT-modified tRNAs, using the peptidyl-tRNA hydrolase, *S.* Typhimurium re-activates translation and resumes growth [119]. The toxin HokB forms pores, which are embedded into the inner bacterial membrane to cause proton leakage and to inhibit the proton-dependent ATP synthesis [168]. To reverse the proton influx and to re-build the membrane potential for ATP generation, HokB is disintegrated into monomers by the periplasmic oxidoreductase DsbC and further degraded by the protease DegQ, allowing the bacteria to resuscitate [166]. However, increasing evidence indicated that ribosomes are involved in persister cell formation and resuscitation [169, 170]. Under nutrient deprivation, the levels of both cyclic adenosine monophosphate (cAMP) and ppGpp raise, resulting in reduced production of ribosomes [171, 172]. ppGpp binds and inhibits diverse GTPases, such as HflX, which is involved in the re-activation of deactivated 100S ribosomes or Era, which promotes synthesis of intact 30S ribosomes [173, 174]. Furthermore, ppGpp induces the expression of ribosome modulation factor (RMF), the hibernation promoting factor (HPF) and the ribosome-associated inhibitor (RaiA) [169, 172]. HPF stabilizes and promotes the formation of inactive 100S ribosomes by RMF while RaiA stabilizes the inactive form of 70S ribosomes, leading to reduced translational activity [172, 175-177]. Hence, it has been suggested that persister cell formation is independent of toxins and that ppGpp induces persistence via the activation of RMF, HPF and RaiA, leading to increased accumulation of inactive S100 ribosomes in persister cells [178]. In addition, an elevated cAMP concentration is also involved in the induction of RMF and RaiA as well as in the repression of HflX [178]. Therefore, persister cell resuscitation is linked to the activation of the ribosomes in which membrane located receptors are involved in the detection of external nutrients if available. The availability of nutrients is propagated into the cytosol, leading to decreased concentration of cAMP and ppGpp, thus stimulating the correct assembly of ribosomes [179, 180]. When the concentration of cAMP and ppGpp drops, the repression of HflX is abolished, leading to the re-activation of intact 100S ribosomes and the induction of chemotaxis genes in order to move the bacteria towards available nutrients [180]. Because the ribosomal content of persister cells varies, the re-awaking of dormant cells is heterogeneous, where higher ribosome levels result in faster resuscitation [181].

8.2 Antibiotic tolerance

8.2.1 General definition of drug tolerance

Tolerance is described as the transient ability of the whole bacterial population (not a subpopulation as for persister cells) to survive lethal concentrations of antibacterial agents due to growth arrest without involvement of resistance mechanisms [182]. Killing of tolerant bacteria requires prolonged exposure to antibiotics to achieve the same killing as for drug susceptible bacteria [183]. However, determination of tolerance by measuring the MIC is insufficient because the MIC of tolerant bacteria is the same as in susceptible strains. Therefore, time killing curves to determine the killing kinetics are a better choice to reveal a tolerant population. In addition, the extended time which is necessary to eradicate a proportion of a bacterial population, is dictated as the minimum duration for killing (MDK) and is far more suitable to characterize a tolerant population [22, 183]. Two forms of tolerance are distinguished, designated as tolerance by “slow growth” and “tolerance by lag” [183]. In both cases, the MDK is higher when compared to susceptible strains.

8.2.2 Tolerance by slow growth

Tolerance and persistence are similar, however, tolerance concerns the whole population, while persistence is only attributed to a small fraction of bacteria. Tolerance is conferred by slow growth, for example, during slow growth the killing of *E. coli* with β-lactams is decelerated as a consequence of reduced cell wall synthesis, the major target of β-lactam antibiotics [184, 185]. The same phenomenon was also observed for the DNA gyrase inhibitor ciprofloxacin, where tolerance and survival was proportional to the growth rate of *P. aeruginosa* [186]. Supposedly, the reduced growth reflects low metabolic activity, which goes along with low target activity of the antibiotic and reduced cellular damage that enables survival. Furthermore, plotting the ratio between growth and MDK of different bacterial species and antibacterial agents supports the assumption of growth rate-dependent tolerance [183]. Tolerance by slow growth can be inheritable due to species-specific low growth rates [183]. For example, the MDK for *M. tuberculosis* is 40-fold higher than for *E. coli*, consistent with their specific growth rates [183]. In addition, poor growth conditions or external stress factors can also reduce the growth rate, as demonstrated for an auxotrophic *E. coli* strain, which was unable to synthesise tryptophan, thereby bacteria in un-supplemented cultures were more tolerant to antibiotics [187]. In addition, extremely low nutrient availability increased drug tolerance of *Bacillus subtilis* due to slow growth, leading to a doubling time of

4 days. In contrast, under optimal conditions *B. subtilis* needs only 26 minutes for one cell division [188, 189]. Nevertheless, despite the extended growth time, these bacteria are still metabolically and transcriptionally active [188]. In a study conducted with *S*. Typhimurium, it was demonstrated that low Mg^{2+} decreased the growth rate of the bacteria, while drug tolerance upon ciprofloxacin treatment was increased because Mg^{2+} is a crucial factor for ribosome assembly and maturation, not only in *S*. Typhimurium, but in a wide range of bacteria [190-192]. Another example for the importance of the doubling time and decreased drug susceptibility, is the induced non-growing state of *M. tuberculosis* as a response to the hostile environment of host cells [193]. Hence, it is important to choose the appropriate antibiotic to kill slow-growing pathogens, because the impact of bacteriostatic agents on tolerant bacteria is negligible, in contrast to bactericidal drugs, such as Streptomycin [194].

8.2.3 Tolerance by Lag

The lag phase is defined as the duration of time needed by bacteria to switch from the non-growing state to exponential growth, for example, when stationary phase bacteria adjust to a new environment with fresh nutrients for growth [195]. It was proposed to divide the lag phase into two parts, called lag1 phase, in which no biomass increase was observed, yet which showed promotor activity for enzymes that utilize carbon sources, and lag2 phase [196]. It was demonstrated that shortly after transfer of stationary phase *Salmonella* into fresh medium, gene expression involved in phosphate uptake was induced. Within an additional 20 minutes, more than 900 genes were upregulated, including genes for transcription, translation, iron-sulfur protein assembly, nucleotide metabolism, lipopolysaccharide (LPS) biosynthesis, and aerobic respiration [197]. During the lag2 phase, bacteria start to gain biomass and prepare for the first cell division to enter the exponential phase [196]. Furthermore, *Salmonella* accumulates iron, calcium, and manganese in the lag phase, which presumably stimulate the formation of ROS, when stationary phase bacteria were transferred into fresh oxygenated medium, explaining the induction of the oxidative stress response by OxyR and SoxRS during the lag phase [197]. Considering the broad range of genes which are initiated during the lag phase, it is conceivable that transition from the lag phase into the exponential phase can vary even within the same clonal population. This makes the lag phase an important factor for drug tolerance, in particular upon exposure to β-lactam antibiotics, targeting cell wall synthesis. In one study, exposure of a drug-sensitive *E. coli* strain to ampicillin with a short lag phase led to the eradication of the bulk population [22]. However,

re-cultivation of the few bacterial survivors and subsequent intermittent treatment with ampicillin decreased the efficiency of the antibiotic and increased the proportion of surviving bacteria due to an elongated lag phase [22]. Afterwards, sequencing data revealed several mutations in coding regions, which were responsible for the extended lag phase. One of the mutations caused an amino acid exchange in methionyl-tRNA synthetase, presumably delaying growth resumption due to an impaired protein synthesis during the lag phase [22]. The MIC of the evolved strain was indistinguishable from the ancestral strain; however, the MDK of the tolerant strain was increased (a hallmark of tolerance). The same phenomenon was also observed for *S. aureus* [198], where cycling treatment with daptomycin resulted in a point mutation within one gene designated as *pitA*, a putative inorganic phosphate transporter. The increased tolerance was accompanied by elevated levels of intracellular phosphate and polyphosphate, which has been suggested to improve bacterial fitness [198, 199].

Drug tolerance does not only result in treatment failure of recalcitrant infections, but can also enhance the evolution of resistance [200]. Daily treatment of *E. coli* with high concentrations of ampicillin showed mutations in *ampC* and partial resistance [23]. However, resistance occurred after 7 to 17 cycles of treatment, while increased tolerance was already observed after 3 to 4 cycles [23]. This is explained by the fact that there are far more genes present which can mutate and contribute to enhanced tolerance than genes which confer resistance. Therefore, tolerance facilitates the fixation of resistance mutations in a bacterial population and promotes its dissemination by increasing the probability that this mutation is not lost during drug treatment. This effect is similar to persister cells, which can also serve as a reservoir of viable bacteria for resistance development as described above [19, 23, 69].

8.3 Heteroresistance

8.3.1 General definition of Heteroresistance

Heteroresistance comprises a small subpopulation of an otherwise susceptible bacterial culture, which can survive antibiotic treatment and, in contrast to persister cells, is able to grow in the presence of antibiotics [201]. Heteroresistance can be the result of either mono- or polyclonal heteroresistance depending on the origin of the resistance subpopulation.

8.3.2 Monoclonal heteroresistance

Monoclonal heteroresistance is the result of either physiological or genetic heterogeneity in a clonal population [202]. This phenomenon is triggered by mutations, such as point mutations, deletions or insertions, which can either be fixed in a bacterial population or get lost depending on the fitness cost of the respective mutation [201]. For example, heteroresistance of MDR *Acinetobacter baumannii* against colistin was the result of increased induction of efflux pumps, which had no impact on the bacterial fitness, thus the evolved heteroresistance became genetically stable [203]. However, more common for (monoclonal) heteroresistance is unstable heteroresistance, as it has been shown for clinical isolates of *E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae* and *A. baumannii* due to tandem gene amplification of resistance genes leading to higher expression levels [204]. These bacteria suffer from slow growth, which is why they are outcompeted by the original non-mutated parent strain when there is no antibiotics for selection pressure.

8.3.3 Polyclonal heteroresistance

A mixed population of distinct genotypes with different resistance levels is described as polyclonal [202]. The same mutational events are responsible for polyclonal heteroresistance as for monoclonal heteroresistance.

8.4 Quinolone action and resistance

Quinolones are wide-spectrum antibiotics used to treat diverse bacterial infections, including salmonellosis, in which ciprofloxacin is the first choice [205]. Indeed, the emergence of quinolone resistant *Salmonella spp*. is a concerning problem nowadays, increasing the risk of treatment failure [206-211].

8.4.1 Structure of quinolones

Nalidixic acid is a hydrocarbon heterocyclic compound with a carboxyl group at position 4. It was discovered in 1962 as the first available quinolone used for treatment of common urinary tract infections (UTIs) [212, 213]. However, nalidixic acid is only efficient at high concentrations, therefore it was desirable to research for improved quinolone drugs. To increase the efficiency of quinolones, the nucleus of 4-quinolone was modified by adding a flourine atom at position 6 and a piperazinyl side chain at position 7, resulting in the formation of the first fluoroquinolone norfloxacin [212]. Further modifications and substituents notably altered antibactericidal activity of fluoroquinolones. For example, the addition of a methyl

group at position 2 and to the piperazinyl side chain have turned norfloxacin into the broad-spectrum antibiotic ofloxacin, increasing the antibacterial efficiency [213, 214]. Ciprofloxacin, the most commonly used fluoroquinolone today, differs in its structure only by one carbon atom from norfloxacin (Fig. 5), nevertheless the additional carbon atom increases the antibacterial activity of ciprofloxacin against Gram-negative bacteria approximately by ten-fold [215]. However, new fluoroquinolones are under development by further modifications of the cyclic structure to enhance the potency against fluoroquinolone resistant pathogens [216].

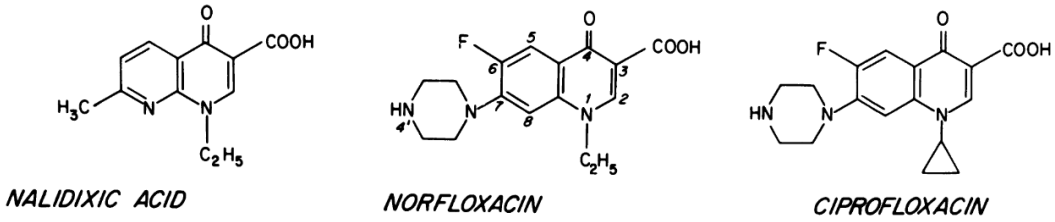

Fig. 3: Structure of nalidixic acid, norfloxacin and ciprofloxacin. The antibactericidal activity of nalidixic acid, as the original quinolone, was enhanced by addition of a piperazinyl side chain at position 7 and a flour atom at position 6. The figure has been adapted from Wolfson and Hooper (1989) [213].

8.4.2 Quinolones action

The major target of fluoroquinolones is the DNA gyrase (topoisomerase II) [213, 217, 218], which is important for negative super coiling of the bacterial DNA and energized by ATP [219, 220], as well as involved in the regulation of transcription of ribosomal RNA [221-223]. DNA gyrase is a heterotetramer, consisting of two subunits, termed as GyrA and GyrB, encoded by the genes *gyrA* and *gyrB*, respectively [224]. Expression of both genes correlates with the super coiling of the DNA, leading to increased expression when the DNA is in an uncoiled conformation [225]. The DNA gyrase envelopes the DNA and guides one segment of the DNA into a cavity, which is formed by GyrA, by twisting the DNA. Upon ATP binding, the cavity closes and the trapped DNA strand is transferred through a cleaved DNA segment (cleaved by GyrB), while one molecule of ATP is hydrolysed [226]. In the last step, the cleaved DNA part is re-ligated and hydrolysis of the second ATP molecule resets the DNA gyrase into the initial state. The re-ligation step is important for the efficiency of fluoroquinolones because the

antibacterial activity is based on the stabilisation of the cleavage complex and the inhibition of this re-ligation step. Therefore, the covalent binding of fluoroquinolones to DNA gyrase will result in double strand breaks, ultimately leading to cell death. Additionally, the fluoroquinolone-DNA gyrase complex blocks the replication machinery, thereby inhibiting the DNA synthesis, which can result in growth arrest at low fluoroquinolone concentration. However, bacteria have evolved a well-regulated DNA repair mechanism, the so-called SOS response, to counteract such DNA damage [227]. In addition, fluoroquinolones target also the topoisomerase IV, a heterotetramer composed of ParC and ParE, which unwinds positive supercoils and particularly plays a crucial role in the segregation of the chromosome after DNA replication (decatenation) in an ATP-dependent manner [228-230]. It was believed that fluoroquinolones target the DNA gyrase in Gram-negative bacteria while in Gram-positive bacteria fluoroquinolones target the topoisiomease IV [231]. However, this assumption has been reconsidered because there is no apparent preference of fluoroquinolones. Instead, the antibactericidal efficiency varies between bacterial species regardless of whether Gram-negative or Gram-positive [231]. To deploy its antibacterial feature, fluoroquinolones have to cross the bacterial cell wall to reach their cytoplasmic located target. Fluoroquinolones are hydrophilic and therefore cannot easily diffuse through the hydrophobic lipid bilayer. Only a fraction of ciprofloxacin is translocated directly through the membrane by forming stacks of ciprofloxacin molecules in which partial charge neutralization allows one ciprofloxacin molecule of the stack to penetrate the membrane [232]. Generally, a substantial amount of fluoroquinolones passes the membrane via outer membrane proteins (Omps) to reach its cytosolic target [233, 234].

8.4.3 Quinolone induced reactive oxygen species

In addition to the primary effect of DNA lesions caused by fluoroquinolones, it has been also demonstrated that exposure to norfloxacin causes metabolic perturbations and the formation of ROS, resulting in cytotoxic changes, such as protein carbonylation and the oxidation of nucleotides [235]. In particular, guanosine is oxidized to oxo-guanine, most likely induced by Fenton oxidants [236, 237], which in turn is incorporated into the DNA, leading eventually to DNA double strand breaks [235, 238]. Furthermore, binding of norfloxacin to the DNA gyrase forces the DNA gyrase to remain in the DNA cleavage complex, with the consequence of increased formation of superoxides. The free oxygen radicals cause oxidative damage of proteinaceous iron-sulfur clusters, releasing iron into the cytosol [239, 240]. The released iron

reacts with hydrogen peroxide via the Fenton reaction to the extreme deleterious hydroxyl radicals, which can damage the DNA or other macromolecules [239-241]. However, this model has been questioned, since, according to some research groups, there is no correlation between ROS formation, antibiotic treatment and bacterial killing [242, 243] whereas others supported drug-induced ROS formation [88, 89, 235, 244-246].

8.4.4 Quinolone resistance mechanisms

8.4.4.1 Drug target modification

In 1969 the first nalidixic acid resistance was reported after observing *E. coli* growing on nutrient agar plates supplemented with high concentration of nalidixic acid [247]. Taking into account that nalidixic acid resistance is easily acquired and that nalidixic acid is only efficient at high concentrations, it was desirable to improve its efficiency. Therefore, the basic structure of nalidixic acid was enhanced, yielding the more efficient fluoroquinolones. Since fluoroquinolones target the DNA gyrase and DNA topoisomerase IV, mutations of their subunits GyrA and GyrB in DNA gyrase or ParC and ParE in topoisomerase IV contribute to resistance against fluoroquinolones. Resistance development usually occurs at the amino terminal domain of GyrA between residue 67 and 106, or in ParC between residue 63 and 102, which is close to the DNA binding domain with its catalytically important tyrosines (Tyr) [248]. This region is designated as the *quinolone resistance determining region* (QRDR) of GyrA and ParC and mutations in this region lead to resistance to nalidixic acid and increases the MIC for fluoroquinolones, such as ciprofloxacin or norfloxacin, which differ only in one carbon atom [99, 218, 249, 250]. Furthermore, a nalidixic acid-resistant strain of *S.* Typhimurium is more tolerant to four-fold the MIC of ciprofloxacin than a nalidixic-sensitive strain [51, 99]. Additional mutations of the target enzymes can also confer higher resistance (up to 100-fold) [231]. The strain ATCC 14028s, used in this project, harbours a mutation in *gyrA*, whereby aspartate (Asp) at position 87 was replaced by tyrosine, making the strain resistant to nalidixic acid and more tolerant against ciprofloxacin [99]. Fluoroquinolones form hydrogen bonds with DNA gyrase, which is mediated by divalent cations, such as magnesium [230]. Therefore, amino acid exchange can diminish the formation of electrostatic interaction between target and drug, rendering the DNA gyrase less susceptible while having only little effect on catalytic activity. In contrast, mutations of GyrB or ParE are less common. Besides mutation of DNA gyrase, other resistance mechanisms exist as well, which confer resistance to

fluoroquinolones, including reduced membrane permeability, increased efflux pump activity and enzymatic modification of fluoroquinolones [230, 248, 251-261].

Noteworthy, it was also demonstrated that in persister cells, similar to resistant bacteria, efflux activity is augmented as well as expression of efflux pump associated genes, resulting in decreased accumulation of antibactericidal antibiotics, emphasizing the close relationship between drug tolerance and resistance [262, 263]. Furthermore, already low level of resistance might promote the formation of persister cells, since resistance correlates with persistence, and persistence might pave the way for further resistances [51, 62].

9 Aims and Objectives

The goal of this project was to investigate the metabolic status of persister cells and the correlation between persistence and the ATP concentration using *Salmonella enterica subsp. enterica* serovar Typhimurium as a model organism. Furthermore, the impact of endogenous phages on drug tolerance upon ciprofloxacin treatment has been scrutinized. The following questions were addressed:

1. Is low ATP always beneficial for persister cell formation?
2. What leads to an increased susceptibility of an ATP-deficient mutant in the presence of ciprofloxacin?
3. Do endogenous phages have an effect on bacterial physiology and how do endogenous phages bias persister cell formation?

10 Publications

The following sections include the accepted publications of the thesis 'Mechanisms of Persister Cell Formation in *Salmonella enterica* subsp. *enterica* serovar Typhimurium'.

10.1 **Publication 1:** The role of ATP pools in persister cell formation in (fluoro)quinolone-susceptible and-resistant strains of *Salmonella enterica* ser. Typhimurium

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Veterinary Microbiology Volume 210, October 2017, Pages 116-123

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10.2 **Publication 2:** *Salmonella* Central Carbon Metabolism Enhances Bactericidal Killing by Fluoroquinolone Antibiotics

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ASM Journals Antimicrobial Agents and Chemotherapy July 2022 Vol. 66, No. 7

https://doi.org/10.1128/aac.02344-21

10.3 **Publikation 3:** Prophage Gifsy-1 Induction in *Salmonella enterica* Serovar Typhimurium Reduces Persister Cell Formation after Ciprofloxacin Exposure

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The role of ATP pools in persister cell formation in (fluoro)quinolone-susceptible and -resistant strains of *Salmonella enterica* ser. Typhimurium

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ABSTRACT

In this study, we investigated the reported dependence on the ATP pools for persister cell formation in fluoroquinolone-resistant variants of the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium. We compared the generation of persister cell populations after ciprofloxacin challenge of wildtype and a nalidixic acid-resistant variant of *S.* Typhimurium with reduced ciprofloxacin-susceptibility, as well as strains containing a deletion of the *atp* operon or harbouring the cloned *atp* genes. A *gyrA* mutation (D87Y) was found to contribute to increased stationary phase formation of persister cells in *S.* Typhimurium. However, in contrast to expectations from prior studies, while treatment with the ATP synthase poison arsenate showed the expected increase in persister cells surviving ciprofloxacin treatment, a more direct approach using a strain of *Salmonella* deleted for the *atp* operon showed severe reductions in persister cell formation. Persister cell formation was recovered after introduction of the cloned *atp* operon which restored the reduced ATP levels. These results suggest either an alternative explanation for previous studies, or that persister cell formation in *Salmonella* is differently regulated.

1. Introduction

Infections caused by serovars of *Salmonella enterica* remain one of the major causes of bacterial zoonotic and food-related gastrointestinal diseases worldwide (World Health Organization, 2015). Although severe systemic forms of human infections by *Salmonella* are generally associated with the human-restricted serovar *S.* Typhi, there has been an alarming increase in the severity and systemic forms of infections observed for non-typhoidal serovars such as *S.* Typhimurium (Kariuki et al., 2015). Likewise, increasing levels of antibiotic resistance and the emergence of *Salmonella* serovars for which there are limited vaccines (Kariuki et al., 2015; Tennant and Levine, 2015), indicates a better understanding of host-pathogen interactions and resistance mechanisms for this important bacterial pathogen is imperative.

Antibiotic treatment due to *Salmonella* infections causing gastroenteritis is not generally recommended, as the infection is usually self-limiting. Antibiotic therapy may be recommended for immuno-compromised, very young or elderly patients, and is necessary in cases of bacteraemia (Parry and Threlfall, 2008). Fluoroquinolone antibiotics are currently the main antibiotics of choice for severe *Salmonella* infections (Parry and Threlfall, 2008). However, *Salmonella* resistance to the first-generation quinolone antibiotics, *e.g.* nalidixic acid, has shown a steady rise in many countries in both livestock and human isolates, and show variable degrees of cross-resistance to the next generation fluoroquinolone antibiotics (Griggs et al., 1994, 1996). Likewise, the subsequent generations of fluoroquinolones such as ciprofloxacin, have also been accompanied by a rise in resistance (Redgrave et al., 2014; Michael and Schwarz, 2016).

In addition to the worldwide increase in antibiotic resistant bacterial pathogens, there has been renewed interest in the formation of persister cells, sub-populations of bacteria which survive antibiotic therapies, but without acquiring resistance and which remain susceptible when re-challenged (Lewis, 2010). Such persister cells can re-emerge once therapy has been discontinued and contribute to chronic or recurring infections (Fauvert et al., 2011). Persister cells arise in bacterial cultures both during the exponential phase of growth, referred to as type II persisters, as well as during stationary phase, referred to as type I persisters (Balaban et al., 2004; Brauner et al., 2016). While a large number of stress- and stationary phase-associated genes and regulatory systems have been found to play a role in generation of the persister phenotype (Fauvert et al., 2011), recent studies have indicated that the intracellular ATP levels is one of the key factors in the formation of persister cells in both the Gram-positive opportunistic pathogen *Staphylococcus aureus* and Gram-negative *Escherichia coli*

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(Conlon et al., 2016; Shan et al., 2017).

