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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