Salmonella serovars are facultative, intracellular pathogens, which are actively invasive for host cells and capable of surviving within macrophage (Figueira and Holden, 2012). Infection or uptake of *Salmonella* by macrophage has been shown to increase the population of persisters resistent to antibiotic treatment (Helaine et al., 2014). Inhibition of phagosome acidification reduced the persister population significantly, and growth of broth cultures at pH 4.5 or amino acid starvation conditions were found to recapitulate the increase in persister cells observed after macrophage uptake (Helaine et al., 2014). Interestingly, acidic conditions have been found to increase the intracellular ATP pools in *E. coli* (Sun et al., 2011). As the ATP pools play not only a central role in the normal metabolic activities of the bacterial cell, but also in the expression and secretion of extra- and intracellular virulence factors of *Salmonella*, we chose to examine the role of the ATP pools in the formation of persister cells in *Salmonella*. Furthermore, we were interested in determining whether a dependence upon the ATP pools for persister formation would be affected in strains with reduced ciprofloxacin susceptibility. In contrast to prior studies (Conlon et al., 2016; Shan et al., 2017), we find strains of *Salmonella* with reduced cytosolic ATP levels due to deletion of the *atp* operon show severe reductions in persister cell generation in both exponential and stationary phase cultures.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. The *Salmonella enterica* serovar Typhimurium strain ATCC 14028 was the background used for all strains in this study. Strain 8640 is a virulent, spontaneous nalidixic acid-resistant (NalR) derivative previously used in our laboratory for mice infection studies. The *atp* operon deletion (Δ*atpIBEFHAGDC:kan*) strain, AT1144, was constructed by λ Red recombinase mutagenesis (Datsenko and Wanner, 2000) and has previously been described (Garcia-Gutierrez et al., 2016). The *atp* operon deletion was introduced into the NalR ATCC 14028 strain 8640 by bacteriophage P22 transduction using P22 lysates prepared on strain AT1144 according to standard protocols, followed by selection for growth on plates containing kanamycin (50 mg/L). Putative transductants were screened for the absence of contaminating bacteriophage by passage on Green plates, and for the correct chromosomal insertion of the deletion mutation by PCR. The kanamycin-resistance cassette was eliminated by introduction of plasmid pCP20, followed by screening for kanamycin-sensitivity, and subsequently for loss of plasmid pCP20 by growth at 37 °C as previously described (Datsenko and Wanner, 2000). Strains harbouring the vector control plasmid pWKS30 (Wang and Kushner, 1991), or pWKS30-*atp* harbouring the entire cloned *atpIBEFHAGDC* operon, were grown on plates or liquid media containing 100 mg/L carbenicillin to maintain selection of the plasmids.

Table 1
Strains and Plasmids used in this study.

Strain	Relevant Features*	Reference/Source
25922	*E. coli* ATCC 25922 antibiotic susceptibility testing	ATCC
1298	*S.* Typhimurium ATCC 14028 wildtype, virulent	ATCC
8640	*S.* Typhimurium ATCC 14028 wildtype, virulent, NalR	Laboratory stock
AT1144	*S.* Typhimurium 4/74 Δ*atpIBEFHAGDC:kan*	Garcia-Gutierrez et al. (2016)
9200	*S.* Typhimurium ATCC 14028 NalR Δ*atpIBEFHAGDC*	This study
9220	8640(pWKS30)	This study
9222	8640(pWKS30-*atp*+)	This study
9224	9200(pWKS30)	This study
9226	9200(pWKS30-*atp*+)	This study
Plasmids		
pWKS30	*bla lacZα* pSC101ori	Wang and Kushner (1991)
pWKS30-*atp*+	*bla atpIBEFHAGDC*+ pSC101ori	This study

*Abbreviations: Nal, nalidixic acid; kan, kanamycin-resistance (*aph*);

2.2. MIC determinations

Minimum inhibitory concentrations (MIC) assays were performed according to CLSI recommendations (CLSI, 2017) in cation-adjusted Mueller-Hinton broth (CAMHB or Mueller-Hinton II) in flat-bottomed, 96-well plates (Corning) with an inoculum of 105 bacteria/well and incubated at 37 °C overnight. For comparison, MIC assays were also performed in L-broth medium. The optical density of the cultures was determined at 460 and 600 nm immediately after inoculation and after overnight incubation using a BioTek Synergy HT plate reader. Controls included *E. coli* strain ATCC® 25922, medium alone and wells without antibiotic additions. In order to obtain sufficient colony-forming units (CFU) for persister assays (antibiotic survival assays), the inoculum for liquid cultures was increased to 106 or 107 CFU/mL. MIC determinations were therefore also performed for the increased inocula of 107 and 106 CFU/mL. However, no major differences were observed in the determined MIC values other than a higher optical density background (data not shown).

2.3. Time-killing/Persister assays

Persister assays were performed by inoculation of 5 ml of culture medium from fresh, single colonies taken from plates streaked out the day before, and grown with aeration (shaking) at 37 °C to an optical density at 600 nm of approximately 0.5 (mid-log), and adjusted to approximately 107 CFU/ml for the assays. Persister studies with stationary phase cultures were inoculated in the same manner as above and grown overnight (18 h). The antibiotic concentrations in the persister assays were four-fold the pre-determined MIC concentrations for the respective strains as determined above. For assays involving stationary phase cultures, 106 CFU/ml for the assays was used to compensate for the higher cell density/OD-unit. For assays involving arsenate, samples from growing cultures were added to medium containing sodium arsenate at the indicated final concentrations 30 min prior to the addition of ciprofloxacin. Samples for bacteria surviving ciprofloxacin treatment (persisters) were taken at the times indicated in the figures, diluted and plated to agar plates for enumeration of surviving CFU/ml of culture. All assays were repeated at least three times from independent cultures.

2.4. ATP determinations

Bacterial ATP concentrations were determined using the BacTiter-Glo Microbial Cell Viability Assay Kit (Promega) according to the manufactureŕs instructions, including standard curves with purified ATP, and culture medium controls. ATP levels are reported in relative fluorescence units (RFU)/cell where the fluorescence for a given volume of bacterial cultures in the reactions has been divided by the total CFU/ml determined in parallel and normalised to the values for the control strain or samples.

2.5. Statistical analyses

Statistical analyses were performed using the GraphPad statistics software (GraphPad Software, Inc.). Where shown, statistical analyses for significance were based on at least three, independent experiments and significance was determined using a two-tailed, unpaired Student́s *t*-test, where $P > 0.05$ is considered non-significant, and P values ≤ 0.05 are considered significant. In the figures, this is indicated

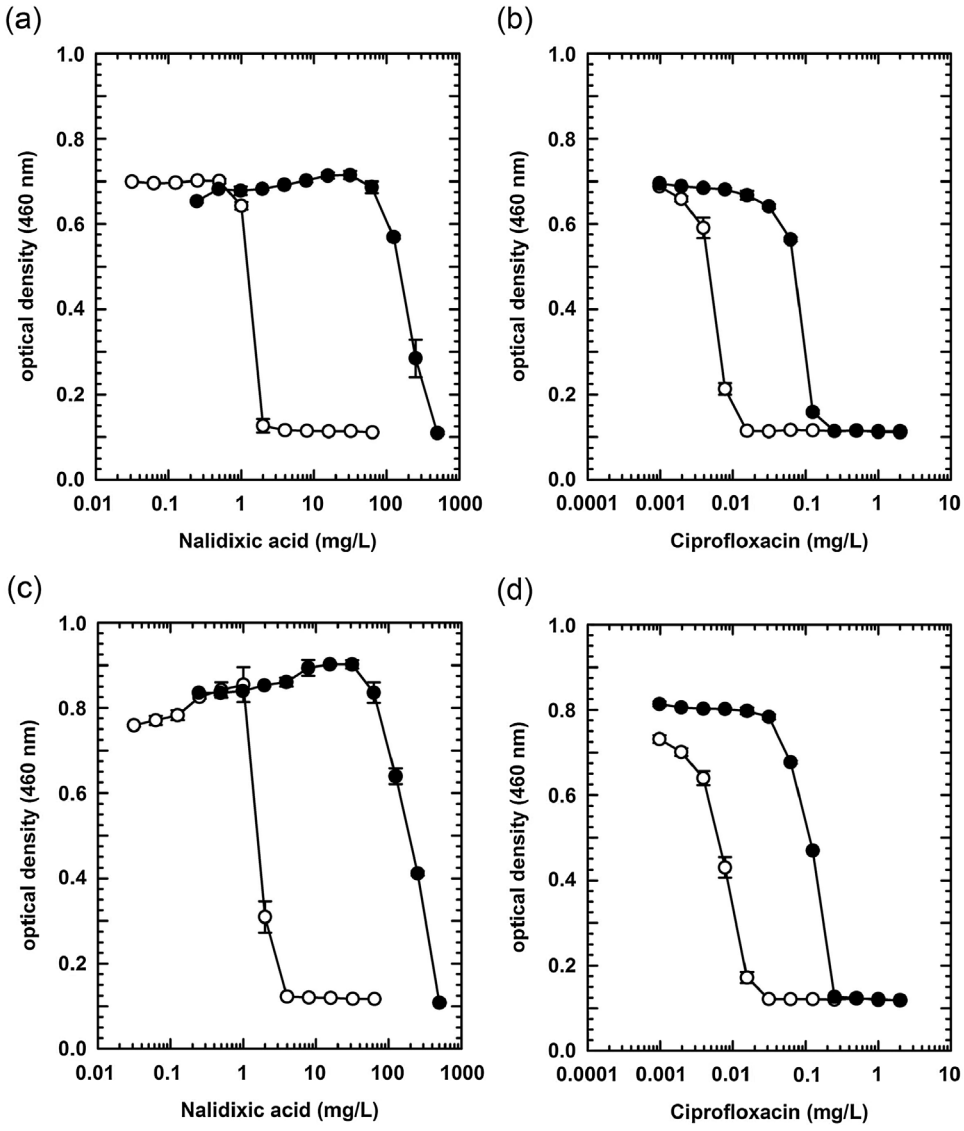

Fig. 1. MIC determinations for wildtype (strain 1298, open symbols) and spontaneous nalidixic acid-resistant strains (strain 8640, filled simbols) of *S.* Typhimurium ATCC 14028. Strains were inoculated at 105 bacteria/well in 0.1 ml of either cation adjusted Mueller-Hinton broth (a and b) or L-broth (c and d) containing either nalidixic acid (a and c) or ciprofloxacin (b and d) at the indicated concentrations and the optical density of the cell suspensions determined at 460 and 600 nm. After overnight incubation at 37 °C, the optical densities were again determined and the minimum inhibitory concentration (MIC) in Mueller-Hinton medium was determined as the concentration at which no growth occurred. The results shown are representative of at least three, independent determinations.

as: $P > 0.05$, non-significant (n.s.); $P \leq 0.05$, *; $P \leq 0.01$, **; or $P \leq 0.001$, ***.

3. Results

3.1. Antimicrobial susceptibility testing of Salmonella strains

As the mechanisms for nalidixic acid and second generation fluoroquinolone resistance are largely shared (Redgrave et al., 2014), we determined the MIC values for ciprofloxacin for both nalidixic acid-susceptible and – resistant derivatives of the otherwise wildtype, prototrophic *S.* Typhimurium strain ATCC 14028. As shown in Fig. 1a, the MIC value for nalidixic acid of the original, susceptible strain was 2 mg/L, and of the spontaneous nalidixic acid-resistant strain, ≥512 mg/L. However, despite the large difference in nalidixic acid MIC determinations, the difference in the MIC values for ciprofloxacin between the two strains was more moderate, for the original and for the susceptible strain 0.016 mg/L, and that for the nalidixic acid-resistant isolate 0.25 mg/L (Figs. 1b and d). Sequence analysis of the *gyrA* quinolone resistance-determining region (QRDR) indicated a mutation of Asp87 to tyrosine (D87Y) in the nalidixic acid-resistant isolate, a mutation previously associated with quinolone-resistance in *Salmonella* serovars Typhimurium and Enteriditis (Eaves et al., 2004; Levy et al., 2004). No sequence differences were found in the QRDR regions of *gyrB*, *parC* or *parE* (data not shown).

3.2. Persister cell populations in wildtype and NalR/CipR strains

We next compared the killing kinetics when challenged with ciprofloxacin in the two strain backgrounds. Here, we were interested to know whether reduced susceptibility to ciprofloxacin would affect the generation of persister cells, *i.e.* the cells capable of surviving high concentrations of ciprofloxacin. Samples of either exponentially growing (mid-log) or stationary phase cultures were transferred to media containing concentrations of ciprofloxacin four-fold higher than the previously determined MIC values (4× the MIC) for the respective strains, *i.e.* 0.0625 mg/L for strain 1298, and 1 mg/L for the nalidixic acid-resistant strain 8640. The choice of 4× the MIC was made to avoid the known bacteriostatic rather than bacteriocidal effects of quinolones at excessively high concentrations (Crumplin and Smith, 1975). As shown in Fig. 2a, the initial killing kinetics for mid-log cultures were the same for both strains, with only a significant difference in surviving CFU at three and four hours post-challenge ($P = 0.0214$, and 0.0208, resp.), but which was no longer significant by 24 h. In contrast, stationary phase cultures showed a much larger difference, with statistically significant differences beginning one hour post-challenge, and a highly significant difference ($P = 0.0007$) 4 h post-challenge. In general, the low numbers of surviving bacteria at 24 h post-challenge

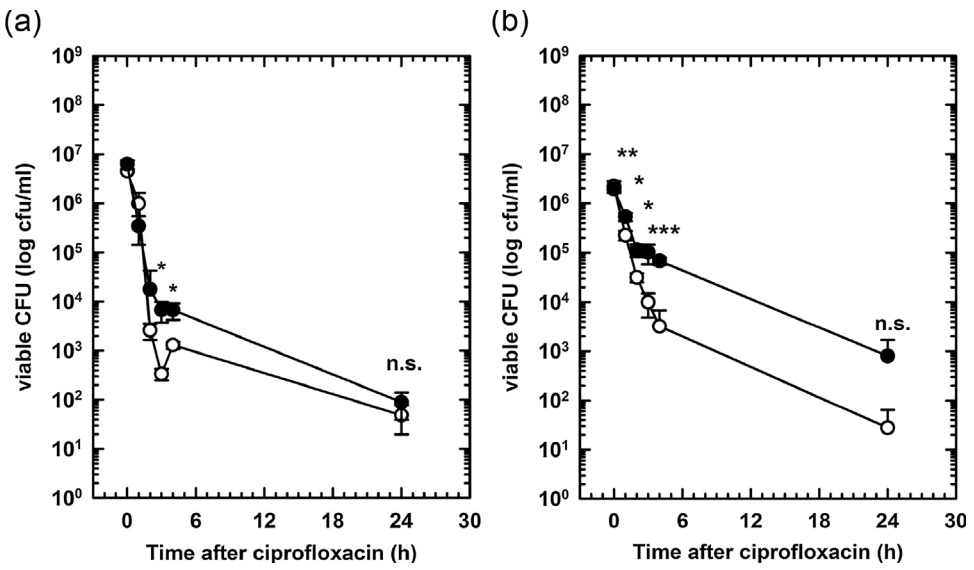

Fig. 2. Determination of the persister cell populations for wildtype and spontaneous nalidixic acid-resistant strains of *S.* Typhimurium ATCC 14028. Survival of the wildtype (open symbols) or nalidixic acid-resistant (filled symbols) strains was determined as viable CFU/ml at different times after transfer to media containing ciprofloxacin at 4× the MIC. Shown are the survival kinetics for mid-log (a) or stationary phase cultures (b). The data shown are the averages of at least three, independent experiments. n.s., non-significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

prevented accurate determinations of significance; nevertheless, the nalidixic acid-resistant strain nevertheless showed up to 30-fold higher surviving bacteria after ciprofloxacin challenge, consistent with the altered killing kinetics. These results suggested that the presence of persister cells in exponentially growing cultures was largely independent of the fluoroquinolone-susceptibility status, but the altered ciprofloxacin-susceptibility due to the *gyrA* mutation in some manner contributed to higher survival rates in stationary phase cultures.

3.3. Persister cell levels in NalR/CipR strains after arsenate treatment

High-level resistance to ciprofloxacin and other fluoroquinolones are conferred by gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) mutations, whereas mutations in efflux systems have been found to confer decreased susceptibility to a wide range of antibiotics (Giraud et al., 2000). In addition to the ATP-dependent ABC-type transporters, other efflux systems involved in transport of toxic compounds, including antibiotics, use the proton motive force of the membrane to drive transport (Amaral et al., 2011). Both types of efflux systems are therefore directly or indirectly connected to the ATP pools, as the F1F0-ATPase uses the flow of protons across the inner membrane for ATP synthesis. We were therefore interested to know whether reduced susceptibility to ciprofloxacin might also affect the reported dependence of persister cell formation on reduced ATP levels in the cell (Conlon et al., 2016; Shan et al., 2017). We compared the persister cell populations in both the original and reduced ciprofloxacin-susceptibility strains after addition of arsenate, an inhibitor of ATP synthesis (Hong and Pedersen, 2008). As shown in Fig. 3, arsenate treatment of cultures prior to ciprofloxacin challenge resulted in similar fractions of persister cell populations surviving ciprofloxacin treatment at four-fold the respective MIC concentration in both strain backgrounds (Fig. 3a, b). Likewise, the effects on the ATP pools were similar in both the wildtype and nalidixic acid-resistant strains (Fig. 3c, d, resp.). These results were consistent with prior studies indicating higher persister cell formation in arsenate-treated strains of *S. aureus* and *E. coli* (Conlon et al., 2016; Shan et al., 2017). Note that the highest concentration of arsenate used here (0.25 mM) was 40-fold lower than that used in the study of Shan et al. (2017). Furthermore, despite an increase in persisters of up to ten-fold with 0.1 mM arsenate, there were only minimal effects on the bacterial ATP pools at this concentration (Figs. 3c and d). These results suggested the correlation between persister cells and the ATP pools was different in *Salmonella*.

3.4. Reduced persister cell levels in an atp operon deletion strain

In order to examine a role for the ATP pools on persister cell formation in the nalidixic acid-resistant strain more directly, we determined the levels of persister cell populations in this otherwise wildtype strain background and a derivative harbouring a deletion of the *atp* operon (Δ*atp*). As shown in Fig. 4c, the Δ*atp* operon mutant showed a reduction in intracellular ATP pools of approximately 40% to 50%, consistent with other studies (Jensen and Michelsen, 1992). However, loss of the ATP synthase in the Δ*atp* operon strain resulted in an approximately 30- to 100-fold reductions in persister cells at 24 h post-challenge relative to the wildtype strain in both mid-log (Fig. 4a, $P = 0.0014$) and stationary phase (Fig. 4b, $P = 0.0008$) cultures, with differences in the killing kinetics apparent at 4 h post-challenge. We also determined the persister cell populations in both strains harbouring a plasmid containing the full-length, cloned *atp* operon under control of the native promoter(s). As shown in Fig. 5, no major differences were observed in the nalidixic acid-resistant strain harbouring the cloned *atp* operon (Fig. 5a). Although the Δ*atp* operon strain harbouring the cloned *atp* operon showed a five- to ten-fold increase in persister cells compared to the Δ*atp* strain harbouring the control plasmid (Fig. 5b), due to the low numbers of bacteria recovered 24 h post-challenge, significant differences could only be determined for the 3 and 4 h time points in the killing curves. Nevertheless, the results were consistent with the observations in Fig. 4, *i.e.* that the correlation between the ATP pools and the generation of persister cells in *Salmonella* is different to that reported for *S. aureus* and *E. coli*.

4. Discussion

In this study, we were interested to know whether the reported dependence on reduced ATP pools in the generation of persister cells was also a contributing factor in *Salmonella*, a facultative intracellular pathogen which is confronted with a very different set of environmental and nutritional conditions compared to extracellular pathogens such as *Staphylococcus* spp. and *E. coli*. Furthermore, due to the increase in quinolone- and fluoroquinolone-resistance in *Salmonella* serovars, we also determined whether changes in the susceptibility to nalidixic acid and ciprofloxacin would affect the generation of persister cells and/or a dependence on the ATP pools. However, while it would appear that reductions in growth in general support the generation of persisters, a direct link to the ATP pools was not apparent in this study. Arsenate treatment showed an increase in persister cells (Fig. 3), consistent with a role for the ATP pools in persister cell formation (Conlon et al., 2016;

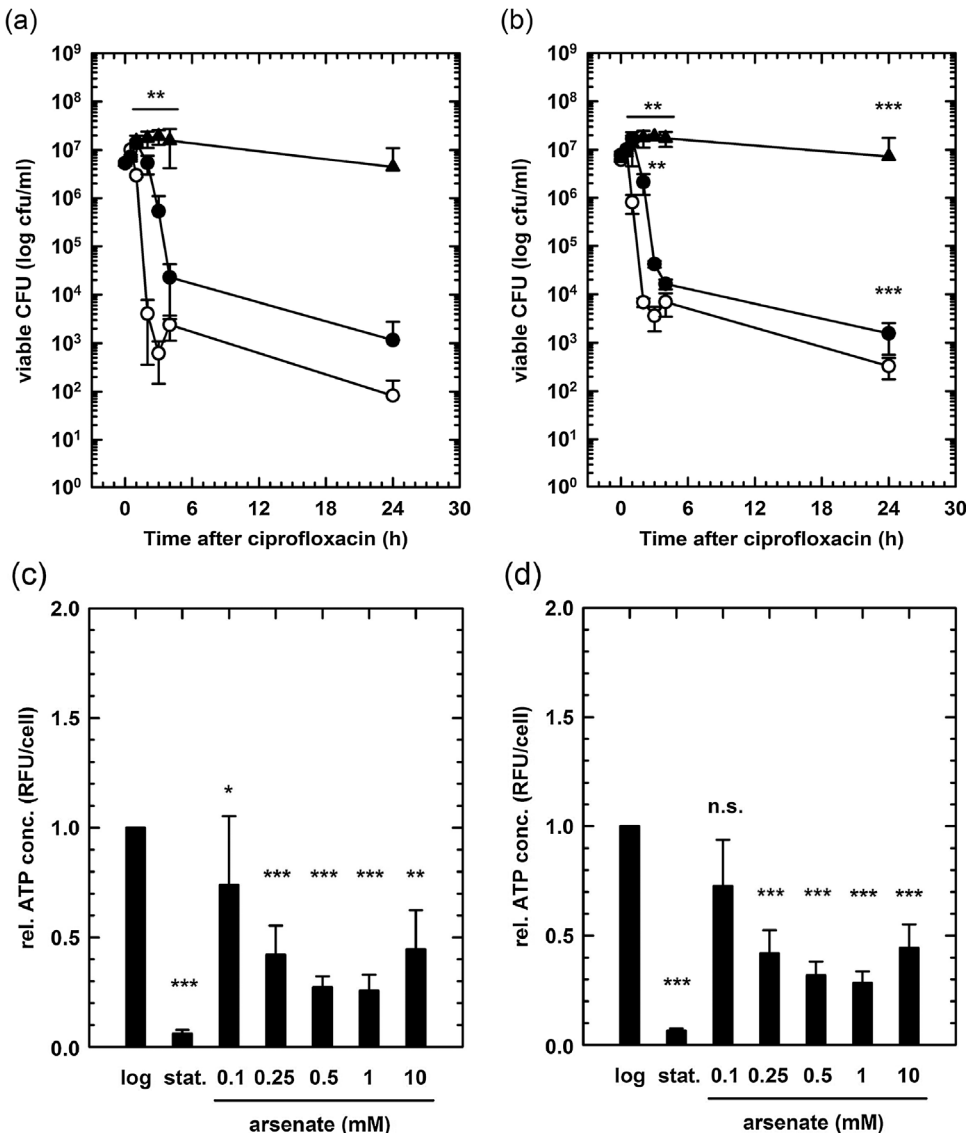

Fig. 3. The effects of arsenate treatment on persister cell formation and ATP levels in wildtype and spontaneous nalidixic acid-resistant strains of *S.* Typhimurium. Shown are the survival kinetics for either the (a) wildtype or (b) nalidixic acid-resistant strains either untreated (open circles) or pre-treated for 30 min with either 0.1 mM (filled circles), or 0.25 mM arsenate (filled triangles) prior to addition of ciprofloxacin at 4× the MIC. The ATP levels of either the (c) wildtype or (d) nalidixic acid-resistant strains of exponential cultures (log), stationary phase (stat.), or after pre-treatment with the indicated concentrations of arsenate as above are shown. Results shown are representative of at least three, independent experiments. Significance determinations were all calculated compared to the ATP levels of untreated, mid-log cultures. n.s., non-significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Shan et al., 2017). However, a more direct approach using an *atp* operon deletion strain showed up to 100-fold reductions in persister cell formation, independent of the growth phase (Fig. 4). Re-introduction of the cloned *atp* operon in the *atp* operon deletion strain increased the levels of persisters surviving ciprofloxacin challenge. These results suggest that the reported correlation between reductions in the ATP pools and the formation of persister cells is more complex, at least in *Salmonella*.

A possible explanation for the apparent discrepancy between the reported dependence on reduced ATP levels for the generation of persister cells and our conflicting observations with the *atp* operon deletion strain might be related to increased respiration in *atp* operon deletion strains and strains over-expressing the F1-ATPase (Jensen and Michelsen, 1992; Soria et al., 2013; Adolfsen and Brynildsen, 2015). Irrespective of how the intracellular ATP levels were reduced in those studies, an increase in respiration, glucose and oxygen consumption, and acetate production was observed, consistent with an increase in substrate level phosphorylation pathways, *i.e.* overflow metabolism. Overflow metabolism refers to the phenomenon whereby organisms use metabolic pathways which only partially oxidise their growth substrates (*e.g.* glucose) even in the presence of oxygen, rather than fully utilising the more energetically favourable respiratory pathways. The acetyl-CoA generated is instead metabolised to acetate and/or lactate, which is excreted (Wolfe, 2005). Furthermore, the increased rates of respiration following reductions in the ATP pools through deletion of the *atp* operon or over-expression of the F1-ATPase are also accompanied by an increase in the expression of genes involved in oxidative stress responses (Soria et al., 2013). A by-product of respiration is the production of superoxide and other reactive oxygen species (Imlay, 2008, 2009). Inhibition or loss of the ATP synthase would therefore increase the dependence on respiratory pathways accompanied by additional production of reactive oxygen species. Although somewhat controversial (Dwyer et al., 2015), it has been proposed that one additional result of the action of bactericidal antibiotics, including fluoroquinolones, is a metabolic shift toward overflow metabolism and the generation of reactive oxygen species (ROS) which contribute to the killing activity (Kohanski et al., 2007; Lobritz et al., 2015). A recent study found that overflow metabolism is also the major route utilized by intracellular *Salmonella* for generation of energy in both murine and human macrophage cell lines (Garcia-Guiterrez et al., 2016). Notably, intracellular *Salmonella* recovered from murine macrophage cell lines show much higher levels of persister cells than bacteria grown in broth culture (Helaine et al., 2014).

Helaine et al. (2014) found that *Salmonella* recovered from macrophage showed up to 100-fold higher levels of persister cells, and the same increase in the persister cell population could be reproduced by

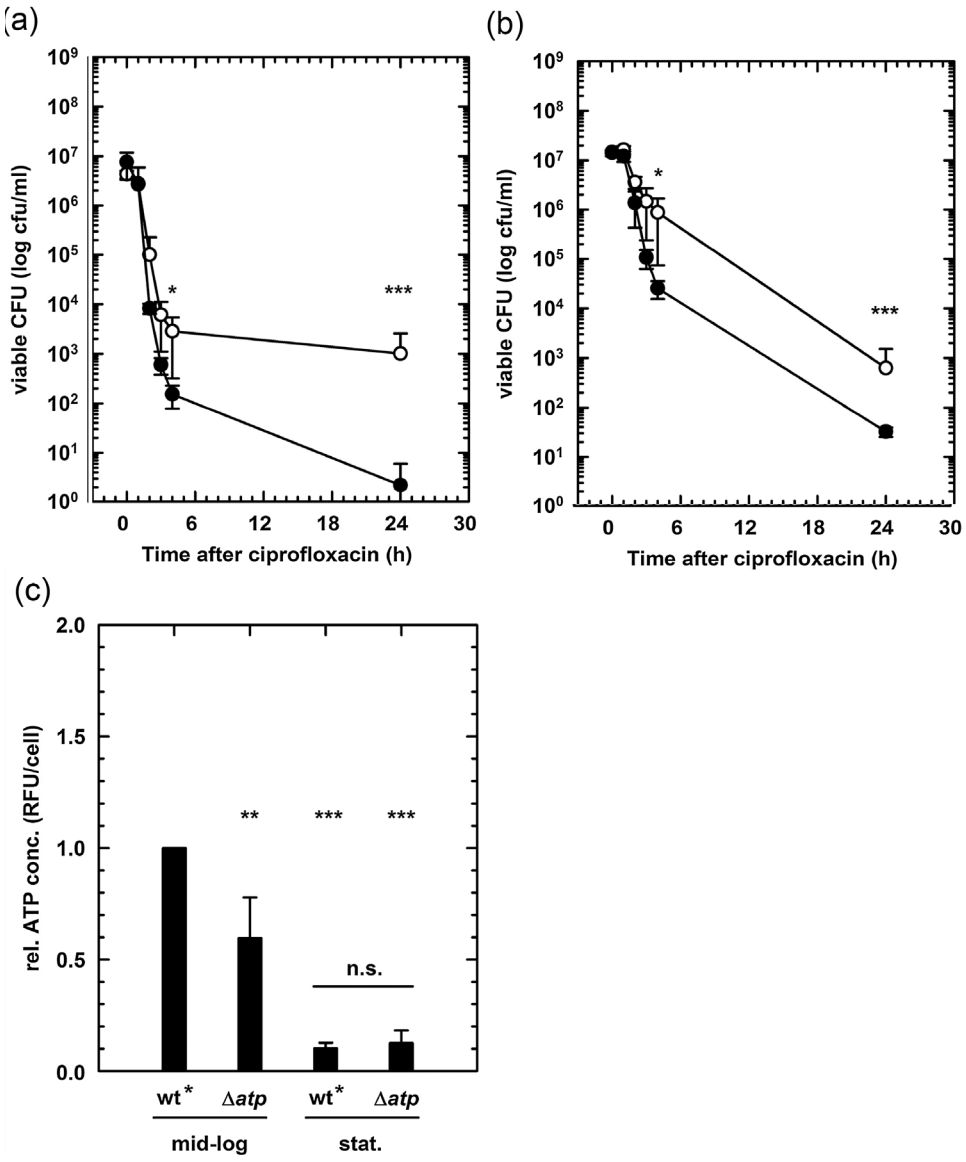

Fig. 4. Determination of persister cell populations in a strain harbouring a deletion of the *atp* operon. Shown are surviving cells of (a) mid-log or (b) stationary phase cultures of either the nalidixic acid-resistant strain 8640 (open symbols) or Δ*atp* operon derivative strain 9200 (filled symbols) after challenge with ciprofloxacin at 4× the MIC in samples taken at the times indicated post-challenge. Results shown are the averages of at least three, independent assays. The ATP levels of the nalidixic acid-resistant strain (wt*) or Δ*atp* operon derivative (Δ*atp*) for exponential cultures (log), stationary phase (stat.) are shown. Results shown are representative of three, independent experiments. n.s., non-significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

growth at low pH or nutrient limitation, conditions likely to prevail in the phagosomal compartment. In contrast, in a recent study in *E. coli* (Shan et al., 2017), acid stress did not result in a significant increase in the persister cell population, suggesting either differences in the mechanisms leading to persister cell formation in the two bacteria, or different metabolic responses to acid stress. A recent study reported that the cytoplasmic pH of intracellular *Salmonella* in macrophage rapidly drops to approximately pH 5.5 after uptake in an OmpR-dependent manner (Chakraborty et al., 2015). The authors further showed that, in addition to repression of the *cadBA* genes which would consume protons, OmpR was apparently also involved in activation of the *atpB* gene, encoding the α F0 subunit of the ATP synthase, thereby maintaining the cytoplasmic acidification in media at pH 5.6. Together, these observations suggest that the killing effect of bactericidal antibiotics due to reactive oxygen species production may be reduced for facultative intracellular pathogens such as *Salmonella*, which have already been "primed" for detoxification of ROS in the intracellular environment, similar to induction of the acid tolerance response (Foster, 1995).

In the studies of Conlon et al. (2016) and Shan et al. (2017), arsenate treatments were used to reduce the ATP pools and resulted in higher levels of persister cells in their assays. However, while arsenate interferes with ATP synthesis, other side effects of arsenate and metabolised forms also include the generation of reactive oxygen species (Shi et al., 2004). We therefore suggest one possible interpretation for the results with arsenate additions could be that arsenate induces the oxidative stress and/or SOS response pathways, either directly through generation of reactive oxygen species after metabolism in the cell, or indirectly, by shifting the bacteria towards respiratory pathways in the absence of a functional ATP synthase. Arsenate treatment would therefore not only result in reductions in the ATP pools, but also activate oxidative stress responses which reduce the efficacy of ciprofloxacin. Further work is clearly required to discriminate between these different possibilities.

5. Conclusions

Here, we show that in *S.* Typhimurium, a *gyrA* (D87Y) mutation conferring nalidixic acid resistance and reduced ciprofloxacin susceptibility appears to contribute to an increase in persister cells in culture, as seen by a reduction in the killing rates between 1 and 4 h post-challenge in the presence of ciprofloxacin. Furthermore, in contrast to recent studies in *S. aureus* and *E. coli*, reductions in the ATP pools in *S.* Typhimurium results in a reduction in the occurrence of persister cells, rather than an increase.

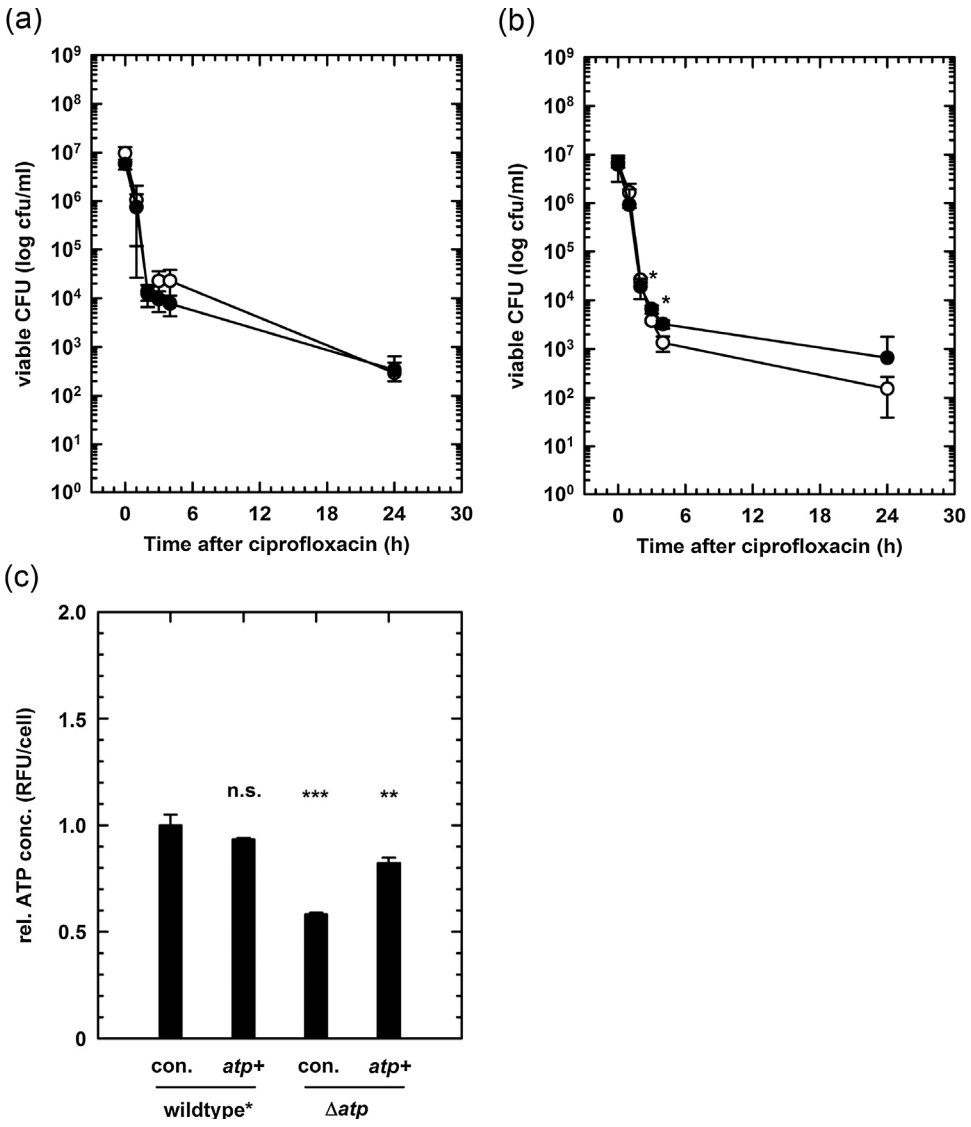

Fig. 5. Determination of persister cell populations in strains harbouring plasmids containing the cloned *atp* operon. Shown are the persister cell populations present after challenge with ciprofloxacin at 4× the MIC for mid-log cultures of either (a) strain 8640, or (b) the *atp* operon deletion strain 9200 (Δ*atp*). In both panels, strains harbour either the vector control plasmid (open symbols) or the cloned *atp* operon (filled symbols). Results shown are the averages of at least three, independent assays. In panel (c) are shown the ATP levels determined for strain 8640 (wildtype*) or the *atp* operon deletion strain 9200 (Δ*atp*) harbouring either the vector control (con.) or cloned atp operon (*atp* +). n.s., non-significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Conflicts of interest

All authors: no conflicts of interest to declare.

Acknowledgments

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MECHANISMS OF RESISTANCE

Salmonella Central Carbon Metabolism Enhances Bactericidal Killing by Fluoroquinolone Antibiotics

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ABSTRACT The efficacy of killing by bactericidal antibiotics has been reported to depend in large part on the ATP levels, with low levels of ATP leading to increased persistence after antibiotic challenge. Here, we show that an *atp* operon deletion strain of *Salmonella enterica* serovar Typhimurium lacking the ATP synthase was at least 10-fold more sensitive to killing by the fluoroquinolone antibiotic ciprofloxacin and yet showed either increased survival or no significant difference compared with the wild-type strain when challenged with aminoglycoside or β-lactam antibiotics, respectively. The increased cell killing and reduced bacterial survival (persistence) after fluoroquinolone challenge were found to involve metabolic compensation for the loss of the ATP synthase through central carbon metabolism reactions and increased NAD(P)H levels. We conclude that the intracellular ATP levels *per se* do not correlate with bactericidal antibiotic persistence to fluoroquinolone killing; rather, the central carbon metabolic pathways active at the time of challenge and the intracellular target of the antibiotic determine the efficacy of treatment.

KEYWORDS *Salmonella*, antibiotic resistance, carbon metabolism, fluoroquinolones

Prior studies have shown that even antibiotics considered bactericidal are unable to completely eliminate sensitive bacteria, a phenomenon referred to as persistence (1, 2). In contrast to antibiotic resistance, the persistence of small populations of bacteria during antibiotic challenges is nonheritable and is believed to involve the activation of toxin/antitoxin modules, the accumulation of toxic metabolites, or the activation of bacterial stress responses (1, 2). Studies on the bactericidal effects of DNA gyrase inhibitors found the generation of highly reactive oxygen species (ROS) contributed to the bactericidal effects (3, 4). This synergistic mechanism of bacterial killing has since been extended to include other classes of bactericidal antibiotics and involves effects on metabolic pathways stimulating ROS production contributing to cell death (4–6). However, other studies found no accelerated formation of ROS after exposure to antibiotics (7–9). Norfloxacin challenge of a quinolone-resistant *gyrA* mutant of *Escherichia coli* was reported to show no ROS production, suggesting that ROS is produced only in response to antibiotic stress (4). However, in the absence of the primary drug target DNA gyrase, quinolone antibiotics act on secondary targets, such as DNA topoisomerase IV (10). Previously, norfloxacin treatment of a *gyrA* mutant of *E. coli* had been shown to produce a robust SOS response, indicating that norfloxacin treatment of a *gyrA* mutant does indeed activate a major stress response pathway (11). Furthermore, the gyrase of *Staphylococcus aureus* is intrinsically resistant to fluoroquinolones, making topoisomerase IV the major target of fluoroquinolone antibiotics in this microorganism (12). These observations suggest that the connections between antibiotic stress and ROS production remain unclear.

Reductions in ATP production have been reported to play a central role in the survival of both *E. coli* and *Staphylococcus aureus* against bactericidal antibiotics (13–15).

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Treatment of bacteria with quinolone/fluoroquinolone antibiotics should activate the SOS response/toxin-antitoxin systems resulting in reduced ATP levels leading to improved survival (16, 17). To investigate the role of the ATP pools and antibiotic persistence in the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium, we recently examined antibiotic killing and persister cell formation in an *atp* operon deletion (Δ*atp*) mutant (18). In contrast to prior studies (13, 14), despite reduced ATP levels, the Δ*atp* mutant showed significant, 10-fold reductions in persistence after a challenge with the fluoroquinolone antibiotic ciprofloxacin. Here, we show that it is not the ATP levels in the cell *per se* which are involved in survival toward bactericidal antibiotics and formation of persister cells but rather the central carbon metabolism pathways which are active at the time of antibiotic challenge and the targeting of DNA gyrase and topoisomerase IV by fluoroquinolones.

RESULTS

Increased fluoroquinolone killing in an *atp* operon deletion (Δ*atp*) strain. To determine how the loss of the ATP synthase would affect cell survival when challenged with different classes of bactericidal antibiotics, we performed antibiotic killing assays with fluoroquinolone (ciprofloxacin), aminoglycoside (kanamycin), and β-lactam (ampicillin) antibiotics. As shown in Fig. 1a, the Δ*atp* mutant showed reduced persistence in response to ciprofloxacin treatment. However, we observed either increased survival of the Δ*atp* deletion mutant with kanamycin treatment or no significant differences in the killing kinetics with ampicillin (Fig. 1b and c, respectively; 4 h posttreatment shown in Fig. 1d). Notably, all three antibiotics belong to groups considered bactericidal (5) and were used at concentrations at least 4-fold higher than the MIC. The results for the fluoroquinolone ciprofloxacin were therefore not consistent with the proposed increase in persistence against bactericidal antibiotics in bacterial cells depleted for ATP (13, 14) and contrasted with the increased killing observed for an Δ*atpA* mutant of *E. coli* for other classes of antibiotics (19). We therefore chose to examine the increased susceptibility of the Δ*atp* mutant to ciprofloxacin more closely.

Under aerobic conditions, in the absence of a functional ATP synthase and/or ATP depletion, bacteria rely on substrate-level phosphorylation/glycolysis reactions for generation of ATP, accompanied by increased oxygen consumption and respiratory chain activities (4, 19–21). In undefined, complex media such as Lennox broth (L-broth), there is essentially no available glucose (22). However, there are variable levels of sugars, such as trehalose, mannose, and maltose/maltodextrins, which can also support growth (23). We therefore examined the effect of increasing the glucose availability on the ATP levels and antibiotic lethality after a challenge with ciprofloxacin. As seen in Fig. 1e, glucose supplementation increased the ATP levels of the Δ*atp* mutant strain to levels equivalent to that of the wild-type strain. However, despite similar ATP levels for both strains, the increased ATP levels did not significantly affect the bactericidal effects of ciprofloxacin (Fig. 1f; see Fig. S1 in the supplemental material), indicating that cell survival and persister cell formation after ciprofloxacin challenge were independent of the intracellular ATP levels.

Metabolic genes involved in increased killing by ciprofloxacin. A characteristic of mutants defective in ATP synthase is an elevated respiration rate (19–21). In prior studies, the *E. coli* respiratory NADH dehydrogenase complex I (encoded by the *nuo* operon) and NADH dehydrogenase II (Ndh) had been implicated in the generation of reactive oxygen species (ROS; superoxide and hydrogen peroxide), using NADH or NADPH as the substrates, suggesting a role for the NAD(P)H pools in ROS production (24–28). To determine whether the metabolic reorganization in the Δ*atp* strain might be involved in the reduced persister cell formation, we performed antibiotic killing assays comparing wild-type and Δ*atp* strains harboring additional mutations in genes involved in central carbon metabolism and respiration, including a number previously reported to play a role in persister cell formation (25, 27, 29). As shown in Fig. 2, for four of the additional mutations, namely, Δ*fre*, Δ*nadB*, Δ*ndh*, and the Δ*nuo* operon deletion, there was no longer a statistically significant difference between the Δ*atp*

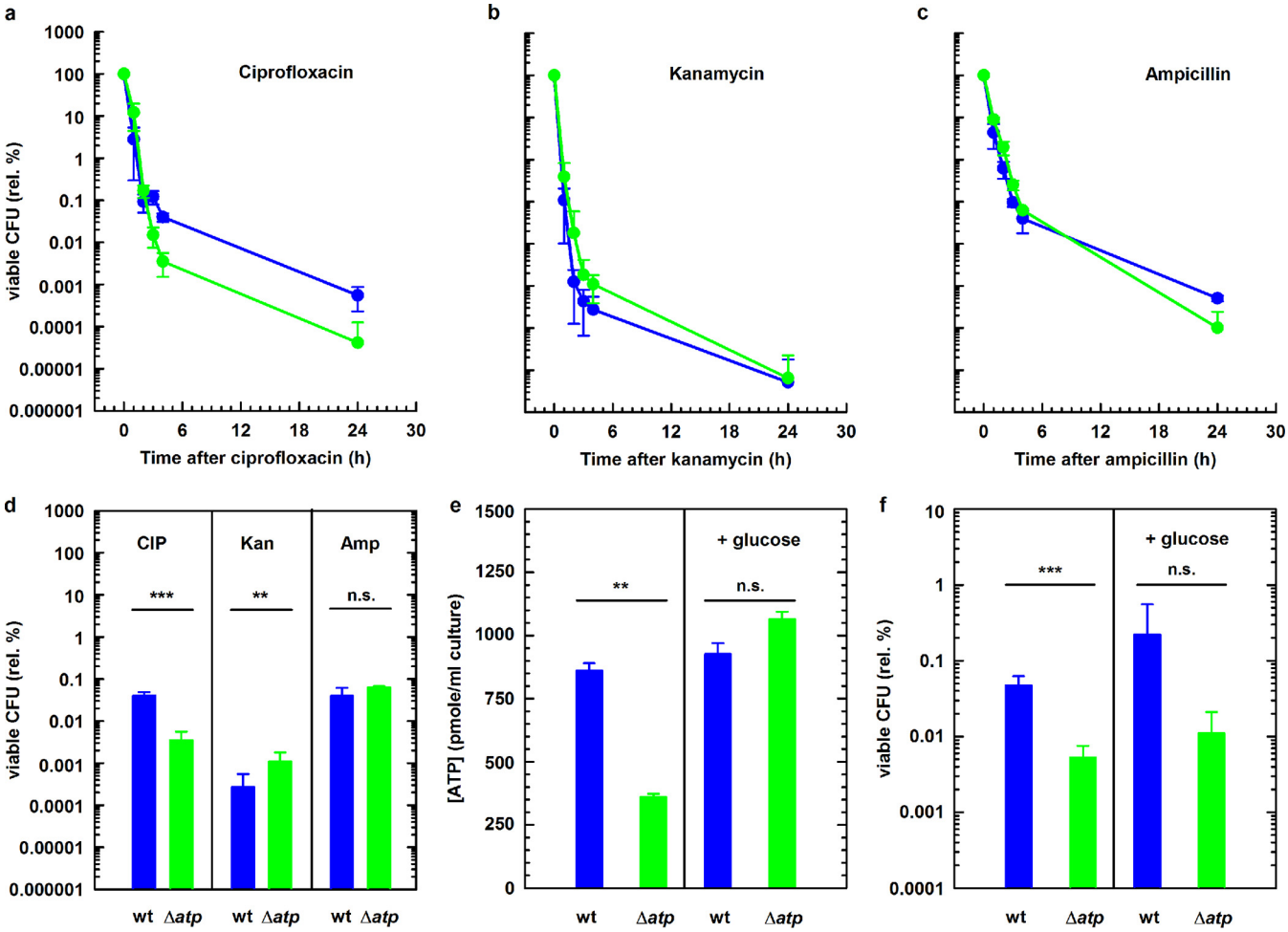

FIG 1 Bactericidal antibiotic killing does not correlate with ATP levels. (a to c) Bacterial cultures of either the wild-type (blue) or Δ*atp* operon deletion (green) strains were grown to an optical density of 0.5 and then diluted into media containing the indicated antibiotics to a final concentration of approximately 10^7 CFU/mL. At the times indicated, samples were removed, and dilutions were plated onto agar plates without antibiotics for the determination of the surviving CFU. (a) The Δ*atp* deletion strain showed a 10-fold increased killing and reduced persister cell population after challenge with 1 μg/mL ciprofloxacin (CIP), but not after challenge with 50 μg/mL kanamycin (b) or 25 μg/mL ampicillin (c). (d) At 4 h post-antibiotic challenge, significant but opposite killing responses were observed for ciprofloxacin and kanamycin treatments but little or no difference for the β-lactam ampicillin. Glucose supplementation (0.2%, wt/vol) of the cultures increased the intracellular ATP concentration of the Δ*atp* mutant to similar levels as the wild-type strain (e) but did not significantly affect survival after ciprofloxacin challenge (f). See also Fig. S1 and Table S2. wt, wild type.

mutant background and the corresponding mutation in the wild-type background, indicating an increase in the persister cell population (see Fig. S2 and Table S2 in the supplemental material). Furthermore, for the Δ*atp* Δ*fre* and Δ*atp* Δ*nuo* strains, there was also no longer a significant difference with respect to the original wild-type strain (see Table S2). These results suggested these gene products or pathways were likely to be involved in the increased cell killing and reduced persister cell formation in the Δ*atp* strain.

The *fre* gene encodes an NAD(P)H-dependent flavin reductase, implicated in the generation of hydroxyl radicals when high rates of reduced flavin ($FADH_2$) production lead to the transfer of electrons to free, ferrous iron (Fe^{2+}), supporting the Fenton reaction and production of hydroxyl radicals (30, 31). However, these reactions are expected to occur only under conditions of high NADH levels, generally present under low respiration rates (26). Similarly, *nadB*, encoding aspartate oxidase, the first enzyme in the nicotinamide (NAD) biosynthetic pathway, contributes to substantial superoxide and H_2O_2 production (27, 29). The *nuo* operon and the *ndh* gene encoding the NADH dehydrogenases NDH-1 and NDH-2, respectively, are both associated with the respiratory chain and are involved in reoxidation of NADH to NAD^+. While the effect on

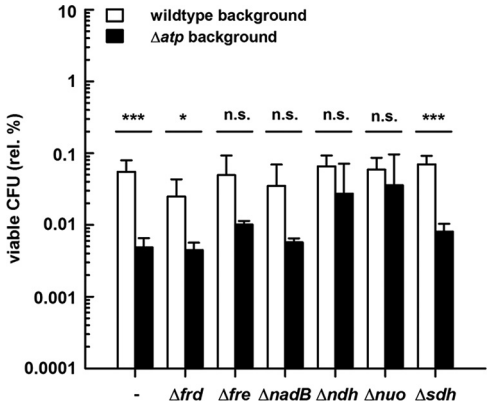

FIG 2 Metabolic genes involved in the increased killing by ciprofloxacin for the Δ*atp* deletion strain. The wild-type (open bars) or Δ*atp* deletion strains (filled bars) harboring additional deletion mutations as indicated were grown and challenged with ciprofloxacin as in Fig. 1. Shown are the results comparing survival (viable CFU) 4 h postchallenge (see also Fig. S2 and Table S2).

bacterial killing was not as great for the *sdhCDAB* operon deletion mutation, succinate dehydrogenase has also been implicated in superoxide and H_2O_2 production (26, 27). These gene products are therefore involved in either contributing to the pools of NADH/NAD^+ (*nadB*, *ndh*, and the *nuo* operon) or generating $FADH_2$ (*fre*) or have been previously implicated in ROS production (*frdABCD* and *sdhCDAB*) and could therefore contribute to hydroxyl radical-generating pathways.

Elevated NADH pools in the Δ*atp* operon deletion strain. Elevated central carbon metabolism reactions fueling substrate-level phosphorylation in the Δ*atp* operon deletion mutant could lead to an increase in the NADH pools driving the flavin reductase to increased rates of $FADH_2$ production with a subsequent transfer of electrons to ferrous iron (32, 33). While the increased respiration of the Δ*atp* mutant might be expected to regenerate NAD^+ to maintain the NADH/NAD^+ ratios of the cell, it remained possible that the increased reliance on other metabolic pathways for ATP synthesis might affect the homeostasis. We therefore determined the NADH pools in the wild-type and Δ*atp* deletion strains. As shown in Fig. 3a, the Δ*atp* deletion mutant showed significantly higher NADH levels than the wild-type strain prior to ciprofloxacin challenge, and it remained 2-fold higher than the wild-type strain within the first

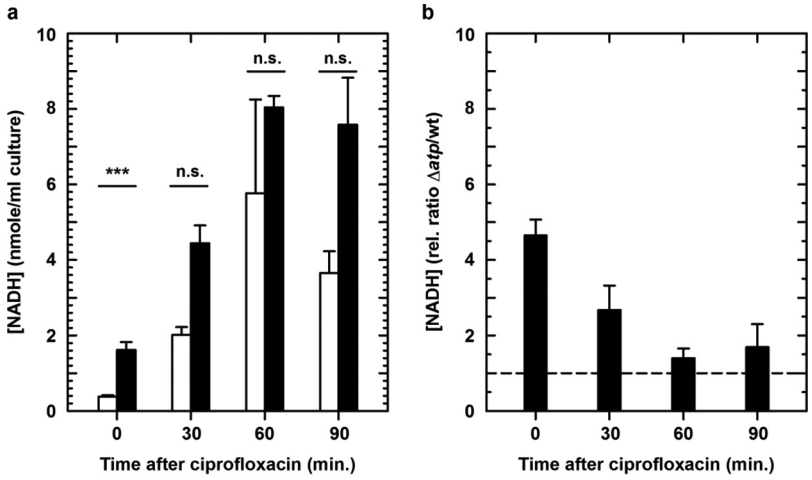

FIG 3 NADH levels are elevated in the Δ*atp* deletion strain relative to the wild-type strain. (a) Cultures of either the wild-type (white bars) or Δ*atp* deletion strain (black bars) were challenged with 1 μg/mL ciprofloxacin, and samples were removed prior to and at the times indicated for determination of the NADH concentrations. (b) Relative ratios of total NADH in the Δ*atp* mutant compared with those of the wild-type strain shown in a. The dashed line indicates a relative ratio of mutant/wild-type of 1.

30 min postchallenge (Fig. 3b). These results indicated that the prerequisite of elevated NADH levels for increased $FADH_2$ production and subsequent hydroxyl radical generation by flavin reductase was likely present in the Δ*atp* deletion strain.

Elevated endogenous ROS implicated in the increased killing by ciprofloxacin. We next examined a number of additional culture conditions to either support or exclude elevated ROS formation as a contributing factor in the increased killing by ciprofloxacin for the Δ*atp* deletion strain. As shown in Fig. 4a, anaerobic growth completely eliminated the differences in cell killing/persister cell formation of the Δ*atp* operon deletion strain relative to the wild-type strain, indicating that oxygen was involved (see Fig. S3 in the supplemental material). Likewise, the addition of the iron chelator 2,2¨-dipyridyl (dipyridyl) to the cultures completely compensated for the reduced cell survival (Fig. 4b and Fig. S3), supporting a role for the intracellular iron pools in the increased cell killing and reduced persistence in the Δ*atp* deletion strain.

As shown in Fig. 4c and Fig. S3, supplementation of the Δ*atp* deletion mutant with NAD^+ also increased the survival of the Δ*atp* deletion strain, although the survival remained significantly lower than that of the wild type supplemented with NAD^+. However, survival of the Δ*atp* deletion strain was significantly increased in the presence of exogenous NAD^+ compared with that of the Δ*atp* mutant grown without NAD^+ additions (Table S2). External NAD^+ itself cannot be taken up by cells; it is normally hydrolyzed in the periplasm to nicotinamide mononucleotide (NMN) and then transported across the inner membrane where it enters the biosynthetic pathways to regenerate NAD^+ and NADP in the cytoplasm (34, 35). Exogenous NAD^+ might be expected to result in an increase in the pools of NADH, which ought to increase the flavin reductase generation of $FADH_2$ and hence additional hydroxyl radicals (see above). However, NAD^+ also inhibits *de novo* biosynthesis and the production of NADH-dependent superoxide by NADH dehydroxygenase (36, 37), and H_2O_2 production by NadB is also inhibited by nicotinamide precursors (27). Supplementation of cultures with NAD^+ did not result in a further increase in survival of the Δ*fre* and Δ*atp* Δ*fre* strains (Fig. 4d and Fig. S3).

As the preceding results appeared to support the idea that elevated NADH levels contributing to ROS production may have been responsible for the increased cell killing/reduced persister cell formation of the Δ*atp* mutant, we also performed ciprofloxacin killing assays in media supplemented with glutathione, the major cellular reductant of reactive oxygen species, such as superoxide and hydrogen peroxide (38–40). The biosynthesis of glutathione is ATP dependent, and the cycling of efflux and uptake has also been found to be sensitive to the ATP concentrations (40, 41). As seen in Fig. 4e, glutathione supplementation increased survival and persister cell formation of the Δ*atp* mutant after ciprofloxacin challenge, indicated by the loss of a significant difference for survival of the Δ*atp* mutant compared with that of the wild-type strain. Similar protection by glutathione addition has also been observed for *E. coli* after ciprofloxacin and norfloxacin treatments (4, 42). Note that prior treatment of the growth medium with catalase did not improve survival of the Δ*atp* mutant compared with that of the wild-type strain, indicating that exogenous H_2O_2 was not involved (43, 44).

Loss of cytosolic superoxide dismutases abrogates the increased cell killing. The cytosolic superoxide dismutases SodA and SodB are involved in the detoxification of superoxide, generating H_2O_2 as an end product, which in turn is detoxified by a number of different catalases in both *E. coli* and *Salmonella* (45). To determine whether H_2O_2 production by the superoxide dismutases might contribute to increased ROS production, we introduced deletion mutations of *sodA* and *sodB* into both the wild-type and Δ*atp* deletion strains and performed ciprofloxacin killing assays. As seen in Fig. 4f, the loss of both SodA and SodB fully complemented for the reduced survival of the Δ*atp* mutant after ciprofloxacin challenge, showing persistence levels significantly higher than the wild-type strain. These results indicated that endogenous H_2O_2 production by the superoxide dismutases was a major factor contributing to the reduced cell survival. In addition, the rate of killing of the Δ*atp* Δ*sodA* Δ*sodB* strain after ciprofloxacin challenge appeared to be reduced, with increased survival at all time points

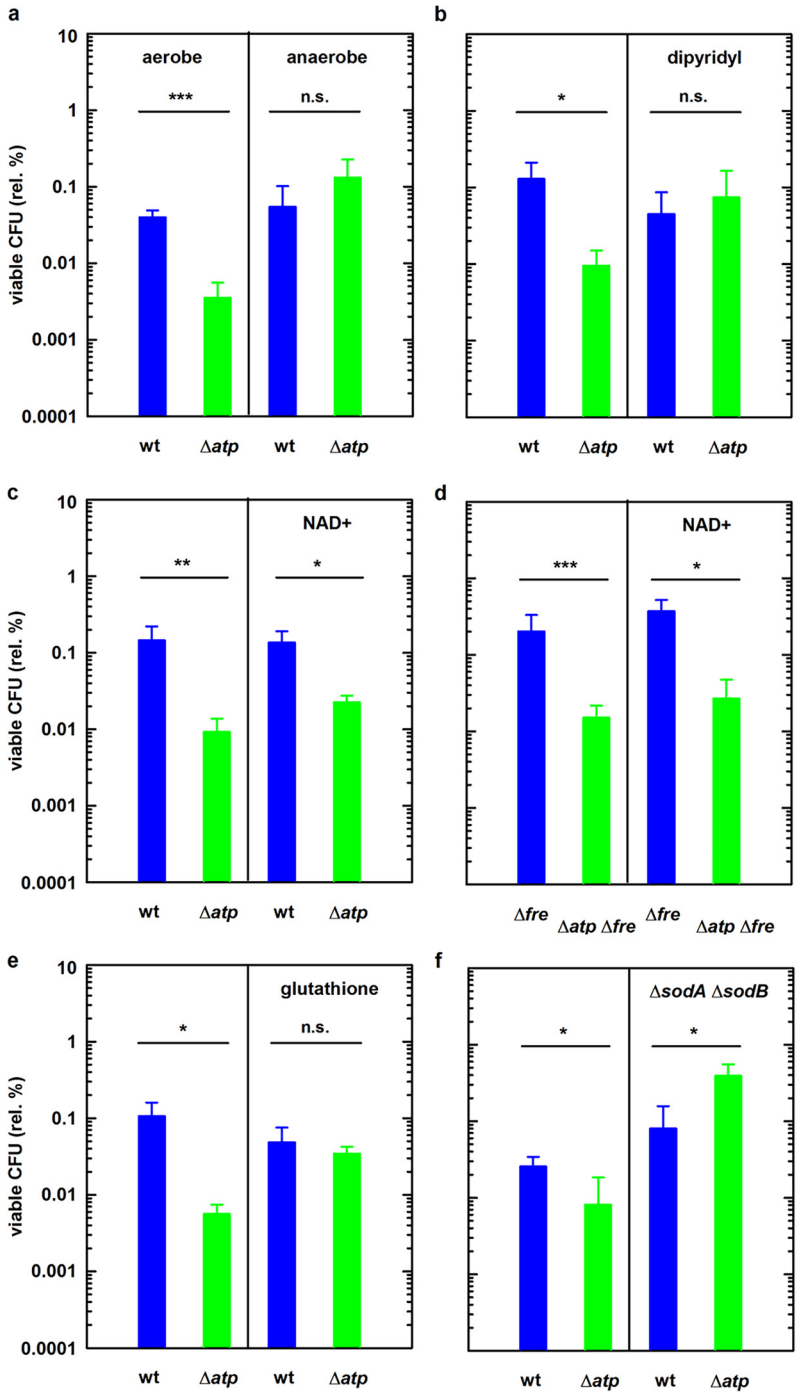

FIG 4 Anaerobiosis, iron chelation, exogenous glutathione, and loss of cytoplasmic superoxide dismutases eliminate the increased ciprofloxacin killing of the Δ*atp* deletion mutant. (a) Aerobic or anaerobic cultures of the wild-type (blue bars) or Δ*atp* deletion mutant (green bars) were challenged for 4 h with 1 μg/mL ciprofloxacin and the surviving, viable CFU/mL were determined. (b) Cultures of the wild-type or Δ*atp* deletion mutant strains were split into 5-mL cultures containing 1 μg/mL ciprofloxacin in the presence or absence of 0.5 mM 2,2′-dipyridyl (dipyridyl) as indicated, and viable CFU/mL 4 h postchallenge were determined. (c) Cultures of the wild-type (blue bars) or Δ*atp* deletion mutant (green bars) strains were split into 5-mL cultures containing 1 μg/mL ciprofloxacin in the presence or absence of 1 mM NAD^+ as indicated, and viable CFU/mL were determined 4 h postchallenge. (d) Cultures of the Δ*fre* (blue bars) or Δ*atp* Δ*fre* (green bars) deletion mutant strains supplemented with 1 mM NAD^+ and challenged with 1 μg/mL ciprofloxacin as in c. (e) Cultures of the wild-type or Δ*atp* deletion mutant strains were challenged with 1 μg/mL ciprofloxacin in the presence or absence of 2 mM glutathione as indicated, with viable CFU/mL determined 4 h postchallenge. (f) Cultures of Δ*sodA* Δ*sodB* (blue bars) or Δ*atp* Δ*sodA* Δ*sodB* (green bars) deletion mutant strains were challenged with 1 μg/mL ciprofloxacin as above, and viable CFU/mL were determined 4 h postchallenge. For all panels (a to f), viable CFU/mL for the full, 24-h duration of the antibiotic killing assays are shown in Fig. S3. See also Table S2.

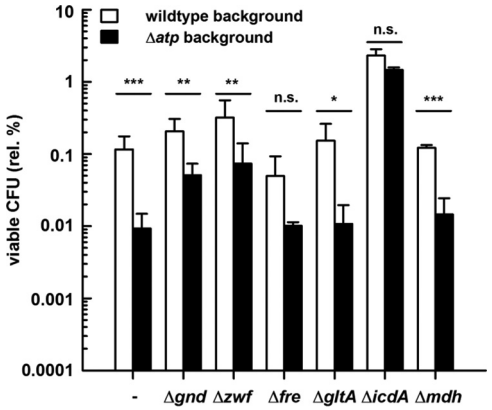

FIG 5 Inactivation of genes involved in NADPH production alleviate the increased killing by ciprofloxacin for the Δ*atp* deletion strain. The wild-type (open bars) or Δ*atp* deletion strains (filled bars) harboring the indicated gene deletion mutations were grown and challenged with ciprofloxacin as in Fig. 1. Shown are the results comparing survival 4 h postchallenge for strains harboring deletion mutations of 6-phosphogluconate dehydrogenase (Δ*gnd*), glucose-6-phosphate-1-dehydrogenase (Δ*zwf*), citrate synthase (Δ*gltA*), the NADP-specific isocitrate dehydrogenase (Δ*icdA*), and malate dehydrogenase (Δ*mdh*). For comparison, assays were also repeated for flavin reductase (Δ*fre*). Viable CFU/mL for the full, 24-h duration of the antibiotic killing assays are shown in Fig. S4. See also Table S2.

relative to the wild-type strain (Fig. S3l and Table S2). Interestingly, in four, independent assays, the Δ*atp* Δ*sodA* Δ*sodB* strain was found to completely recover by 24 h postciprofloxacin challenge, whereas the isogenic wild-type Δ*sodA* Δ*sodB* strain did not. Notably, both strains have identical MIC values for ciprofloxacin (Table S2), and while the Δ*atp* Δ*sodA* Δ*sodB* strain appeared to have a slightly greater minimum duration of killing (MDK) suggestive of a ciprofloxacin-tolerant phenotype, growth recovery at 24 h would indicate resistance, which is clearly not the case based on the killing curve (Fig. S3l). Furthermore, determinations of the minimum bactericidal concentration (MBC) showed the same bactericidal effects for both the *atp*$^+$ Δ*sodA* Δ*sodB* and Δ*atp* Δ*sodA* Δ*sodB* strains for concentrations of ciprofloxacin up to 8 μg/mL (16$\times$ MIC), ruling out antibiotic-induced persistence as an explanation for this phenotype (Table S2). The behavior of the Δ*atp* Δ*sodA* Δ*sodB* strain at 24 h postchallenge would therefore appear to defy a simple categorization as either tolerant, resistant, or persistent (see Discussion).

Elevated NADPH pools also contribute to increased bacterial killing. While the results shown in Fig. 3 indicated elevated NADH pools in the Δ*atp* mutant strain, both NADH and NADPH can serve *in vitro* as the substrates for the flavin reductase Fre (30, 46). We therefore examined the effects of mutations in genes involved in NADPH production on survival and persister cell formation in the wild-type and Δ*atp* deletion strain. The *gnd* and *zwf* gene products provide NADPH in the first set of reactions in the pentose phosphate pathway. Another major source of NADPH in the cell, however, is isocitrate dehydrogenase, encoded by the *icdA* gene, which is part of the tricarboxylic acid (TCA) cycle and responsible for approximately 20% to 25% of the NADPH pool in *E. coli* (47). As shown in Fig. 5, introduction of Δ*gnd*, Δ*zwf*, or Δ*icdA* mutations all increased the survival and persister cell formation of the Δ*atp* mutant 10- to 100-fold. Furthermore, introduction of the Δ*icdA* mutation into the Δ*atp* mutant background increased the persistence to levels equivalent to that of the *atp*$^+$ Δ*icdA* strain, which also showed a greater than 10-fold increase relative to the wild-type strain. Introduction of the Δ*gnd* and Δ*zwf* mutations still showed significant differences between the wild-type (*atp*$^+$) and Δ*atp* deletion strain backgrounds; however, deletion of these genes significantly increased survival compared with the Δ*atp* deletion strain, strongly suggesting the NADPH pools also contributed to the loss of persistence (Table S2).

Notably, mutations in *icdA* leads to the accumulation of not only isocitrate but also citrate (48), and high levels of citrate are known to cause growth defects, likely by sequestering intracellular iron. Furthermore, mutations in *icdA* are associated with resistance to

nalidixic acid and other antibiotics, and the export of iron-citrate complexes has been proposed to protect bacteria from hydroxyl radicals generated through the Fenton reaction (49, 50). Deletion mutations in *gltA*, encoding citrate synthase, or *mdh*, encoding malate dehydrogenase, showed no significant effects on either the wild-type strain or on the increased cell killing of the Δ*atp* operon deletion strain. These observations therefore suggested an additional involvement of the NADPH pools in the reduced cell survival and persistence of the Δ*atp* mutant after ciprofloxacin challenge.

Elevated ROS and DNA damage contribute to the increased killing by ciprofloxacin. The preceding observations suggested that the activities of the central carbon metabolic/glycolytic pathways during aerobic, exponential growth resulted in a metabolic situation in which the *S.* Typhimurium Δ*atp* mutant would be more susceptible to elevated ROS. As shown in Fig. 6a, assays for ROS production using CellROX DeepRed indicated a small (ca. 20%), but reproducible elevated ROS production in the Δ*atp* mutant during normal, exponential growth prior to ciprofloxacin treatment. Despite a 15- to 20-fold increase in ROS levels after ciprofloxacin challenge, no significant difference in the final levels between the wild-type and Δ*atp* mutant was apparent (see Table S2). Similar assays using hydroxyphenyl fluorescein (HPF), intended to be specific for hydroxyl radicals (51), also showed an elevated (ca. 20%), steady-state hydroxyl radical concentration in the Δ*atp* mutant relative to the wild-type strain, but which was not statistically significant (Fig. 6b). After ciprofloxacin challenge, the slight increase observed prior to the treatment was still observed but was now statistically significant.

The apparent increased levels of endogenous ROS in the Δ*atp* mutant suggested a possible explanation for the reduced persistence seen after challenges with ciprofloxacin. However, the Δ*atp* strain does not show obvious growth defects, either on rich medium-based agar plates or in liquid media, other than a slightly smaller colony size and an earlier entry into post-exponential growth. Nevertheless, one might expect some form of cell damage would be associated with elevated, basal levels of ROS, despite the apparent ability of the Δ*atp* strain to compensate during normal growth. We therefore also performed terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assays for determination of DNA fragmentation. As shown in Fig. 6c, despite assay-to-assay variability in the incorporation, the amount of fluorescein-dUTP labeling in the Δ*atp* deletion mutant was significantly higher than the wild-type strain, both prior to and after challenge with ciprofloxacin, indicating the presence of increased DNA strand breaks.

Exogenous glutathione was capable of increasing the survival of the Δ*atp* deletion strain after ciprofloxacin challenge (Fig. 4e), suggesting a role for limitations in glutathione pools in the increased sensitivity of the Δ*atp* deletion strain to ciprofloxacin. As shown in Fig. 6d, determinations of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) for the wild-type strain prior to ciprofloxacin addition was consistent with values previously reported for *E. coli* (52). In contrast, the GSH:GSSG ratio of the Δ*atp* strain was approximately half that of the wild-type strain when sampled prior to ciprofloxacin treatment. Although the GSH:GSSG ratio was reduced for both the wild-type and Δ*atp* deletion strains post-ciprofloxacin challenge, there was no longer a significant difference between the two strains. Large reductions in the GSH:GSSG ratios after challenges with the oxidant menadione have also been observed in *E. coli*, consistent with a role for glutathione in maintaining the cytosolic redox status under conditions of increased ROS (39).

Expression of genes involved in oxidative stress are elevated in the Δ*atp* strain. As the results seen in Fig. 6 indicated elevated, endogenous ROS and DNA damage in the Δ*atp* operon deletion strain prior to ciprofloxacin challenges, we also examined the expression levels of promoters for genes involved in oxidative stress and SOS response pathways in the wild-type and Δ*atp* deletion strains. As shown in Fig. 7, consistent with elevated hydrogen peroxide and oxidative stress conditions, we observed approximately 2-fold elevated levels of expression of the OxyR-dependent *ahpC* and *oxyS*, and SoxR-dependent *soxS* gene promoters in the Δ*atp* operon deletion strain compared to the wild-type under normal, aerobic growth conditions (45, 53). While the

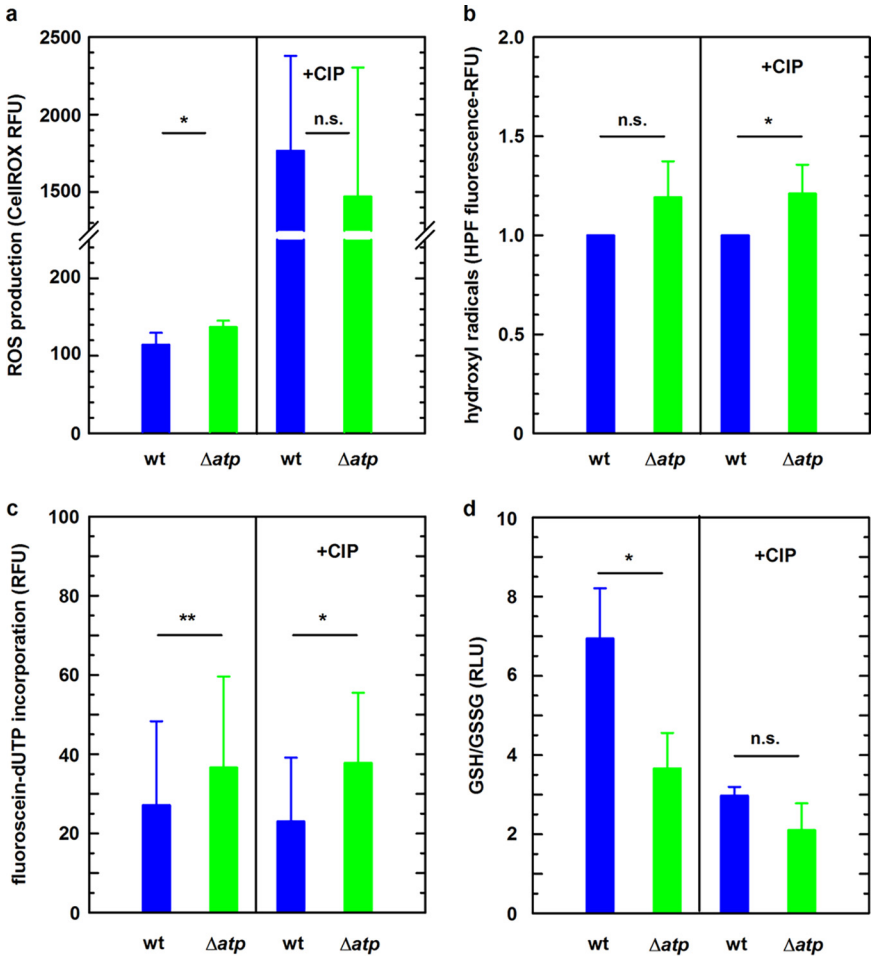

FIG 6 The Δ*atp* deletion strain shows increased levels of endogenous superoxide and hydroxyl radical production, DNA damage, and reduced levels of reduced glutathione during normal, exponential growth. (a) Reactive oxygen species (ROS) was determined by incubation of mid-log cultures of either wild-type (blue bars) or Δ*atp* operon deletion (green bars) strains in the presence of CellROX in the absence (left half-panel) or presence of ciprofloxacin (+CIP), followed by determination of the relative fluorescence by flow cytometry. (b) Hydroxyl radical production was determined by incubation of cultures of either wild-type (blue bars) or Δ*atp* operon deletion (green bars) strains with HPF in the absence (left half-panel) or presence of ciprofloxacin (+CIP), followed by determination of the relative fluorescence by flow cytometry. Due to the high variability in the fluorescence on different days, the fluorescence data were normalized to that for the wild-type strain. (c) The presence of DNA strand breaks was determined by incorporation of fluorescein-dUTP in TUNEL assays performed with cultures of wild-type (blue bars) or Δ*atp* operon deletion (green bars) strains in the absence (left half-panel) or presence of ciprofloxacin (+CIP), and the level of incorporation was determined by flow cytometry. (d) The ratio of reduced (GSH) to oxidized glutathione (GSSG) was determined from mid-log cultures of wild-type (blue bars) or Δ*atp* operon deletion (green bars) strains in the absence (left half-panel) or presence of ciprofloxacin (+CIP), and the ratios of the relative intracellular pools were reported as relative luminescence (RLU). For additional details, see Materials and Methods and Table S2.

expression of the *ahpC* promoter was significantly higher in the Δ*atp* operon deletion strain at all times during growth (Fig. 7g), significantly higher expression was observed for the *oxyS* and *soxS* promoters beginning at 4 h postinoculation, corresponding to the time and growth phase when the cultures were challenged with ciprofloxacin in the persister assays. These observations indicated that the activation status of the regulators involved in responses to oxidative stress (OxyR and SoxR) was higher in the Δ*atp* strain. Notably, genes involved in the SOS response (*recA*, *sulA*, *dinP*, and *umuDC*) showed either the same or reduced levels of expression compared to the wild-type strain. A control promoter fusion of the *mgtCB* promoter responsive to the ATP levels (54, 55) showed significantly higher expression in the Δ*atp* deletion strain (Fig. 7a), consistent with the reduced ATP levels (Fig. 1d). However, the reduced intracellular

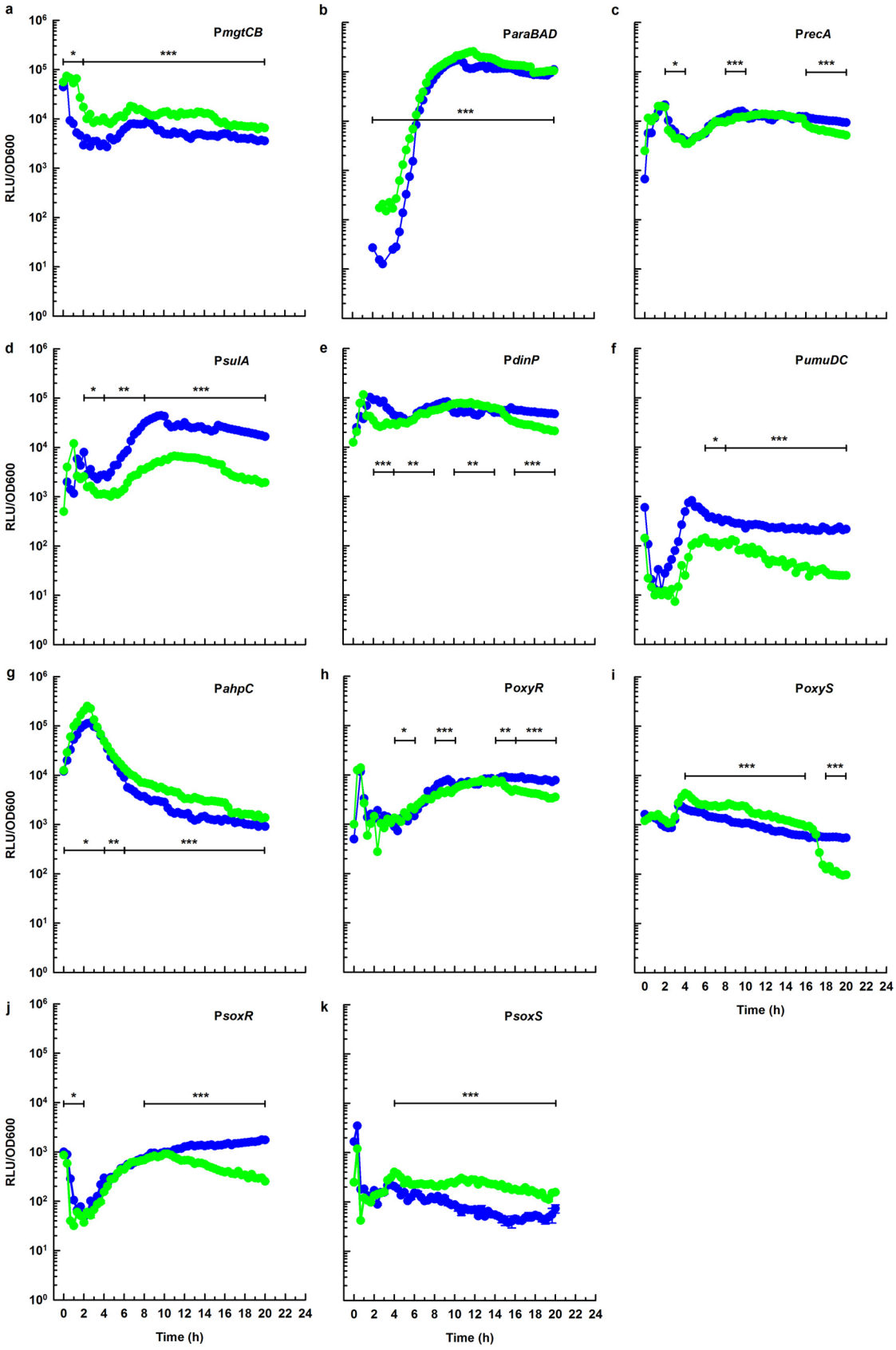

FIG 7 Genes involved in oxidative stress show elevated expression levels in the Δ*atp* operon deletion strain. Promoters for the expression of genes involved in oxidative stress (*ahpC*, *oxyR*, *oxyS*, *soxR*, and *soxS*) or the SOS response (*recA*, *sulA*, *dinP*, and

(Continued on next page)

ATP levels did not affect expression of the arabinose-induced *araBAD* promoter, which showed essentially the same induction kinetics in both the wild-type and Δ*atp* deletion strain backgrounds (Fig. 7b), verifying that the reduced ATP levels in the Δ*atp* operon deletion strain did not detrimentally affect either the promoter reporter fusions, or transcription and translation in general.

DISCUSSION

A number of publications have suggested that reduced ATP levels in both Gram-negative and -positive bacteria is a key metabolic trait evoking increased survival and persistence to bactericidal antibiotics (13–15, 56). Here, we show that despite significantly reduced ATP levels in the *S.* Typhimurium Δ*atp* operon deletion strain, the strain shows an approximately 10-fold lower persistent population of bacteria which survive a challenge with the fluoroquinolone antibiotic ciprofloxacin. Increasing the ATP levels by glucose addition did not affect the increased susceptibility (Fig. 1c and d). As the initial rate of killing of both strains appears to be identical (Fig. 1; Fig. S1 to S4 in the supplemental material), with the exception of the Δ*atp* Δ*sodA* Δ*sodB* strain (Fig. S3l, see below), we conclude that reduced ATP levels leads to a reduction in the spontaneously occurring persister cell population during normal, exponential growth. Notably, these results are also consistent with a prior study with *S.* Typhimurium in which elevated ATP levels also did not result in increased bactericidal killing by ciprofloxacin (57).

The results of our study indicate that it is not the reduced ATP levels *per se* which are involved in increased survival/persistence when challenged with bactericidal fluoroquinolone antibiotics; rather, it is the central carbon metabolism pathways generating NAD(P)H which influence bactericidal antibiotic killing. The introduction of additional mutations in the *fre*, *ndh*, *nuo*, and *sdhCDAB* operons, of which all have been implicated in the generation of ROS, resulted in decreased killing and increased persister cells in the Δ*atp* operon deletion strain (Fig. 2). Likewise, the introduction of mutations affecting NADPH production also reduced the killing of the Δ*atp* deletion strain (Fig. 5), likely due either to a role in ROS production by the respiratory NDH-I (28) or as a substrate for the flavin reductase Fre (26, 30, 46). Together, these observations are consistent with a model in which the elevated NAD(P)H pools in the Δ*atp* deletion strain led to an increase in the adventitious reduction of flavins (FAD, FMN, and riboflavin) to increase the pool of $FADH_2$. $FADH_2$ can transfer electrons to ferric iron ($Fe3^+$) to yield ferrous iron ($Fe2^+$), which in turn, in the presence of H_2O_2, can generate hydroxyl radicals (26, 32, 33). This conclusion is supported by the observations that the deletion of the *fre* gene encoding flavin reductase (Fig. 2) or chelation of iron by 2,2′-dipyridyl (Fig. 4) led to the recovery of persister cell formation by the Δ*atp* strain.

The H_2O_2 most likely originates as a superoxide through the increased respiration and oxygen consumption rates characteristic for such *atp* mutants (20, 21) and subsequently detoxified through the cytosolic superoxide dismutases SodA and SodB to H_2O_2. The introduction of Δ*sodA* and Δ*sodB* mutations eliminated the reduced persistence of the Δ*atp* deletion strain (Fig. 4f), suggesting that apparently even small increases in the basal levels of superoxide and H_2O_2 shift the Δ*atp* operon mutant precariously close to the limit of internal H_2O_2 levels which can be effectively detoxified rapidly enough to avoid hydroxyl radical production. This suggestion is consistent with the observations from the promoter expression assays shown in Fig. 7. During exponential growth in the same medium used for the persister assays, the promoter activities

FIG 7 Legend (Continued)
umuDC), as well as controls for ATP levels (*mgtCB*) or transcriptional activation (*araBAD*), were cloned upstream of a promoterless *luxCDABE* operon in the plasmid pDEW201 (86) and introduced into the wild-type (blue curves) or Δ*atp* operon deletion (green curves) strains. The luminescence in relative light units (RLUs) and optical density (OD_{600}) of the cultures was determined at 30-min intervals. The wild-type and Δ*atp* operon deletion strains harboring the *araBAD* promoter (P*araBAD*) fusion with the *lux* operon were inoculated into media supplemented with 0.1% arabinose to verify that the reduced ATP levels in the Δ*atp* operon deletion strain did not limit either the induction kinetics or duration of gene expression for the various promoter-*lux* operon fusions. For reasons of clarity, regions of the growth curves with nonsignificant differences between the two strain backgrounds are not shown. The results shown are representative of at least two, independent determinations. See also Table S2.

of genes involved in oxidative stress (*ahpCF*, *oxyS*, and *soxS*) showed significantly higher expression levels relative to the wild type. However, promoters for genes of the SOS response regulon (*sulA*, *dinP*, and *umuDC*) showed either unchanged or lower levels of expression, which is an unexpected result considering the apparently higher levels of DNA damage seen in the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays (Fig. 6). In prior studies, it has been estimated that the endogenous H_2O_2 concentration in *E. coli* is around 20 nM. At 100 nM (0.1 μM), there is an increase in mutagenic effects, but growth inhibition is seen only at concentrations of 1 to 2 μM (58, 59). In *E. coli*, 1 μM H_2O_2 has been reported to produce "crippling" levels of DNA damage, and submicromolar levels show DNA damage (51, 60). In *E. coli*, OxyR is activated at levels of H_2O_2 of $>$0.1 μM. As seen in Fig. 7g, *ahpCF* promoter expression is significantly elevated at all times in the Δ*atp* strain, strongly suggesting that the 0.1 μM level of H_2O_2 has already been reached (*ahpCF* expression is OxyR regulated). However, if DNA damage due to oxidative stress accumulates, then why are the promoters involved in the SOS response not active?

We suggest that this apparent contradiction may be explained by the reduced ATP pools of the Δ*atp* strain. Quinolone antibiotics are bactericidal due to the inhibition of gyrase and topoisomerase IV leading to lethal double-stranded DNA (dsDNA) breaks (61). Repair of dsDNA breaks requires the sequential activities of two protein complexes, namely, RecBCD and RecA, of which both have ATP-dependent activities. RecBCD generates single-strand DNA (ssDNA) at the ends of the dsDNA breaks, which in turn is recognized by RecA. RecA-ATP binds to the ssDNA, and the RecA-ATP filaments search for regions of homology for homologous recombination repair of the damage (62–64). ATP (hydrolysis) is thought to speed up the homology search and repair process (65, 66). In addition, the RecA-ATP-ssDNA complex (RecA* coprotease) is also involved in the self-cleavage of LexA, which in turn leads to the expression of genes of the SOS response, including *dinP*, *umuDC*, and *sulA* (62, 67–69). During fast growth in rich media, as used here, the bulk of the ATP consumed is used for ribosome and protein synthesis, i.e., cell mass accumulation (70, 71). With this in mind, it is possible that the reduced ATP levels in the Δ*atp* operon deletion mutant (Fig. 1e), coupled with the high demand for ATP by protein synthesis to maintain the growth rate in rich media as used here, may limit the rate at which either RecBCD generates ssDNA or RecA-ATP-ssDNA formation, leading to slowed repair of DNA breaks. Concomitantly, the RecA* coprotease activity needed for LexA cleavage and SOS response gene expression would also be reduced, resulting in the lower expression of LexA-regulated promoters. This proposal would explain the reduced persister cell population in the Δ*atp* strain. The increased DNA damage due to increased levels of endogenous superoxide and H_2O_2 production, coupled with a slower recombination repair and SOS response would result in the Δ*atp* mutant being more vulnerable to the effects of fluoroquinolone antibiotics. During rapid growth, a challenge with a gyrase/topoisomerase IV inhibitor, such as ciprofloxacin, could simply overwhelm a larger fraction of the population of the Δ*atp* mutant which is dependent on glycolysis and other NAD(P)H-generating reactions for ATP production compared with the wild-type strain.

The increased antibiotic susceptibility and reduced persister cell population shown in this study was observed only for the fluoroquinolone ciprofloxacin but not for other classes of antibiotics also considered bactericidal. The decreased survival of the Δ*atp* operon mutant is likely related to the action of ciprofloxacin on the gyrase and/or topoisomerase IV and subsequent DNA damage. The ciprofloxacin killing/persister assays in this study were performed in a *gyrA* mutant background of *S.* Typhimurium. As pointed out in the Introduction, prior studies have suggested that the production of ROS by bactericidal antibiotics is likely the result of metabolic perturbations in response to antibiotic stress (4). In that study, the authors found no significant ROS production when an *E. coli gyrA* mutant was challenged with norfloxacin, although it is known that (fluoro)quinolone antibiotics inhibit secondary targets, such as topoisomerase IV, when the primary target (gyrase) is resistant (10–12). This observation appeared somewhat unusual, as the MIC for the *gyrA*(D87Y) mutant (approximately 250 ng/mL) is $>$10-fold higher than that

for the wild-type strain (16 ng/mL); but there is nevertheless a complete growth inhibition of the *gyrA*(D87Y) mutant at the MIC, which we would also interpret as antibiotic stress and which is accompanied by substantial bacterial killing. Likewise, the lethal effects due to the accumulation of DNA strand breaks at bactericidal concentrations of fluoroquinolones are essentially the same in both *gyrA*$^+$ and *gyrA* mutant strains (61). Consistent with this idea, the killing curves and persister assays performed with ciprofloxacin for *gyrA*$^+$ and *gyrA*(D87Y) strains and their Δ*atp* operon deletion counterparts showed reduced persisters in both respective Δ*atp* deletion strain backgrounds, indicating the reduced persistence was independent of the status of the *gyrA* gene (Fig. S1). An additional reason for examining persistence in a *gyrA* mutant background here is the observation that *gyrA* mutations are the most frequent cause of (fluoro)quinolone resistance both *in vitro* and in clinical isolates (72, 73). As our persister assays were all performed for a duration of 24 h, the use of the *gyrA*(D87Y) strain background avoided the accumulation of spontaneous resistance during the course of the assays.

As shown in Fig. 1 and the Fig. S1 to S4, ciprofloxacin is clearly bactericidal at a concentration of 4× MIC (1 μg/mL) in both the *S.* Typhimurium *gyrA*(D87Y) and *gyrA* (D87Y) Δ*atp* operon deletion strains for the full 24 h of the persister assays. An interesting exception to this finding was the results for the Δ*atp* Δ*sodA* Δ*sodB* strain, which showed recovery 24 h after ciprofloxacin challenge, in contrast to the otherwise wild-type (*atp*$^+$) Δ*sodA* Δ*sodB* strain which showed a continuous loss of viable CFU/mL over 24 h (Fig. S3l). The results for the Δ*atp* Δ*sodA* Δ*sodB* strain are reminiscent of what Balaban et al. have referred to as "drug-induced persistence," where the signal for the generation of persisters is the antibiotic itself (74). As indicated in Table S2, the MIC values for the Δ*atp* Δ*sodA* Δ*sodB* and *atp*$^+$ Δ*sodA* Δ*sodB* strains are the same, namely, in both cases, 500 ng/mL, which is slightly higher than that of the wild-type and Δ*atp* strains (250 ng/mL). Furthermore, the determination of the minimum bactericidal concentrations (MBCs) showed the same, low (≤0.001%) viable CFU recovery for both the *atp*$^+$ Δ*sodA* Δ*sod* and Δ*atp* Δ*sodA* Δ*sodB* strains at concentrations up to 8 μg/mL (Table S2), indicating the concentration of ciprofloxacin used in the persister assays (1 μg/mL) was indeed bactericidal, and argues against drug-induced persistence (74).

In the case of ciprofloxacin, it was found that the induction of expression of the toxin TisB by ciprofloxacin leads to persister formation in *E. coli* by lowering the proton motive force (PMF) of the membrane, thereby reducing ATP synthesis (17). The authors also found that the expression of *tisB* was activated as part of the SOS response, i.e., that the DNA damage caused by ciprofloxacin leads to TisB-dependent reductions in ATP for the generation of persistent bacteria. Our observations that full recovery after a 24-h challenge with ciprofloxacin occurred only for the Δ*atp* Δ*sodA* Δ*sodB* and not for the *atp*$^+$ Δ*sodA* Δ*sodB* strain would imply that this type of induced persistence is independent of the ATP synthase. Furthermore, the observation that genes involved in the SOS response were expressed at lower levels in the Δ*atp* strain than those in the wild-type (Fig. 7) would indicate that antibiotic-induced persistence through *tisB* expression does not explain the recovery of the Δ*atp* Δ*sodA* Δ*sodB* after challenges with ciprofloxacin.

The simplest explanation for the growth of the Δ*atp* Δ*sodA* Δ*sodB* after 24 h in the presence of ciprofloxacin would be that elevated superoxide production in the Δ*atp* mutant background exacerbates the previously reported increase in mutagenesis in Δ*sodA* Δ*sodB* strains lacking the cytosolic superoxide dismutases (75), leading to the generation of resistance. In support of this idea, in preliminary studies, we also performed persister assays comparing the *atp*$^+$ Δ*sodA* Δ*sodB* and Δ*atp* Δ*sodA* Δ*sodB* strains, followed by plating of samples prior to and post-challenge not only with ciprofloxacin for the enumeration of surviving (persistent) bacteria but also with rifampicin for the determination of the occurrence of spontaneous rifampicin-resistance mutations. Consistent with the proposition of elevated mutagenesis, we observed a significantly higher recovery of rifampicin resistance in the Δ*atp* Δ*sodA* Δ*sodB* strain than that in the *atp*$^+$ Δ*sodA* Δ*sodB* strain, although the Δ*atp* strain showed no significant

difference relative to the wild-type strain (see Fig. S5 in the supplemental material and Table S2). Based on these observations, we conclude that the loss of SodA and SodB eliminates a major source of endogenous H_2O_2, allowing for the recovery of the reduced persister cell population in the Δ*atp* operon deletion strain. However, the increase in the persistence in the Δ*sodA* Δ*sodB* background apparently comes at a cost, namely, increased mutagenesis in the Δ*atp* deletion background due to the missing superoxide detoxification activities. This interpretation is also consistent with the elevated ROS determinations seen in Fig. 6. We would also note that the type of DNA damage which accumulates in *sodA sodB* mutants apparently does not activate the SOS response (75). Although the endogenous production of H_2O_2 would appear to be the major source of the increased killing and loss of persistence after ciprofloxacin treatment in the Δ*atp* operon deletion strain, further studies will be required to clarify the sources and role of superoxide with regard to persistence and generation of resistance.

An open question remains as to why our study shows a clear enhanced killing and reduced persister cell formation for an ATP synthase operon deletion strain, whereas prior studies have indicated that reduced ATP levels improve survival and persistence in both *E. coli* and *Staphylococcus aureus* (13, 14). We suggest the answer lies in the preculture conditions used for experimental cultures. Our study concerns the subpopulation of spontaneous persistent bacteria present during steady-state, exponential growth, referred to as type II persister cells (2, 74). An inherent difficulty in determining such persister cell populations is the problem of carryover inoculation of experimental cultures with "triggered" (type I) persister cells which accumulate under stress conditions, including saturated, overnight cultures and stationary phase. This problem is avoided generally by diluting the overnight cultures 100- to 1,000-fold and sampling these cultures under the assumption that the resulting generations (bacterial doublings) of the experimental cultures is sufficient to dilute out the prestressed, type I persisters prior to sampling. As detailed in the Materials and Methods section, we reasoned that one could avoid the stationary phase of growth completely if the precultures were inoculated directly with an exponentially growing single colony which is then challenged during the mid-log phase of growth (here, an optical density at 600 nm [OD_{600}] of ≤ 0.5) by dilution into fresh media containing the antibiotic. Colonies of *E. coli* have been found to show exponential growth for up to 24 h on agar plates, with minimal changes in respiration throughout the colonies (76, 77), i.e., an estimated $\geq$30 generations of exponential growth. In this manner, the pre-experimental and experimental cultures do not enter stationary phase and are not confronted with the accumulation of toxic metabolites (e.g., acetate and lactate) or nutrient deprivation, due to the higher diffusion capacity of agar plates compared with increasing cell densities in a fixed volume of liquid medium. We would point out that the growth conditions we have used here are essentially the same as those described in the study cited in Balaban et al. (74) for discrimination of antibiotic-induced persisters. As noted in the Introduction, contradictory results in persistence have been observed for mutants in the ATP synthase in prior publications, with some authors making the same observation of enhanced killing of *atp* mutants as we have made, but the reasons for the reduced persistence was not investigated (19, 57). We suggest that greater attention should be given to the preculture conditions of bacterial cultures where steady-state conditions are important for the experimental work, especially in the case of persister studies.

While our results do not address the possibility that other classes of bactericidal antibiotics (aminoglycosides and β-lactams) may also have effects resulting in increased ROS, the observation that the Δ*atp* deletion strain did not show the same increased susceptibility for inhibitors of protein synthesis and cell wall biosynthesis suggests either (i) insufficient ROS is generated by these antibiotics to result in increasingly lethal damage or (ii) the type of damage generated by inhibitors of translation or cell wall biosynthesis is not

exacerbated by elevated ROS to become increasingly lethal in the Δ*atp* operon deletion strain compared with DNA strand breakage events.

MATERIALS AND METHODS

Bacterial growth media and reagents. Bacterial strains were grown in Lennox broth (78) with supplements as indicated in the text. The antibiotics ciprofloxacin, ampicillin, or kanamycin (Carl Roth) were used at the concentrations and for the times indicated in the figures. Glutathione (Merck) was added at a concentration of 2 mM and 2,2′-dipyridyl (Sigma-Aldrich) at 0.5 mM.

Strains. Unless otherwise indicated, the strain background for all strains was a nalidixin-resistant, *gyrA*(D87Y) derivative of *S.* Typhimurium ATCC 14028 (strain 8640) used previously in our laboratory (18). Gene or operon deletions were constructed using the λ red recombinase replacement method (79, 80). Experimental strains, including the Δ*atpIBEFHAGDC* operon (Δ*atp*) deletion mutant (81), were constructed by bacteriophage P22HT*int105* (82) transductions into strain 8640 using standard protocols with selection on plates containing kanamycin (50 μg/mL). Putative transductants were repurified on Green plates (83), and the chromosomal deletions were verified by PCR. The kanamycin resistance was eliminated by transformation with the plasmid pCP20 (84), followed by screening for a loss of kanamycin resistance. Plasmid pCP20 was then eliminated by growth at 37°C, and individual clones were screened for a loss of ampicillin and chloramphenicol resistance at 30°C. Strains and plasmids used in this study are listed in Table S1 in the supplemental material.

MIC and minimum bactericidal concentration (MBC) determinations. MIC determinations were performed for the wild-type and Δ*atp* operon deletion strains in L-broth with a starting CFU of 10^5 bacteria/well in 96-well, flat-bottomed plates (Corning) according to Clinical and Laboratory Standards Institute (CLSI) recommendations (18, 85). Minimum bactericidal concentration (MBC) assays were performed essentially as recommended by the CLSI guidelines (86) with modifications. To allow a direct comparison with the persister assays, the MBC assays used cultures pregrown in the same manner as for the persister assays and using the same final concentration of bacterial suspensions (10^7 CFU/mL) rather than the recommended 10^5 CFU/mL. The bacterial suspensions were added in replicate to wells of 48-well plates (Corning) containing L-broth medium and ciprofloxacin in a final volume of 1 mL to yield final concentrations of bacteria at 10^7 CFU/mL and ciprofloxacin concentrations as indicated in Table S2. The plates were incubated at 37°C for 24 h with intermittent shaking in a BioTek Synergy HT plate reader. The following day, bacteria in each well were collected by centrifugation, the supernatants were discarded, and the bacterial pellets were resuspended in 1× phosphate-buffered saline (PBS) to remove residual ciprofloxacin. Dilutions were then plated in replicate to L-broth agar plates for enumeration of surviving bacteria. Untreated bacterial cultures and uninoculated bacterial growth media served as positive and negative/blank controls, respectively. Data for the MIC and MBC assays are given in Table S2.

Antibiotic killing/persister assays. Antibiotic killing/persister assays were performed in accordance with recommendations in which the culture conditions, treatment regimens, and enumeration of persister cells are defined clearly (74). In this study, persister assays were performed using bacterial cultures grown in 6 mL of L-broth inoculated with a single colony from a plate streaked out the day before and incubated overnight (≤18 h) at 37°C. Prior studies have shown that bacterial colony growth on agar plates shows exponential growth characteristics similar to batch cultures up to 24 h postinoculation (76, 77). Consistent with this observation, single colonies used to inoculate 6-mL liquid cultures showed no significant lag phase as determined by optical density measurements when grown with aeration at 37°C. In addition, due to the comparatively larger volume of surrounding medium on agar plates, diffusion reduces the accumulation of toxic metabolites (e.g., acetate and lactate) and nutrient limitations during growth. Likewise, we avoided carryover of stationary-phase cells from saturated, overnight liquid cultures.

Cultures were incubated with aeration (shaking at 200 rpm) or under oxygen-deprivation conditions as standing cultures in anaerobe jars containing AnaeroGen 2.5L packs (Thermo-Scientific), as indicated. When cultures reached mid-log growth phase (defined here as an OD_{600} of ≤0.5), the bacteria were transferred into 6 mL of L-broth at a final concentration of approximately 10^7 CFU/mL and containing antibiotics at 4-fold the previously determined MIC values. In experiments examining the effects of glucose (0.2%) or NAD^+ (1 mM), bacteria were grown in the presence of supplements prior to the shift to ciprofloxacin-containing media. In persister assays with glutathione (2 mM) or dipyridyl (0.5 mM), the compounds were added immediately prior to the addition of ciprofloxacin. In killing assays with catalase, culture tubes containing 1 μg/mL ciprofloxacin were pretreated for 20 min at 37°C with 100 μg/mL catalase (Sigma) prior to inoculation with the experimental strains as described above. Cultures were incubated at 37°C either with aeration or as standing cultures in an oxygen-free environment in a vessel with Oxoid AnaeroGen 2.5L sachet (ThermoFisher). At regular intervals, aliquots were removed and washed by centrifugation, the bacterial pellets were resuspended in 1× PBS, and dilutions were plated to L-broth plates for CFU/mL determinations. Unless otherwise indicated in the text, antibiotic survival for the various strains is shown for time points 4 h postantibiotic challenge. The full, 24-h antibiotic killing assays are shown in Fig. S1 to S4.

Bacterial intracellular ATP determinations. ATP concentrations were determined using the BacTiter-Glo microbial cell viability assays (Promega; G8231), with luminescence determined in a Synergy HT plate reader (BioTek). Standard curves for the determination of the intracellular ATP levels were performed with purified ATP in parallel to determine the relative luminescence (RLU)/ATP concentration. Strains were grown in L-broth with aeration to the mid-log growth phase (OD_{600}, ≤0.5), with or without supplements as indicated. Results shown are given in nmole ATP/mL of culture. Based on CFU/mL of

culture determinations performed in parallel, the nmol/mL culture determinations have been corrected for minor (<10%) differences in CFU/mL to normalize the determinations to allow a comparison of ATP concentrations for equivalent numbers of bacteria.

NADH determinations. NADH extraction and determination has been described previously (5). Briefly, 50 mL of Lennox broth in a 250-mL flask was inoculated with a single colony from an agar plate and incubated at 37°C with aeration until the cultures reached an OD_{600} of ≤0.5. Cultures were adjusted to an OD_{600} of 0.1, and samples for CFU determinations and triplicate 1.5-mL samples for NADH determinations were removed. Ciprofloxacin (final concentration, 1 μg/mL) was added to the flasks, and incubation was continued at 37°C with aeration. At the indicated time points, triplicate 1.5-mL samples of the cultures were removed for NADH determinations as described previously (5). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and phenazine ethosulfate were added last to the reaction mix in 96-well plates. The plates were incubated for 3 min at 30°C in a BioTek Synergy HT plate reader prior to the addition of 6 μL of fresh yeast alcohol dehydrogenase (Sigma). The plates were further incubated at 30°C for 2 h, and the change in absorbance was determined at 570 nm. For each measurement, a standard curve with NADH (Sigma) was prepared using concentrations ranging between 62.5 nM and 1000 nM.

Determination of glutathione/glutathione disulfide (GSH/GSSG) ratios. Reduced and oxidized glutathione determinations were performed using the GSH/GSSG-Glo assay kit (Promega). For the assay, 6 mL of L-broth was inoculated with a single colony from a plate streaked out the day before. Cultures were grown with aeration to an OD_{600} of ≤0.5 at 37°C. Aliquots of both wild-type and Δ*atp* operon strains were then transferred into 6 mL fresh L-broth containing 1 μg/mL ciprofloxacin and incubated for 3 h at 37°C with aeration. Cells were harvested by centrifugation, resuspended in 1× PBS buffer, and further treated according to the manufacturer's recommendations. Cultures grown in the same manner but without ciprofloxacin addition were used as controls. Luminescence was determined using a BioTek Synergy HT plate reader.

Detection of reactive oxygen species (ROS). For the detection of superoxide production, the CellROX DeepRed kit (Thermo-Fisher) was used. The wild-type and Δ*atp* operon deletion strains were grown to an OD_{600} of ≤ 0.5 and then diluted to an OD_{600} of 0.1 in a 1.5-mL microcentrifuge tube. A total of 100 μL of the bacterial suspension was then diluted into 100 μL L-broth to yield a CFU of approximately 10^7/200 μL in the presence of CellROX DeepRed reagent (5 μM). In parallel, bacterial cell suspensions were also exposed for 3 h to ciprofloxacin (1 μg/mL) in the presence of CellROX DeepRed reagent (5 μM). The assay was incubated at 37°C with shaking in the dark for 3 h. The tubes were then centrifuged (5 min at 16,000 × *g*), and bacterial pellets were resuspended in 1× PBS followed by flow cytometric analyses (excitation, 644 nm; emission, 665 nm) using a CytoFLEX flow cytometer (Beckmann/Coulter).

For the detection of hydroxyl radicals, bacterial strains were incubated at 37°C with aeration until an OD_{600} of ≤0.5 and then diluted to an OD_{600} of 0.02 in a volume of 200 μL, and 1 μg/mL ciprofloxacin was added and incubated for 2 h at 37°C with shaking. Hydroxyphenyl fluorescein (HPF; Thermo-Fisher) was added to the bacterial cell suspensions (final concentration, 100 μM) and further incubated for an additional 2 h. The bacteria were centrifuged, and the pellets were resuspended in 1× PBS. Accumulated fluorescence reflecting endogenous hydroxyl radicals was determined by flow cytometry of the cell suspensions (excitation, 490 nm; emission, 515 nm) using a CytoFLEX flow cytometer (Beckmann/Coulter).

DNA fragmentation/TUNEL assays. DNA fragmentation was determined with the *in situ* cell death detection kit using fluorescein-dUTP (Sigma-Aldrich). From mid-log phase (OD_{600}, ≤0.5) cultures of the wild-type or Δ*atp* strains, 6 mL of fresh L-broth was inoculated with exponentially growing bacteria at a final concentration of 10^7/mL and treated with 1 μg/mL ciprofloxacin for 3 h. A control without antibiotics was prepared in the same manner. A total of 1 mL of culture was centrifuged, the resulting pellets were resuspended in 1× PBS and recentrifuged for 7 min at 4,000 × *g*, and the pellets were resuspended in ice-cold 4% paraformaldehyde (PFA). The samples were then incubated overnight at 4°C. The bacterial suspensions were collected by centrifugation (5 min at 12,000 × *g*), resuspended in 1× PBS, recentrifuged, and resuspended in precooled 0.1% Triton X-100 and incubated for 2 min at room temperature. The samples were washed with 1× PBS (5 min at 12,000 × *g*), recentrifuged, and resuspended in 50 μL of TUNEL reaction mixture provided by the manufacturer. All samples were incubated for 1 h at 37°C protected from light. Finally, the tubes were centrifuged for 5 min at 16,000 × *g* and resuspended in 1× PBS, and the fluorescein isothiocyanate (FITC) signal was analyzed using the CytoFLEX flow cytometer (Beckmann/Coulter) with an excitation wavelength of 488 nm.

Promoter expression assays. Promoters for the expression of genes involved in oxidative stress (*ahpC*, *oxyR*, *oxyS*, *soxR*, and *soxS*) or the SOS response (*recA*, *sulA*, *dinP*, and *umuDC*), as well as controls for ATP levels (*mgtCB*) or transcriptional induction/activation (*araBAD*), were cloned as PCR fragments which amplified the published promoter regions using primers containing BamHI, EcoRI, or BamHI and EcoRI restriction site sequences. After digestion of the PCR fragments and purification, the fragments were ligated upstream of a promoterless *Photorhabdus luminescens luxCDABE* operon in plasmid pDEW201 (87), which was also digested with the respective enzymes. Ligation products were used to transform *E. coli* strain DH5 by electroporation using standard protocols and plated to agar plates containing carbenicillin (100 μg/mL) for selection. Putative positive clones were identified by direct examination for luminescence using a Bio-Rad ChemiDoc XRS+ imaging system. Plasmid DNA preparations from a minimum of four putative clones were verified by sequencing, and one sequence-validated plasmid of each promoter fusion was introduced into the wild-type or Δ*atp* operon deletion strains. The luminescence in relative light units (RLUs) and OD_{600} of the cultures were determined after inoculation of triplicate wells in white, flat-bottomed, 96-well plates (Corning) with approximately 10^5 CFU in a volume

of 0.1 mL/well (10^6 CFU/mL). Plates were incubated at 37°C with intermittent shaking overnight in a Biotek Synergy HT plate reader with continuous determinations of RLUs and OD_{600} every 30 min.

Statistical analyses. Statistical analyses were performed using statistical software (GraphPad Software), using a two-tailed, Student's *t* test and unpaired data sets. For the promoter expression studies in Fig. 7, paired (dependent sample) data sets for 2-h intervals were used for the statistical analyses. Significant differences are indicated in the figures by the following symbols: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; or n.s., *P* value not significant. The number of independent assays and calculated significance values for all figures are given in Table S2.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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RESEARCH ARTICLE

Prophage Gifsy-1 Induction in *Salmonella enterica* Serovar Typhimurium Reduces Persister Cell Formation after Ciprofloxacin Exposure

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ABSTRACT Persister cells are drug-tolerant bacteria capable of surviving antibiotic treatment despite the absence of heritable resistance mechanisms. It is generally thought that persister cells survive antibiotic exposure through the implementation of stress responses and/or energy-sparing strategies. Exposure to DNA gyrase-targeting antibiotics could be particularly detrimental for bacteria that carry prophages integrated in their genomes. Gyrase inhibitors are known to induce prophages to switch from their dormant lysogenic state into the lytic cycle, causing the lysis of their bacterial host. However, the influence of resident prophages on the formation of persister cells has only been recently appreciated. Here, we evaluated the effect of endogenous prophage carriage on the generation of bacterial persistence during *Salmonella enterica* serovar Typhimurium exposure to both gyrase-targeting antibiotics and other classes of bactericidal antibiotics. Results from the analysis of strain variants harboring different prophage combinations revealed that prophages play a major role in limiting the formation of persister cells during exposure to DNA-damaging antibiotics. In particular, we present evidence that prophage Gifsy-1 (and its encoded lysis proteins) are major factors limiting persister cell formation upon ciprofloxacin exposure. Resident prophages also appear to have a significant impact on the initial drug susceptibility, resulting in an alteration of the characteristic biphasic killing curve of persister cells into a triphasic curve. In contrast, a prophage-free derivative of *S.* Typhimurium showed no difference in the killing kinetics for β-lactam or aminoglycoside antibiotics. Our study demonstrates that induction of prophages increased the susceptibility toward DNA gyrase inhibitors in *S.* Typhimurium, suggesting that prophages have the potential for enhancing antibiotic efficacy.

IMPORTANCE Bacterial infections resulting from antibiotic treatment failure can often be traced to nonresistant persister cells. Moreover, intermittent or single treatment of persister cells with β-lactam antibiotics or fluoroquinolones can lead to the formation of drug-resistant bacteria and to the emergence of multiresistant strains. It is therefore important to have a better understanding of the mechanisms that impact persister formation. Our results indicate that prophage-associated bacterial killing significantly reduces persister cell formation in lysogenic cells exposed to DNA-gyrase-targeting drugs. This suggests that therapies based on gyrase inhibitors should be favored over alternative strategies when dealing with lysogenic pathogens.

KEYWORDS Gifsy-1, *Salmonella*, bacteriophage, persister cells

Bacterial persister cells are generally considered to be a slow-growing, metabolically inactive subpopulation with high tolerance to otherwise lethal concentrations of bactericidal antibiotics and which can be responsible for recalcitrant infections (1, 2). Persistence is a phenotypic variant of resistance; however, in contrast to resistant and

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heteroresistant bacteria, persister cells do not proliferate in the presence of antibiotics (1). In general, persister cells represent only a small subpopulation of a bacterial culture, generally less than 0.1% of a growing culture. Persistence is observed in killing assays in which the majority of the drug-sensitive bacteria are rapidly killed with increasing time of treatment until only a plateau of bacterial survivors (persister cells) remain. This rapid killing of drug-sensitive bacteria results in a biphasic killing curve, with a rapid decline of viable cells during an initial phase followed by a second phase characterized by slow rates of killing, representing the phenotypically resistant persister cells (3). Currently, there are two hypotheses as to how bacterial persister cells become established: stochastically during the exponential growth phase and triggered or induced by stress (3). Numerous factors have been reported which can have an influence on persister cell formation, including the involvement of efflux pumps, oxidative stress, energy (ATP) levels, toxin-antitoxin modules, protein aggregation, or the SOS response (4–10). However, the impact on persister cell formation by genes or products encoded by endogenous prophages is less well understood, although prophages are often considered a natural part of bacterial DNA (11).

Prior studies have reported both beneficial and adverse effects of endogenous prophages on bacterial survival after antibiotic exposure (12–14). For example, Sandvik et al. concluded that phage induction was involved in the maximal killing of *Staphylococcus aureus* by ciprofloxacin, suggesting that resident prophage enhanced antibiotic susceptibility (14). In another study, it was found that deletion of 9 cryptic prophages in *Escherichia coli* increased antibiotic susceptibility, including quinolone antibiotics, indicating beneficial effects of prophages toward antibiotic resistance (12). More recently, it was found that both endogenous cryptic prophages and *E. coli* K-12 strains infected and lysogenized with the lambdoid phage φ80 resulted in increased killing of antibiotic-tolerant persistent bacteria due to induction of the prophage by antibiotics (15). These prior studies suggest that the role of endogenous prophages in bacterial susceptibility to antibiotics can be influenced by the status of endogenous prophages, e.g., whether cryptic or inducible, or by prophage gene expression in the absence of active phage replication.

Salmonella enterica serovar Typhimurium harbors a variable assortment of prophages, including Fels-1, Fels-2, Gifsy-1, Gifsy-2, Gifsy-3, and ST64B (16–18). These temperate prophages generally remain dormant as lysogens, integrated into the chromosome of the host. Activation of the prophages to switch from the lysogenic to the lytic cycle occurs as part of the SOS response, which can be triggered when bacteria are exposed to DNA-damaging agents such as mitomycin C or drugs that block DNA replication (e.g., DNA gyrase inhibitors) (19, 20). In a temporally regulated manner, after activation leading to excision of the prophages from the chromosome, the expression of lysis proteins is also induced as part of the late gene expression. In the lambdoid phages such as bacteriophage λ in *E. coli*, host cell lysis occurs after expression of the pore-forming holin (*S*) proteins, the cell wall-degrading endolysin (*R*), and the auxiliary spanins (*RzRz1*) at the end of the lytic cycle between 50 and 60 min postinduction, resulting in phage release (21).

In this study, we investigated the impact of the four resident prophages of *S.* Typhimurium strain ATCC 14028s on antibiotic susceptibility to the bactericidal fluoroquinolone antibiotic ciprofloxacin. We show that deletion of the four endogenous prophages in this *S.* Typhimurium strain changes the biphasic killing curve which is characteristic for persister cell formation into a triphasic killing curve characterized by a delayed entry into the killing phase. The results indicate that Gifsy-1 has a significant role in the initial killing and final levels of surviving bacteria after ciprofloxacin treatment, where reduced survival was attributed to the expression of the gene homologues of the bacteriophage λ lysis proteins S, R, and RzRz1 (22). In contrast, the contribution of Gifsy-2, Gifsy-3, and ST64B to killing by ciprofloxacin was negligible.

RESULTS

Gifsy-1 lysis proteins reduce persister cell formation. In order to investigate a possible role for the endogenous prophage in killing and persistence toward bactericidal antibiotics, we chose to work with *S.* Typhimurium ATCC 14028, a well-characterized strain

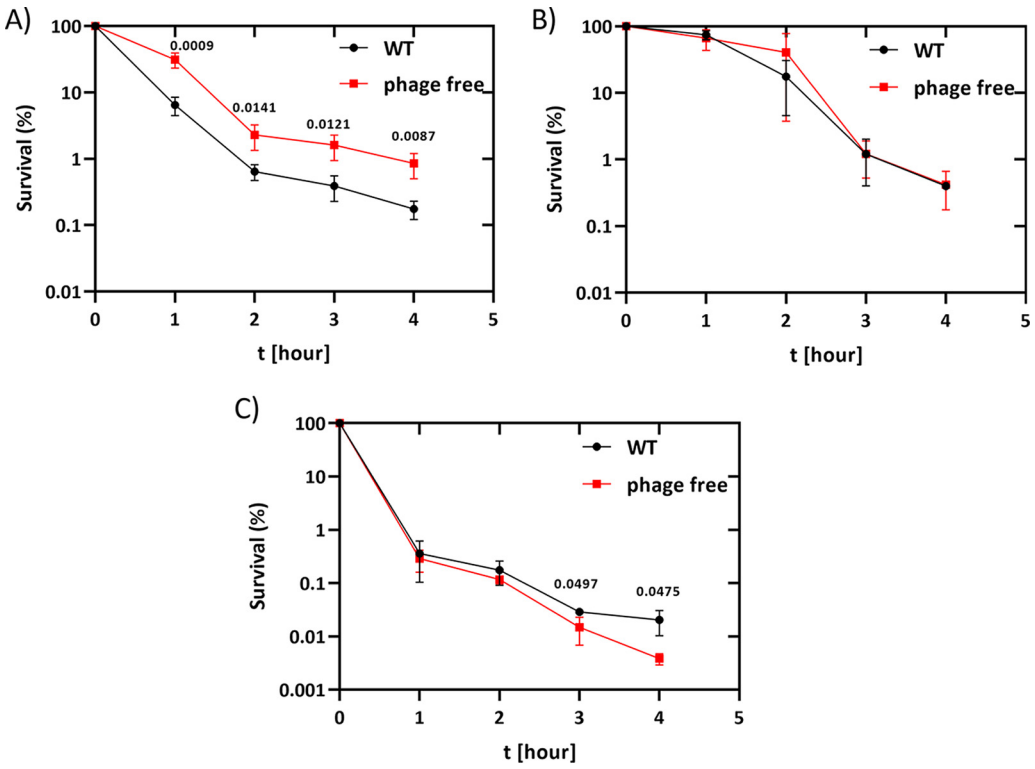

FIG 1 Persister assays with ciprofloxacin, carbenicillin, and kanamycin. (A to C) *S.* Typhimurium 14028s (8640) and its prophage-free variant (11126) were treated with (A) ciprofloxacin, (B) carbenicillin, and (C) kanamycin with 4-fold MIC, and survival was determined at the indicated time points by plating the bacteria on LB plates. Persister assays were performed at least three times, and significance was calculated using the unpaired two-tailed Student's *t* test.

used in both *in vitro* and *in vivo* virulence and infection models (18, 20). To investigate the role of prophages in antibiotic tolerance and/or persistence, we generated derivatives of *S.* Typhimurium ATCC 14028 in which the resident prophages were sequentially deleted from the chromosome (see Table S1 in the supplemental material). We then compared the survival of the wild-type with a prophage-free variant of *S.* Typhimurium ATCC 14028s after a challenge with the gyrase inhibitor ciprofloxacin (1 μg/mL) at the indicated time points. As shown in Fig. 1A, deletion of all four prophages significantly delayed the initial killing phase and increased persister cell formation approximately 3- to 5-fold compared to the wild-type strain harboring all four prophages. These results were very similar to the effects of UV irradiation, where the prophage-free strain was also more tolerant to UV than the wild type (Fig. 2). In contrast, deletion of the prophages did not affect the killing kinetics or formation of persister cells after treatments with either carbenicillin or kanamycin (Fig. 1B and C). These results were consistent with the DNA-damaging effects of both UV irradiation and gyrase inhibitors, leading to induction of the SOS response and phage induction (23–26).

As the initial killing kinetics appeared to be reduced and the final levels of persister cells were higher in the prophage deletion strain, we examined the killing kinetics after addition of ciprofloxacin more closely. The initial slope of the killing curve of the prophage-free variant indicated a delay during the first hour post-ciprofloxacin challenge compared to the wild-type strain, leading to a triphasic killing curve (Fig. 1A). After 1 h of treatment, the slope of the killing curves of both strains were comparable again for up to 2 h posttreatment. However, the final plateau of surviving bacteria also appeared to show the same kinetics, i.e., the appearance of persister cells occurred in both strains at approximately 2 h postchallenge, resulting in more viable bacteria for the prophage-free strain and a significantly increased persister cell population after entry into the plateau phase (Fig. 1A). These results suggested that a large fraction of the bacterial killing during the first hour of ciprofloxacin treatment was dependent on the endogenous prophage.

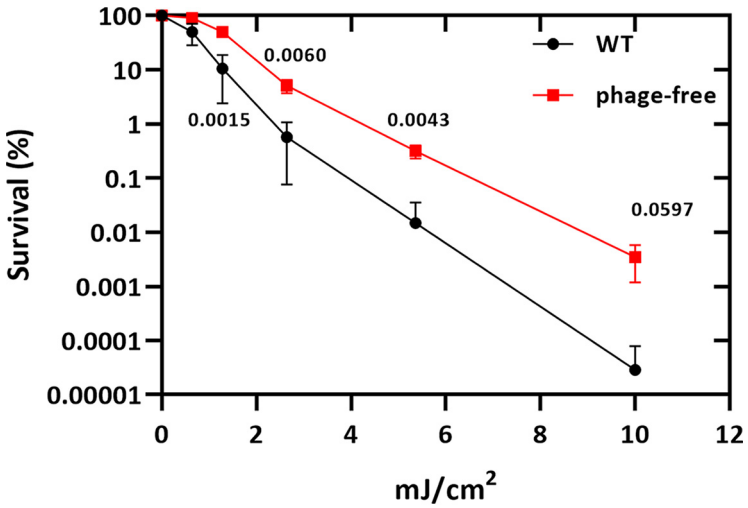

FIG 2 UV survival assay. Both strains, *S.* Typhimurium (8640) and the phage-free variant (11126), were incubated to the mid-log phase before being exposed to UV. Experiments were conducted at least three times, and significance was calculated using the unpaired two-tailored Student's *t* test.

To investigate the involvement of the phages in more detail, we also performed persister assays after ciprofloxacin challenge with strains harboring deletions of the individual prophages. Only the strain harboring a deletion of Gifsy-1 showed a reduction in the killing kinetics and increased persistence similar to that of the strain deleted for all four prophages relative to the wild-type strain (Fig. 3). In contrast, the contribution of Gifsy-2, Gifsy-3, and ST64B to survival of *Salmonella* was negligible (Fig. 3). To determine the level of induction for the four endogenous prophages, Gifsy-1, -2, and -3, and ST64B, we performed real-time quantitative PCR (qRT-PCR) to examine the induction kinetics of each of the prophages

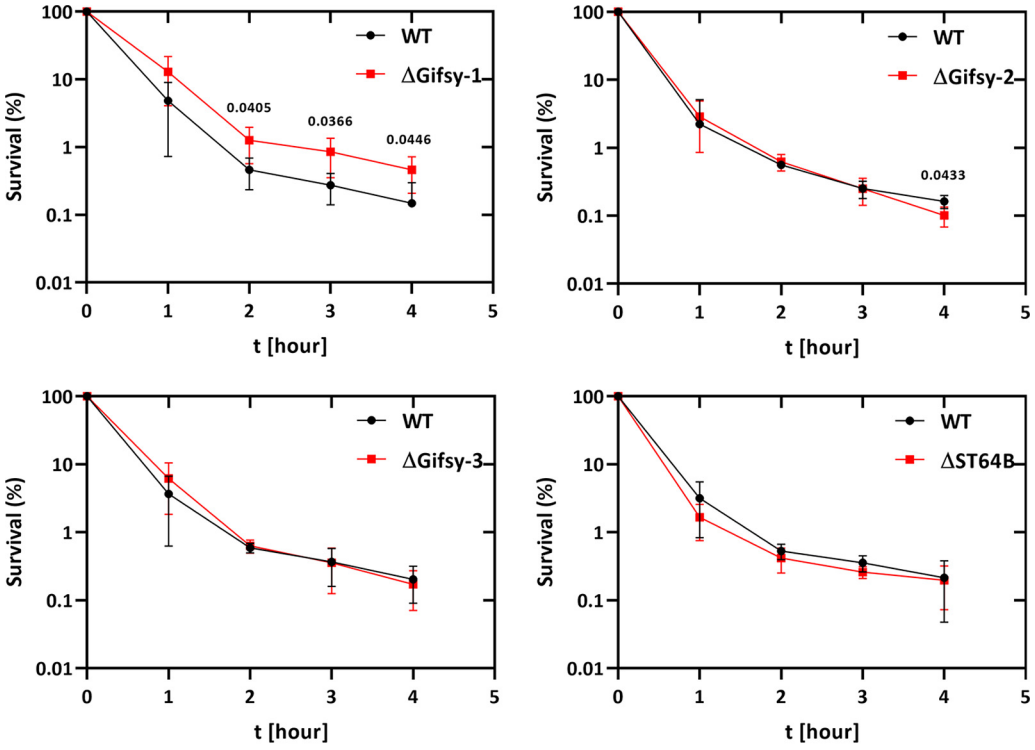

FIG 3 Persister assays with single prophage deletions. The *Salmonella* wild type 14028s (8640) and the corresponding single prophage deletion mutants (11160 = ΔGifsy-1; 11162 = ΔGifsy-2; 11224 = ΔGifsy-3; 10926 = ΔST64B) were incubated to the mid-exponential phase before being challenged with 4-fold the MIC of ciprofloxacin. At least three independent experiments were performed, and significance was calculated using the unpaired two-tailed Student's *t* test.

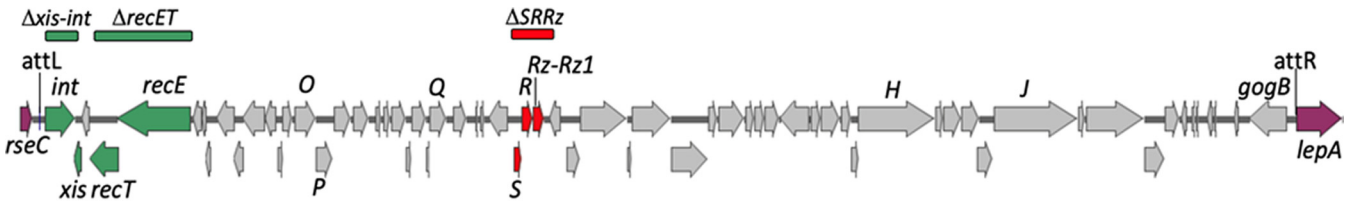

FIG 4 Schematic diagram of the Gifsy-1 prophage showing the positions of the relevant genes and corresponding deletions (overlying bars) analyzed in this study. Single capital letters denote genes named on the basis of their similarity to genes in bacteriophage λ.

after ciprofloxacin challenge. Real-time PCR confirmed that among the four prophages, Gifsy-1 showed the highest level of induction, whereas induction of Gifsy-2 and Gifsy-3 was more modest (Fig. S1). We were unable to detect a signal for ST64B in these assays (data not shown).

The preceding results suggested that induction of the lytic cycle of the prophage Gifsy-1 in *S. Typhimurium* contributed to the killing kinetics and final persister cell population after ciprofloxacin treatment. In order to understand the mechanisms behind the apparent Gifsy-1-dependent effects on killing and persistence, we tested various gene deletions within Gifsy-1 to determine which prophage functions might play a role. One of the earliest events in phage induction and a requirement for viral genome replication is the excision of the phage genome out of the bacterial chromosome. We therefore repeated the ciprofloxacin killing and persister assays using derivatives of Gifsy-1 harboring deletions of the integrase/excisionase (*int/xis*) genes, involved in the excision of the phage from the host chromosome (27), and the *recET* genes encoding the bacteriophage recombination proteins RecET (Fig. 4). As the recombination proteins RecFOR of *E. coli* have been proposed to bias the induction of the SOS response (28), it was therefore possible that the bacteriophage recombination proteins might show a similar effect. In addition, we also examined the role of the lysis proteins S, R, and RzRz1, which are active during the last phase of prophage induction and are responsible for host cell lysis and phage release (21, 29) (Fig. 4). As seen in Fig. 5, only deletion of the Gifsy-1 homologs of the bacteriophage λ *S*, *R*, and *Rz* lysis genes (annotated STM14_3196, STM14_3195, and STM14_3194, respectively, in the *S.* Typhimurium ATCC 14028 genome [22]) showed the same increased persister cell formation after ciprofloxacin treatment as seen for the prophage-free (Fig. 1A) and ΔGifsy-1 deletion strains (Fig. 3). Neither loss of excision/integrase functions in the Gifsy-1 Δ*xis-int* prophage nor the deletion of *recET* significantly affected persister cell formation (Fig. 5). Furthermore, the strain harboring the Gifsy-1 Δ*SRRz* homolog deletions showed up to 10-fold higher levels of surviving bacteria (persisters) after exposure to ciprofloxacin at 24 h postchallenge, supporting the idea that the endogenous Gifsy-1 prophage plays a significant role in both the formation and magnitude of the final persister cell population surviving a challenge with ciprofloxacin (Fig. S2).

DISCUSSION

This study demonstrated that the endogenous prophages of *S.* Typhimurium can have a significant impact on antibiotic tolerance and persistence toward bactericidal antibiotics. Both UV irradiation and ciprofloxacin challenge resulted in up to 10-fold higher levels of bacterial survivors in the *S.* Typhimurium strain deleted for the four endogenous prophages. In particular, the induction of the prophages during the initial killing phase during the first hour postchallenge played a crucial role, as seen by the delayed onset of killing in the prophage-free variant compared to the wild-type strain (Fig. 1A). This observation strongly suggests that the fluoroquinolone-mediated killing mechanism is the same in both strains and differs only in the additional host lysis events due to induction of prophages in the wild-type strain. In the absence of the endogenous prophage, up to 10-fold more bacteria survived the initial killing and contributed to the final persister cell population. That the difference in the killing kinetics and final levels of persister cells results from prophage induction is most clearly shown by comparing the persister cell levels between the wild-type and the Gifsy-1 strain after deletion of its lysis genes (Fig. 3 and Fig. 5). Here, loss of the expression of the holin, endolysin, and spanin significantly increased persister cell formation after

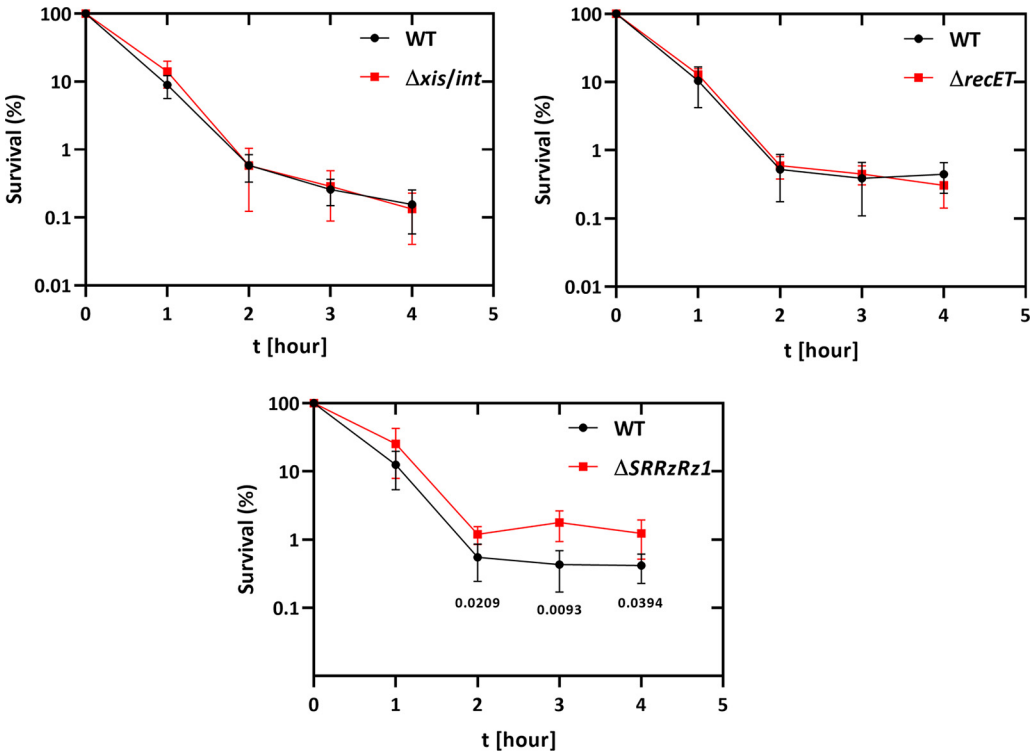

FIG 5 Persister assay with strains harboring specific gene deletions within Gifsy-1. The corresponding strains (8640 = *S. Typhimurium* wild type; 11976 = Δ*recET*; 11958 = Δ*xis/int*; 12074 = Δ*SRRzRz1*) were incubated to the mid-log phase before treatment with 1 μg/mL ciprofloxacin. The genes *xis/int* are responsible for the integration and excision of Gifsy-1 from the bacterial chromosome, while *recET* catalyzes homolog recombination. The bacteriophage λ gene homologues S, R, and RzRz1 are responsible for the lysis of their host. Experiments were conducted at least three times, and significance was calculated using the unpaired two-tailored Student's *t* test.

ciprofloxacin treatment, indicating that other phage functions do not appear to influence the generation of persister cells. These results also indicate that Gifsy-1 does not need to be excised from its host chromosome to initiate lysis since the killing curve of the Gifsy-1 Δ*xis/int* mutant, which remains integrated in the bacterial chromosome, was indistinguishable from the wild type (Fig. 5). These results are also consistent with earlier observations in *E. coli* for bacteriophage λ *int* lysogens, which show severe reductions in phage burst size but nevertheless result in host cell killing and lysis after induction, despite the inability for phage excision (30, 31).

These observations may explain discrepancies between our observations regarding reduced killing and increased persister cell formation in the phage-free and ΔGifsy-1 strains and the results of Wang et al., who found that the endogenous, cryptic phages of *E. coli* conferred resistance to quinolone antibiotics (12). In their study of an *E. coli* strain deleted for all 9 cryptic prophages (referred to as the Δ9 strain), the authors describe the *E. coli* cryptic phage as inactive in terms of cell lysis. In addition, at least two of the cryptic phages (CP4-6 and Rac) were found to express inhibitors of cell division involved in antibiotic resistance (12). Therefore, it is likely that the cryptic phages described in the study of Wang et al. had lost their lytic functions, rendering them permanently inactive with regard to host cell lysis. In addition, the prophages e14, CP4-6, and Rac showed low levels of excision under normal growth conditions, but only e14 was inducible by mitomycin C addition or oxidative stress.

On the other hand, our study is consistent with the observations of Harms et al. in which strains of *E. coli* harboring lysogens of the lambdoid phage φ80 were found to be more sensitive to killing and showed reduced persisters in response to ciprofloxacin (15). φ80 (also known as Lula) is a temperate lambdoid phage which is easily triggered to induction by DNA damage (32). The role of the inducible φ80 prophage in antibiotic resistance and persistence was discovered inadvertently following a study of the role of toxin-antitoxin (TA) systems in antibiotic tolerance and persistence in *E. coli* (33). The authors of that study found

that successive deletion of 10 of the toxin-antitoxin systems present in the *E. coli* genome (Δ10 strain) reduced the persistence of the strains against the antibiotics ciprofloxacin and ampicillin, consistent with a role for toxin-antitoxin systems in bacterial persistence against bactericidal antibiotics. Further studies by this group, however, revealed that many of the TA deletion strains had been lysogenized by φ80, which was induced by the antibiotic treatments used to determine the effects of TA gene deletions (15). Although the φ80 infections and lysogenization of experimental strains led to unfortunate misinterpretation of results regarding the TA systems (33), we suggest the findings of the follow-up studies investigating the role of prophage induction on persistence to antibiotics (15) is an equally, if not more, important observation.

It has been previously observed that high doses of bactericidal antibiotics, including quinolones and fluoroquinolones, are less efficient in bacterial killing and yield higher levels of surviving persister cells (34, 35). This observation has been partially explained by inhibition of RNA and protein synthesis at high concentrations of antibiotics. We suggest that an additional effect involves the induction of endogenous prophages, which also require host transcription and translation activities for induction, synthesis, and phage assembly (17). Inhibition of translation could also promote persister cell formation by interfering with phage induction, which synergistically contributes to the lysis of the host (21, 29).

The results of our study demonstrated that endogenous phages have the potential to increase the efficiency of DNA-damaging agents, consistent with previous studies in which a nonlysogenic strain of *E. coli* for the phage lambda λ was also more tolerant against danofloxacin than the lysogenic counterpart (13). These results are reminiscent of a phenomenon called phage-antibiotic synergy (PAS), in which sublethal concentrations of antibiotics (β-lactam and fluoroquinolone) increased phage production and phage-mediated burst size (36). This was later attributed to a delayed lysis of the host allowing prolonged assembly and higher numbers of infectious phages (37). This suggests that phages can be used as natural adjuvants to increase the efficiency of antibiotics. However, the prerequisite is that the bacteria must have their own prophages integrated in the bacterial chromosome.

Bacteriophages were considered for use to treat bacterial infections long before the discovery of penicillin and later during the cold war in eastern Europe (38). Due to the worldwide increase in antibiotic resistance, there has been renewed interest in bacteriophages as an alternative to antibiotics to treat bacterial infections (39). In recent years, several promising approaches have been reported for using phages to kill pathogens, including the usage of phage cocktails or purified phage proteins (holins/endolysins), a combinatorial treatment with phages and antibiotics, or application of bioengineered phages as vaccines (40). Lysogenic as well as lytic phages could therefore offer new strategies to eradicate drug-persistent bacteria as natural antibacterial agents or increase the efficiency of available antibiotics.

MATERIALS AND METHODS

Media and antibiotics. Lysogeny broth (LB Lennox, Carl Roth) was used for growth of bacterial cultures. Where appropriate for selection or screening, the antibiotics kanamycin (50 μg/mL), carbenicillin (100 μg/mL), and chloramphenicol (15 μg/mL) (Carl Roth) were added to either liquid or solid agar medium. The MICs for the strains were determined for kanamycin (3.12 μg/mL), carbenicillin (3.12 μg/mL), and ciprofloxacin (0.125 μg/mL; Sigma-Aldrich) in microplate assays according to CLSI recommendations. Standard MIC assays are performed in 1:1 dilution steps. Here, the designation 4× MIC refers to the number of dilution steps beyond the determined MIC value rather than 4-fold the determined MIC. For our strain, the MIC for ciprofloxacin was determined as 0.125 μg/mL. Four dilution steps back from this value in the MIC assays starting from the MIC of 0.125 μg/mL corresponds to 0.125 (1× MIC), 0.25 (2× MIC), 0.5 (3× MIC), and 1.0 μg/mL (4× MIC). For persister assays, cultures were treated with the indicated antibiotics at 4-fold the MIC.

Bacterial strains. All strains used in this study are summarized in Table S1, including the original strains for P22 lysate production. The strain background used in this study is *Salmonella enterica* serovar Typhimurium strain ATCC 14028s harboring a *gyrA*(D87Y) mutation conferring nalidixin resistance. Note that while the *gyrA* mutation increases the MIC for ciprofloxacin, it does not affect the killing kinetics (41, 42). The *gyrA* strain background was chosen to avoid accumulation of primary *gyrA* mutations, which occur relatively frequently (43, 44) and which would grow overnight in the persister assays which were carried out for up to 24 h.

The strains harboring deletions of the Gifsy-1 (ΔGifsy-2::*kan*), Gifsy-2 (ΔGifsy-2::*cat*), and ST64B (ΔST64B::*kan*) prophage have previously been described (45). Construction of the ΔGifsy-3::*kan* deletion strain

(MA14253) was performed using the λ Red mutagenesis/gene replacement method (46). Briefly, the primers ppAG72 and ppAG73 containing homologous sequences upstream and downstream of the Gifsy-3 integration site in the *S.* Typhimurium *icdA* gene were used to PCR amplify the kanamycin resistance cassette in plasmid pKD13 (46). The primers were designed to regenerate a wild-type *icdA* gene sequence after recombination, which would otherwise be lost when generating a full deletion of Gifsy-3. The purified PCR product was then used to transform competent cells which had been induced for expression of the bacteriophage λ Red recombinase as previously described (46), with selection for kanamycin resistance. Putative recombinants were screened by PCR using the primer pairs pp599-ppR25 and pp587-pp549 to verify the left and right recombination junctions, respectively. Isolates which were positive in the PCR screening were further sequenced to verify the deletion of Gifsy-3 from the chromosome. The resulting strain, MA14253, harboring the ΔGifsy-3::*kan* deletion/replacement was then used as a donor for P22 transductions into other strains using standard protocols. Additional gene deletions of the Gifsy-1 endoloysin homologs (Δ*SRRz*::*kan*), recombinase (Δ*recET*::*kan*), and excision/integrase genes (Δ*xis-int*::*kan*) were constructed in a similar manner, but using the kanamycin resistance cassette of pKD4 (46). All primers used in this work are listed in Table S3.

To construct the prophage-free strain, phage P22 lysates were first prepared on donor strains harboring deletions of the respective prophage in which the prophage had been replaced with a kanamycin or chloramphenicol resistance cassette (see Table S1). The phage deletions were introduced into our wild-type background (8640) using bacteriophage P22 transductions followed by plating on the appropriate antibiotic selection plates according to standard protocols. Putative transductants were then purified as single colonies on Green plates to avoid carryover of infectious P22 (47) and screened for deletion of the respective prophage by PCR. Isolates harboring the correct prophage deletion were then transformed with the FLP-recombinase plasmid pCP20 to eliminate the antibiotic resistance cassette (46, 48). After screening for loss of kanamycin or chloramphenicol resistance, plasmid pCP20 was subsequently eliminated by growth at the nonpermissive temperature (37°C) for plasmid replication and screened for loss of ampicillin and chloramphenicol resistance at the permissive temperature (30°C) (46). The strain harboring a deletion of one of the prophage was then used as a host for P22 transduction using lysates prepared on a second prophage deletion strain, and the procedure as described above was repeated.

Persister assays and UV killing. Strains for the persister assays were streaked out to Lennox agar plates and incubated overnight at 37°C. The following day, a single colony was used to inoculate 6 mL of Lennox broth in a 16 by 160-mm glass culture tube and were incubated at 37°C with shaking (200 rpm) in a fixed rack with tubes at an angle of approximately 45° to ensure aeration. At an optical density at 600 nm (OD_{600}) of 0.5, the CFU/mL of each culture was adjusted to approximately 5×10^6/mL for the persister assays, or bacterial suspensions were adjusted to an optical density (OD_{600}) of 0.01 for UV killing in 6 mL Lennox broth. For the persister assays, ciprofloxacin (1 μg/mL), kanamycin (25 μg/mL), and carbenicillin (25 μg/mL) were used at 4-fold (4×) the MIC as described above. After addition of the antibiotics, the cultures were further incubated at 37°C with aeration. The bacterial survival and persistence was determined by removing aliquots at the time points indicated in the figures. The aliquots were centrifuged, and the bacterial pellets were resuspended in 1× PBS and plated on Lennox agar plates for colony counting. For UV killing assays, the bacterial suspensions were transferred to a petri dish and exposed to UV light using a mercury vapor discharge lamp (50 W/m^2). After irradiation, the bacteria were plated on LB plates and incubated overnight at 37°C. All assays were conducted at least three times, independently.

Real-time PCR (qPCR). The wild type was incubated to the mid-log phase as described above and subsequently treated with 1 μg/mL ciprofloxacin for 60 min. After treatment, the RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer' recommendations. The extracted RNA was transcribed into 1,000 ng cDNA as follows: a reaction mixture containing 2 μL of 50 μM oligo(dT)18 (Eurofins Genomics), 2 μL of 10 mM deoxynucleoside triphosphates (dNTPs; Invitrogen), 1 μL of 200 U/μL reverse transcriptase (Thermo Scientific), and 0.5 μL of 40 U/μL RiboLock RNase inhibitor (Thermo Scientific) was brought to a final volume of 20 μL with sterile water. Subsequently, 25 ng/well cDNA was used to measure the expression of the phage genes, using 0.2 μL of 10 pmol/μL primers from Table S2, and mixed with 10 μL (10×) SYBR green master mix (Thermo Fisher). The fluorescence signal was determined using an Applied Biosystems StepOnePlus device, using the following protocol: denaturation at 95°C for 2 min, elongation at 60°C for 30 sec, and denaturation at 95°C for 10 sec (cycle 40×). After 40 cycles, elongation was carried out at 60°C for 1 min followed by the final denaturation step at 95°C for 15 sec and the final elongation step at 60°C for 1 min.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, DOCX file, 0.02 MB.

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11 General Discussion and Conclusion

11.1 Discussion

Several publications have highlighted the importance of ATP concentration in the context of antibiotic susceptibility and persister cell formation in Gram-negative as well as in Gram-positive bacteria [95, 96]. It was postulated that low ATP decreases the target activity of the antibiotics, protecting the bacteria from lethal cellular damage. After cessation of antibiotic treatment, bacteria replenish their ATP pools and become active again. Low ATP as a signal for persister cell formation is supported by the fact that increased ATP levels interfere with drug persistence [97]. However, in this project, which includes publications 1 and 2 (**10.1** and **10.2**), we demonstrated that low ATP does not result in increased persister cell formation in *S*. Typhimurium when the operon encoding the ATP synthase, is deleted [99, 100]. Despite reduced ATP pools, persister cell formation was significantly reduced after ciprofloxacin treatment, indicating a more complex relationship between ATP and antibiotic susceptibility. Furthermore, our results suggest that the bacterial ATP-synthase is an attractive target for future drug developments.

In addition to antibiotics, (bacterio)phages can be used to treat bacterial infections [264, 265]. Furthermore, endogenous phages have an as yet to be characterized influence on persister cell formation upon drug exposure. Therefore, another aim of this thesis, which has been described in publication 3 (**10.3**), was to gain a better insight of phage induction during treatment with ciprofloxacin. In this work, I found that challenging *S*. Typhimurium and the phage-free variant with ciprofloxacin resulted in different initial killing kinetics. Deletion of all four endogenous phages decelerated the initial lethal effect of ciprofloxacin, leading eventually to increased persistent bacteria. In particular, Gifsy-1 and its lysis proteins were found to be responsible for decreased persister cell formation in the wild type, while the remaining endogenous phages of *S*. Typhimurium have had a negligible effect.

11.1.1 ATP synthase: an antimicrobial target

We demonstrated that the ATP synthase of the electron transport chain in *S.* Typhimurium is a promising candidate to increase the efficiency of fluoroquinolones and to decrease the formation of persister cells [99, 100]. For *E. coli* a similar effect was observed, whereas the mutation of *atpA*, an important subunit of ATP synthase, increased the susceptibility to various classes of antibiotics [88]. It was suggested, by several research groups, that the

increased oxygen consumption during drug treatment lead to high doses of ROS, resulting in metabolic perturbation and increased cellular damage [89, 101, 235]. This makes the ATP synthase an attractive target to interfere with persister cell formation. So far, deletion of *atpA*, the catalytic domain of the ATP synthase, increased the consumption of oxygen and concomitantly increased the susceptibility against ampicillin, gentamicin and norfloxacin [88]. These results are largely concurrent with our results after deletion of the *atp* operon in *S.* Typhmurium. Apparently, mutations of the ATP-synthase enhance the efficacy of aminoglycosides, because the MIC of kanamycin for *S.* Typhimurium after deletion of the ATP-synthase was significantly reduced. Furthermore, deletion of *atpG* or *atpA* in *E. coli* increased the susceptibility against gentamicin [88]. The augmented sensitivity against aminoglycosides could be the consequence of an increased membrane potential, as a result of an increased respiratory activity. Uptake of aminoglycosides, such as gentamicin and kanamycin, are dependent on the proton motive force, generated by the electron transport chain [91, 266], hence uptake in ATP-synthase mutants should be higher. However, to investigate the impact on persister cells formation, four-fold the MIC for kanamycin was used either for the wild type or the *atp* operon mutant. When using the respective concentrations, treatment resulted in complete eradication of the wild type, while the mutant was, after initial killing, able to regrow over-night, indicating the acquisition of resistance. This phenomenon resembles a variant of resistance, the so-called heteroresistance, in which a small subpopulation acquires a transient genotypic resistance [267]. However, heteroresistance disappears after several generations, when there is no antibiotic pressure anymore [201]. One target of genotypic resistance could be the mutation of *ubiF*, a gene, which encodes for 3-demethoxyubiquinol 3-hydroxylase, involved in ubiquinone synthesis, an important electron carrier of the ETC [268]. An inoperable enzyme would severely disrupt the electron transport chain and the formation of the membrane potential [269]. The lowered membrane potential ultimately results in decreased uptake and decreased intracellular accumulation of aminoglycosides [270, 271].

In contrast to kanamycin, no difference was observed for the β-lactam antibiotic ampicillin, whether for the MIC nor for persister cell formation. It is known that mutations of the ATP-synthase reduce the growth rate [272], hence the process of peptidoglycan synthesis, the major target of β-lactams, is less active, representing a less efficient target. However, as mentioned above, mutation of the ATP synthase in *E. coli* resulted in increased susceptibility after treatment with the β-lactam antibiotic ampicillin [88], in contrast, in our study, where

we observed no difference after ampicillin treatment. Uptake of β-lactam antibiotics is preferentially mediated by outer membrane proteins, in particular by OmpF [273], however, the *atp* operon mutant suffers from oxidative stress, and under these conditions a small RNA, encoded by *micF*, is activated which post-transcriptionally inhibits the mRNA of OmpF by complementary binding [274]. Thus, differences in gene expression or translation and the extent of the oxidative stress could result in varying strong inhibition of OmpF in *E. coli* and in *S.* Typhimurium, resulting in distinct accumulation of β-lactam antibiotics in both species.

Nevertheless, the major question was, why is the mutant more susceptible to ciprofloxacin, one of the most frequently used antibiotics and also applied during severe salmonellosis [45, 205]? To address this question, we performed additional experiments. As already mentioned, mutation of the ATP synthase in *E. coli* increased oxygen consumption and respiratory activity as well as increased the formation of ROS [88, 101]. However, the reason for the augmented ROS production was never completely elucidated. So far, it was only discussed that deletion of the ATP synthase could result in high levels of NADH due to higher uptake of sugar and glycolytic activity, which would in turn lead to a comparable low TCA cycle activity, presumably as a protective counteraction to prevent excessive NADH formation [275]. In order to inhibit the accumulation of NADH, which in turn would stimulate the formation of ROS, NADH has to be recycled to NAD^+ via the respiratory chain. Indeed, the increased respiratory activity goes along with an increased uptake of oxygen and the risk of superoxide formation. Superoxide preferentially targets proteins with (iron-sulfur) Fe-S clusters and therefore biases the activity of the TCA cycle with its Fe-S cluster containing enzymes, which is why the activity of the TCA cycle is reduced during oxidative stress [276, 277]. As proposed for *S.* Typhimurium, accumulation of NADH, or alternatively of NADPH, results in the formation of hydroxyl radicals via the flavin reductase, which uses NADH/NADPH as electron donor to reduce FAD^+ to $FADH_2$, which in turn strongly reacts with ferric iron (Fe^{2+}) to form ferrous iron (Fe^{3+}) [241, 278]. Fe^{3+} eventually reacts with H_2O_2 to generate the short-lived but extremely deleterious hydroxyl radicals. Therefore, deletion of flavin reductase in the *atp* operon background had a significant impact on persister cells formation upon ciprofloxacin treatment, demonstrating that the augmented level of hydroxyl radicals, which is generated in the mutant, mostly arose from the activity of the flavin reductase. However, the wild type phenotype could not be completely restored, indicating other metabolic pathways, which contributed to increased ROS formation. A second potential source of increased ROS formation could be respiratory activity,

because deletion of the NADH dehydrogenase or succinate dehydrogenase slightly, but not significantly, increased persister cell formation. However, most likely several metabolic processes in the *atp* operon mutant have an additive effect on the increased susceptibility after ciprofloxacin treatment.

The understanding of the killing process of an antibiotic could be useful to improve its effectiveness or to develop new therapeutic strategies against bacterial infections. The ATP synthase could be used as potential target, both in Gram-negative and Gram-positive bacteria. For example, bedaquiline is the first developed anti-mycobacteria drug since 40 years, which targets the ATP synthase of active, dormant, intracellular or extracellular mycobacteria with high efficiency [279, 280]. The mode of action involves the binding of bedaquiline to *atpE*, a subunit of the ATP-synthase, which augments the consumption of oxygen, similar to *E. coli* when *atpA* was deleted [88, 281-283]. Furthermore, the inhibition of the ATP synthase causes depletion of ATP and, concomitantly with the exhaustion of ATP, increases the respiration as well as the glycolytic activity [283]. Low ATP levels disturb the homeostasis of the energy household of bacteria because depletion of ATP interferes with the feedback inhibition of the glycolytic pathway, while high ATP levels allosterically impede key enzymes of the glycolysis or TCA cycle. This has been shown for *E.coli* as well as in *Corynebacterium glutamicum*, when low ATP pools stimulated the glycolytic activity and respiration [284-286]. Furthermore, depletion of ATP results in another metabolic phenomenon, the so-called overflow metabolism, which hallmark is an increased glycolytic activity [287]. Overflow metabolism is the conversion of sugar into energy using the central carbon metabolism instead of the electron transport chain, despite the availability of oxygen [288]. This strategy results in the accumulation of fermentation products, such as lactate and in particular acetate, and might result in increased levels of NADH [289]. The reason for switching from respiration to fermentation, is due to the decreasing surface-to-volume (S/V) ratio in fast-growing bacteria. The energy demand of a cell increases, which can`t be further supported by solely respiration, because the ETC reaches its saturated membrane occupancy at a growth rate at 0.3 h^{-1}, thus a further increase of the growth rate is only possible by shifting ATP generation to fermentation although, paradoxically, less ATP is generated [287, 290]. However, fermentation needs less proteins to generate the same amounts of ATP as by respiration, therefore proteomic allocation from respiration to fermentation allows a further increase of biomass [288]. The *atp* operon mutant is forced into overflow metabolism by gene deletion,

hence it can only produce ATP via substrate phosphorylation. The low ATP stimulates the glycolytic activity and concomitantly reduces the activity of the pentose phosphate pathway, which is involved in NADPH formation [284]. NADPH is a crucial cofactor for restoring oxidized glutathione in its reduced form, which in turn catalyses the conversion of H_2O_2 into H_2O before it can react with ferric iron to hydroxyl radicals [87, 291-293]. The *atp* mutant suffers from a reduced ratio of reduced/oxidized glutathione (GSH/GSSG) before treatment with ciprofloxacin, thus aggravating the oxidative stress in the mutant during fluoroquinolone treatment when ROS is induced [100]. Interestingly, treatment of *M. tuberculosis* with bedaquilin, in combination with clofazimine, increased the formation of ROS, potentiating the efficiency of both antibiotics, likewise when the respiration was artificially augmented in *M. tuberculosis* using N-acetylcysteine [90, 283]. This could indicate a similar ROS dependent killing mechanism in the ATP-synthase deficient *S.* Typhimurium mutant and *M*. *tuberculosis*, in particular when considering the fact that both species possess a flavin reductase, which oxidizes NADH while concomitantly transferring the electrons on flavins [294].

Another class of ATP-synthase inhibitors are polyphenols, natural occurring phytochemical compounds [295-298]. There exist a wide variety of different polyphenols with distinct inhibitory efficiencies in which some polyphenols prevent either the hydrolysis or generation of ATP by the ATP-synthase, or both processes simultaneously. For example, the polyphenol resveratrol is considered as a pro-oxidant, in which treatment of *S*. Typhimurium led to DNA fragmentation, membrane depolarization, cell division inhibition, glutathione depletion and increased oxidative stress [299]. These phenotypes resemble the *atp* operon mutant used in this project, when we also observed augmented levels of oxidized glutathione, a hallmark of oxidative stress, and increased DNA damage [100]. However, it has been also reported that resveratrol can reduce the efficiency of antibacterial drugs, such as ciprofloxacin, kanamycin, moxifloxacin, ampicillin and oxolinic acid, resulting in increased survival of *E. coli* and *S. aureus* due to diminished ROS formation [300]. Therefore, it is possible that the concentration, antibiotic, or the investigated strain play a critical role, whether resveratrol is beneficial or not. This assumption is supported by another study, in which resveratrol sensitized *E. coli* and *K. pneumonia* against polymyxin B [301]. Furthermore, a high resveratrol concentration could interfere with ATP synthesis, leading to decreased metabolic activity and a reduced DNA-gyrase activity, the major target of fluoroquinolones, thus increasing survival [95, 96, 220]. Besides polyphenols, venoms from animals, such as spiders, snakes, frogs and bees, are also

known to have a strong antibacterial activity [302]. These venoms are a mixture of biological active compounds, amongst other things, containing antimicrobial peptides (AMPs), enabling animals to survive in microbial-infested environments. One of their bacterial targets is the ATP-synthase, for example, cathelicidin, excreted from the king cobra (*Ophiophagus hannah*), or lycotoxin, produced by wolf spiders (*Lycosa carolinensis*), caused up to 85 % inhibition of *E. colis* purified and membrane-bound ATP-synthase along with inhibition of bacterial cell growth [302]. Another AMP with ATP-synthase inhibitory effect is melittin, the major component of bee venom (*Apis mellifera*), with good antibacterial activity against various pathogens, including *S. enterica*, *S. aureus*, *Lactobacillus casei*, *Listeria monocytogenes*, *Enterococcus faecalis* or diverse *Streptococcus* strains [303, 304]. It was also shown that treatment of *E. coli* with the human cathelicidin LL-37 disrupted the electron transport chain and increased the formation of ROS [305]. It was speculated that LL-37 binds to the outer leaflet of the cytoplasmic membrane and interferes with the cytochrome oxidase-*bd* complex, inducing the formation of superoxides. Alternatively, it might be that the disturbance of the ETC resulted in the accumulation of NADH because regeneration to NAD^+ by respiration should have been abrogated, thereby, similar to the *atp* operon mutant used in this project, accumulated NADH could enhance the formation of hydroxyl radicals. However, more research will be necessary to investigate the impact of AMPs on the ATP-synthase and their possible consequences.

Treatment failure is also often the result of non-resistant drug-tolerant persister cells, which unfortunately can become resistance either after a single treatment or intermittent treatment with antibiotics [15, 19, 22, 23, 69]. Therefore, it is important to eradicate all bacteria during an antibacterial therapy. One promising method is to exploit the energy generating metabolic pathways by inhibition of the bacterial ATP synthase, for example, either by antibiotics or natural occurring chemicals. As shown, this will increase the formation of ROS in a NADH dependent manner and increases the susceptibility against fluoroquinolones.

11.1.2 Drug-induced prophages

The impact of phages on drug tolerance has been largely overlooked, and only recently have scientists started to focus on this matter. In addition, the missing awareness led also to misinterpretations of results by various researchers [106, 306, 307]. A conclusive explanation for persister cell formation and the underlying molecular mechanisms which explains how

drug-sensitive bacteria can survive exposure to antibacterial agents, despite the lack of resistance, has been elusive. In prior studies, it was suggested that the accumulation of the master regulator (p)ppGpp, catalysed by the enzymes SpoT and RelA, led to the formation of polyphosphate, which in turn activates the protease Lon. Subsequently, Lon degrades multiple antitoxins, causing the release of toxins into the cytosol and the deceleration of growth of exponential growing bacteria as well as an increase in drug tolerance against ciprofloxacin [106]. It was believed that the degradation of the antitoxins resulted in a diminished translation due to the activity of 10 RNA endonucleases [306]. However, this model was challenged by several research groups [95, 308], which led Gerdes and colleagues to retract their prior publications [106, 306, 307] and re-evaluate the proposed model. The authors later found that the mutants used in their studies which led to the deduction of RNA endonuclease mediated persister cell formation, were inadvertently contaminated with the phage ϕ80 [108]. Nevertheless, after re-evaluation of their previous published results, they confirmed at least the involvement of (p)ppGpp and the Lon protease in persister cell formation upon ciprofloxacin and gentamicin treatment, though not as previously described via RNA endonucleases. The importance of phage induction in correlation with drug sensibility was also demonstrated in another study with *Pasteurella haemolytica* when bacteria were exposed to the fluoroquinolone danofloxacin [309]. Treatment with danofloxacin caused increased cell lysis, whereby 20 minutes of treatment were at least necessary to trigger activation of the phages and lysis of the host. In the same study a λ positive *E.coli* strain was also treated with danofloxacin and compared to a λ negative strain with the result that removal of λ de-sensitized the λ negative strain. However, it was not determined how the phages reduced the number of viable bacteria upon treatment with danofloxacin. Therefore, in paper 3 (**10.3**), the goal was to find a comprehensible explanation as to which genes were responsible for host-killing. So far, it is known that phages can carry a number of virulence, resistance, or other beneficial genes, which could become useful for the host under certain environmental conditions [158, 310]. In addition to this, it is also obligatory for many phages to encode for lysis proteins because for phage dissemination the lysis of the host is required [152]. Our wildtype strain, *S.* Typhimurium ATCC 14028s, harbours four phages in its chromosome, which are all inducible [144, 146-148, 157], but, as the results indicate, to various extents. According to our real-time polymerase chain reaction (PCR), Gifsy-1 is by far the strongest induced phage when *S.* Typhimurium was exposed to ciprofloxacin. Gifsy-1

encodes for homologs of holin (*S* = STM2613.Gifsy1), endolysin (*R* = STM2613.2n.Gifsy1) and spanins (*RzRz1* = STM2613.1n.Gifsy1), whereby holin accumulates in the cytoplasmic membrane until a critical amount is reached, causing the formation of small pores [152]. These channels allow endolysin to diffuse into the periplasm to degrade the cell wall and together with the auxiliary spanins, it results in the bursting of the host cells. However, by deleting these lysis genes, the killing of the respective mutant was decelerated and resulted in more persister cells after challenging the bacteria with ciprofloxacin. The conclusion was as follows, the phages (predominantly Gifsy-1) were activated, whereby the responsible genes for lysis were expressed as well. However, without disruption of the membrane, the reassembled phages could not leave the cells and the host survived. It is not known, what happens to the phages in the cytosol, when the cell membrane is still intact. In future, it would be interesting to determine the fate of cytosolic trapped phages. It may be conceivable that either the phages are digested or diluted after successive cell divisions. However, a phenotype was only observed upon treatment with ciprofloxacin, but not with carbenicillin, a β-lactam antibiotic. That is interesting because it was reported that ampicillin, another β-lactam antibiotic, was able to trigger the SOS response in *E. coli* [311], similar in *S. aureus* when SOS response and endogenous phages were induced [312]. It might be that generation of ROS, due to ampicillin treatment, triggered the SOS response, whereby the ROS mediated DNA damage could be comparably higher in *E. coli* and *S. aureus* as in *S*. Typhimurium, which would explain why the SOS response was triggered. Another explanation could be that the repressors of the phages in *S. aureus* are directly cleaved by RecA, while the repressor of Gifsy-1 needs the expression of additional anti-repressors [148]. This could lead to a more effective induction of phages because it circumvents at least one transcription and translation event. The wild type and the phage-free variant were also treated with kanamycin, an aminoglycoside, which targets the bacterial translation [313, 314]. However, in contrast to carbenicillin and ciprofloxacin, persister cell formation was significantly reduced in the phage-free strain upon kanamycin exposure. Therefore, to get a better insight of the metabolic status of the phage-free strain, cell lysates were prepared and the proteinaceous part of it analysed (these results were not published in paper 3). The analyses of the proteome revealed only minor differences between the wild type and the phage-free variant. However, these minor differences could be a good explanation for the disturbed persister cell formation after kanamycin treatment. Apparently, the phage-free strain has increased amounts of chaperones and heat-shock proteins, which

could indicate the accumulation of misfolded proteins. Further analyses also revealed that the phage-free strain possesses clearly less LepA, a translational elongation factor with proof-reading ability, which is also crucial for translation under high osmotic stress and low temperature [315, 316]. It may be that the decreased efficiency of the translation, in combination with kanamycin, resulted in increased amounts of misfolded proteins and ultimately into cell death due to accumulation of misfolded proteins in the membrane, forming unspecific pores [317]. Alternatively, the high levels of misfolded proteins cause the formation of protein aggregates in the cytosol, leading to deep dormancy that would impair the resuscitation of persistent cells [318]. However, the involvement of LepA, in the context of bacterial fitness, is currently under investigation in our lab.

This study (described in paper 3 (**10.3**)) provided a new killing mechanism upon treatment with ciprofloxacin. It is currently well accepted that bactericidal antibiotics induce the formation of ROS. Therefore, in addition to the main targets of the respective antibiotics, ROS also contributes significantly to drug lethality [88-90, 235, 240, 245, 246, 319, 320]. Considering the fact that many bacteria harbour endogenous phages, it is conceivable that ROS mediated killing, in the aforementioned publications, is supported by the induction of phages, which eventually contributes to bacterial killing. The fact is inadvertently strengthened by the study of Keren and colleagues [243] where moderate concentrations of norfloxacin were more efficient than high concentrations, and that the addition of the antioxidant thiourea increased only survival at moderate concentrations, but not at high concentrations, because no ROS formation was detected at higher concentrations. Apparently, they expected a correlation between antibiotic concentration and ROS formation. However, previous studies demonstrated that low concentrations of nalidixic acid were more efficient than high concentrations, due to the fact that high concentration interferes with DNA and RNA synthesis (the antibiotic paradox) [159]. In turn, interference with DNA and RNA synthesis would logically reduce the metabolic activity and ROS-mediated DNA damage as well as phage induction. On the other hand, the activation of phages at low concentrations, when metabolic activity is still allowed, would result in the expression of lysis genes and subsequently into the death of the host. How phages can be exploited to combat bacterial infections is currently under investigation, whereby lysins of phages are particularly regarded as a promising alternative to antibiotics [321-324]. Besides endogenous phages, which are integrated into the bacterial chromosomes, purified endolysins could be used to reduce

persister cell formation. For example, the purified lysin LysSTG2, extracted from *S.* Typhimurium, which is stable under various conditions, amongst other things under high salt concentration, effectively kills planktonic and biofilm *Salmonella* as well as *E. coli* and *P. aeruginosa*, demonstrating its potential as therapeutic agent [325]. However, there is still one hitch, killing was only observed after pre-treatment with chloroform for permeabilization of the outer membrane. Therefore, a more suitable chemical for convenient application was used in another study when citric acid and malic acid were used to improve the diffusion of the endolysin Lys68 through the outer membrane of *S.* Typhimurium [326]. The increased permeabilization of the outer membrane caused by both acids, resulted in a 3 to 5 log reduction of *S.* Typhimurium viability, whereby the low pH even boosted the muralytic activity of the lysin. Alternatively, hydrophobic sidechains can be added to lysins to increase the diffusion through the outer membrane to gain access to the peptidoglycan layer [327]. It would be feasible to purify and characterize the endolysin encoded by Gifsy-1 to test a possible medical application, because as demonstrated in paper 3 (**10.3**), the induction of the lysis genes had a highly antibactericidal effect on *S.* Typhimurium.

Nevertheless, the dark side of phage dependent bacterial killing has to be discussed as well. As it has been shown in paper 3 (**10.3**), DNA damage induces the phages and enhances the antibactericidal effect of ciprofloxacin, in contrast to carbenicillin and kanamycin. Therefore, one might suggest that fluoroquinolones would be the first choice for treatment of non-resistant bacteria to exploit inducible phages, which would increases the lethality of the antibiotic. However, despite beneficial effects, phage-mediated killing should be applied with caution, because phages are one of the most efficient vehicles to transfer foreign DNA between bacteria [310]. In this manner, phages are able to transduce virulence or resistance genes by encapsulating host DNA when new phage particles are formed, which are than transduced into new bacteria upon infection. Several examples exist for phage-mediated resistance gene transduction in *Salmonella* [328], such as the transduction from the MDR donor *S.* Typhimurium DT104 to the recipient *S.* Typhimurium DT17 via a P22-like phage, whereby the recipient acquired resistance against chloramphenicol, tetracyclin or/and ampicillin [329]. This may also happen under “real world conditions” when a bacterial infection is treated with antibiotics, as in the case of fluoroquinolones, which in turn could induce the SOS response and RecA regulated endogenous phages. In another example, ciprofloxacin induced phage-dependent lysis of *S.* Typhimurium DT120, causing the release of

a plasmid, encoding for kanamycin resistance, which was transferred to a kanamycin susceptible *Salmonella* strain via transduction [330]. In a microorganism rich environment like the intestines, this could lead to fatal consequences when drug susceptible bacteria receive antibiotic resistant plasmids. Thus, it should always be considered that treatment of bacterial infections with fluoroquinolones may have adverse effects, resulting theoretically in the promotion of antibiotic resistance genes via phages, be it by lysis and release of plasmids or via direct transmission of chromosomal DNA.

Besides resistance genes, phages can also carry virulence genes [310]. This leads directly into another dilemma because exposure to DNA damaging antibacterial agents can result in the activation of toxins located on phages, resulting in unexpected complications during antibiotic therapy. Treatment of Shiga-Toxin encoding *E. coli* with ciprofloxacin in mice, led to the activation of Shiga-Toxin and increased lethality, whereby treatment with fosfomycin, which targets the cell wall synthesis, did not [331]. Concomitantly with Shiga-Toxin induction, phages caused lysis of the host and infected Shiga-Toxin negative strains in the gastrointestinal tract of mice, thus ciprofloxacin has the potential to support the spread of virulence genes [331, 332]. The endogenous phages of our wildtype strain, *S.* Typhimurium ATCC 14028, carry virulence genes as well, which are also important during infection in mice [151]. How and whether these virulence genes would be more strongly induced by fluoroquinolone treatment is still unknown, which is why this aspect requires further investigation in the future.

11.2 Conclusion

11.2.1 ATP pools, ROS formation and phage induction

Stimulation of the metabolism by carbon sources and the provision with a terminal electron acceptor, such as oxygen, can cause the formation of ROS during drug treatment [74]. In addition, blocking the ATP-synthase also enhances the metabolic activity due to low ATP, leading to interference with the feedback inhibition of the TCA-cycle and glycolysis, thus making bacteria more susceptible to antibiotics [283]. On the other hand, it was reported that low ATP was beneficial to persister cell formation [95, 96], while others demonstrated the opposite [88, 99, 100]. Therefore, the question remains, why is low ATP advantageous in one study but not in the other? The best explanation is that the respective studies have used different approaches to reduce the ATP levels, whereby Shan *et al*. used arsenate to artificially lower the ATP concentration [95], whereas Braetz *et al*. deleted the complete ATP-synthase

operon [99, 100]. The deletion of the *atp* operon did not stop the ATP formation but significantly disturbed it, allowing growth and increased oxygen uptake, presumably as a result of augmented TCA activity and glycolysis. However, the addition of arsenate, led to the binding of ADP by arsenate, impeding with the synthesis of further ATP and therefore forced the bacteria into a dormant state without metabolic activity and oxygen consumption. Apparently, there is some kind of threshold behaviour because as long as the ATP concentration does not drop under a certain level to interrupt metabolic activity, the bacteria are more susceptible to antibiotics, despite having less ATP, because bacteria try to restore the intracellular ATP homeostasis. Nevertheless, depletion of ATP quickens the formation of protein aggresomes [318]. Thus, very low ATP levels could result into strong protein aggregation, therefore significantly reducing the metabolic activity to a point, where it is not possible anymore for bacteria to restore ATP homeostasis. This would diminish the formation of ROS and the lethality of antibiotics due to reduced target activity of the antibiotics. Moreover, a further side effect of accumulated protein aggresomes is a reduced translational activity, hence the transcription and translation of phage genes would be significantly reduced as well. Thus, very low ATP would diminish the amount of phage proteins, resulting in incomplete phage assembly and no host lysis due to the lack of lysis proteins. Therefore, low ATP and a diminished formation of ROS would result in low DNA damage and a reduced phage induction. In addition, RecA needs ATP for DNA binding and LexA cleavage, in which both proteins also regulate the induction of phages [148, 333-335]. It also implies that low ATP would interfere with phage induction on a transcriptional level. This is further supported by the fact that overexpression of the toxin TisB inhibits the activation of SOS genes [32]. It was reported that overexpression of TisB resulted in decreased transcription and translation, as a consequence of membrane embedded TisB, causing proton leakage, thus interfering with ATP synthesis [336]. TisB was also brought into connection with disturbed persister cell formation upon ciprofloxacin treatment [33]. However, the question is whether *tisB* induction inhibits DNA gyrase, which should reduce DNA damage in the presence of fluoroquinolones, or whether TisB suppresses activation of SOS-dependent phages and thus lysis of the bacteria. Interestingly, *tisB* is also under the control of RecA and LexA, as the phages λ in *E. coli* or Gifsy-1, Gifsy-2, Gifsy-3 and ST64B in *S.* Typhimurium [147, 148, 335, 336]. May it be that *tisB's* major function is to control the SOS response and to interfere with strong phage induction? In the future, further studies will show if there is any connection between the ATP concentration and

phage induction, which could also explain, why some research groups reported that low ATP is beneficial for persister cell formation, while other demonstrated the opposite.

12 References

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14 Contribution to conception, implementation and publication

Publication 1: The role of ATP pools in persister cell formation in (fluoro)quinolone-susceptible and-resistant strains of *Salmonella enterica* ser. Typhimurium

The mutants and plasmids used in this study were constructed by Dr. Karsten Tedin, Dr. Arthur Thompson and Peter Schwerk. In addition, Peter Schwerk also performed MIC assays with nalidixic acid (Fig. 1 a) and 1 c) in the publication). The remaining experiments were conducted by Sebastian Braetz. The intellectuel input came from Dr. Karsten Tedin, Prof. Dr. Marcus Fulde and Sebastian Braetz. The paper was written by Dr. Karsten Tedin and Sebastian Braetz.

Publication 2: *Salmonella* Central Carbon Metabolism Enhances Bactericidal Killing by Fluoroquinolone Antibiotics

The mutants and plasmids used in this study were constructed by Dr. Karsten Tedin, Dr. Arthur Thompson and Peter Schwerk. In addition, Peter Schwerk also tested the plasmids carrying promoters for the SOS and oxidative stress response (Fig. 7 in the publication). The remaining experiments in this study were conducted by Sebastian Braetz. The intellectuel input came from Dr. Karsten Tedin, Prof. Dr. Marcus Fulde and Sebastian Braetz. The paper was written by Dr. Karsten Tedin and Sebastian Braetz.

Publication 3: Prophage Gifsy-1 Induction in *Salmonella enterica* Serovar Typhimurium Reduces Persister Cell Formation after Ciprofloxacin Exposure

The mutants used in this study were constructed by Dr. Karsten Tedin and Peter Schwerk. The ΔGifsy-3 deletion strain was constructed by Dr. Nara Figueroa-Bossi as well as the diagram showing the gene organization of Gifsy-1 (Fig. 4 in the publication). The remaining experiments were performed by Sebastian Braetz. The intellectuel input came from Dr. Karsten Tedin, Prof. Dr. Marcus Fulde and Sebastian Braetz. The paper was written by Sebastian Braetz edited by Dr. Karsten Tedin and Dr. Nara Figueroa-Bossi.