Increased survival of antibiotic resistant *Escherichia coli* inside the macrophages

Migla Miskinyte\(^1\) and Isabel Gordo\(^1\)#

\(^1\)Instituto Gulbenkian de Ciência, Oeiras, Portugal

# Corresponding author: igordo@igc.gulbenkian.pt

**Abstract**

Mutations causing antibiotic resistance usually incur a fitness cost in the absence of antibiotics. The magnitude of such cost is known to vary with the environment. Little is known about the fitness effects of antibiotic resistance mutations when bacteria confront the host’s immune system. Here we study the fitness effects of mutations in the *rpoB*, *rpsL* and *gyrA* genes, which confer resistance to rifampicin, streptomycin and nalidixic acid, respectively. These antibiotics are frequently used in the treatment of bacterial infections. We measured two important fitness traits – growth rate and survival ability – of twelve *Escherichia coli* K-12 strains, each carrying a single resistance mutation, in the presence of macrophages. Strikingly, we found that 67% of the mutants survived better than the susceptible bacteria in the intracellular niche of the phagocytic cells. In particular, all *E. coli* streptomycin resistant mutants exhibit an intracellular advantage. On the other hand, 42% of the mutants were costly when bacteria were allowed to divide outside macrophages. This study shows that single non-synonymous changes affecting fundamental processes in the cell can contribute to prolonged survival of *E. coli* in the context of an infection.
Introduction

A great deal of bacterial adaptation in the context of infectious diseases is their rapid evolution to tackle the immune system and antibiotics. *Escherichia coli* is both a commensal and a versatile pathogen, that can cause death (15). Given these characteristics, it is an ideal organism to study the transition of commensalism to pathogenicity. *E. coli* colonizes the infant gastrointestinal tract within hours after birth, and typically a mutualistic relation builds up. However, even the harmless *E. coli* can cause an infection when gastrointestinal barriers are broken (43) or in immunosuppressed hosts (27). Healthy hosts are also susceptible to highly adapted *E. coli* pathogenic clones, which can cause many different types of infections. There is evidence that some of the pathogenic strains evolved from the commensal *E. coli*, through the acquisition of new genes and mutations (15). A fundamental part of the ecology of *E. coli* during infection process is its interaction with the host immune system cells, in particular with macrophages. It is however not known if *E. coli* harbouring antibiotic resistance can have an advantage or disadvantage in the context of an interaction with the immune system. This knowledge is important given the high frequency of antibiotic resistance within commensal *E. coli* in healthy individuals (3, 30), which may lead to an increased risk of treatment failure during an infection process, because of limited therapeutic options.

Mutations that cause antibiotic resistance often produce associated fitness costs in bacteria (2, 34). When the environment contains an antibiotic, resistant bacteria exhibit an advantage. However when the antibiotic is absent, resistant bacteria typically have reduced growth rates, although this depends on the genetic background (19, 50). This is
not surprising, since mutations which cause antibiotic resistance often target physiologically important functions in the cell, such as transcription and protein synthesis, cell wall synthesis or nucleic acid synthesis (2). Interestingly, the fitness effect of a resistance mutation can be detrimental in one environment and beneficial in another (6, 22, 35, 39, 49). For example, Trindade et al. showed increased variation in fitness effects of resistant mutations in *E.coli* with increased environmental stress. Similarly, Hall et al. demonstrated that the costs of 24 different *rpoB* mutations vary greatly among 41 environments with different carbon source. Having in mind that fitness effects of resistant mutations exhibit strong genotype-by-environment interactions, it is important to determine the effects of resistance in an environment imposed by the host. Despite its importance, to our knowledge there are only a few studies that explicitly address fitness effects of antibiotic resistant under conditions that are closer to the growth conditions in a host (1, 9). Furthermore, it has been shown that fitness effects of antibiotic resistant mutations vary substantially in the different *in vivo* and *in vitro* models (8, 17, 21, 33).

One important interaction that bacteria face in natural conditions is the interaction with cells from the immune system that are able to phagocytise them. There is little information available on fitness effects of antibiotic resistance in this important context. The aim of the research reported here is to determine whether or not single point mutations conferring rifampicin, streptomycin and nalidixic acid resistance can affect reproduction and survival of commensal *E.coli* in the face of professional phagocytes. This study shows that commensal bacteria carrying specific resistance mutations can survive better in the intracellular environment of professional phagocytes.
This may have important consequences in designing therapeutic treatments and may be important to understand the spread of drug resistance.

**MATERIALS AND METHODS**

**Media and growth conditions**

The RAW 264.7 murine macrophage cell line was maintained in an atmosphere containing 5% CO$_2$ at 37°C in RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 mM hepes (Invitrogen), 100 U/ml penicillin/streptomycin (Gibco), 50 µM 2-mercaptoethanol solution (Gibco), 50 µg/ml gentamicin (Sigma), with 10% heat-inactivated FCS (standard RPMI complete medium). Before infection assays, M₆s were maintained in the same conditions, but in antibiotic-free RPMI media (without penicillin/streptomycin and gentamicin). Bacterial strains were grown and competed in antibiotic-free RPMI media in an atmosphere containing 5% CO$_2$ at 37°C or in Luria-Bertani (LB) medium at 37°C, with aeration (Grant-bio PHP-4 type Thermo-Shaker at 700 rpm).

**Construction of strains**

Susceptible MG1655-YFP and MG1655-CFP strains (MG1655, galK::CFP/YFP, ΔlacIZYA) containing yellow (YFP) and cyan (CFP) fluorescent proteins under constitutive expression were created by moving yfp or cfp chromosomal inserts by P1 transduction from previously described strains (MC4100, galK::CFP/YFP, ampR (pZ12), strR (rpsl150)), that were kindly given by R. Kishony (24). To ensure constitutive expression of YFP or CFP fluorescent proteins the lac operon was deleted from MG1655 background. Ampicillin resistance (pZ12) was removed from the yfp or cfp locus using Wanner and Datsenko method (13). Mutations conferring
resistance to rifampicin (in \textit{rpoB} gene), streptomycin (in \textit{rpsL} gene) and nalidixic acid
(in \textit{gyrA} gene) were previously constructed in \textit{Escherichia coli} K-12 MG1655
background (see Table S1, (50)). General transduction using P1 bacteriophage was
performed as previously described (48) in order to place resistance mutations in the new
\textit{E.coli} K-12 MG1655-YFP and MG1655-CFP background. To confirm mutations, each
antibiotic resistance target gene was amplified and then sequenced. The primers used to
amplify part of \textit{rpoB} gene were: 5′-CGTCGTATCCGTTCCGTTGG-3′ and 5′-
TTCACCCGGATAACATCTCGTC-3′; for \textit{rpsL} gene: 5′-
ATGATGGCGGGATCGTTG-3′ and 5′-CTTCCAGTTCAGATTTACC-3′ and for
\textit{gyrA} gene: 5′-TACACCGGTCCACATTGAGG-3′ and 5′-
TTAATGATTGCCGCGTCGG-3′. Each resistant clone was grown from a single
colony in LB medium supplemented with the respective antibiotic and stored in 15%
glycerol at -80°C.

**Competitive fitness in conditions where bacteria can divide: test for effects on
reproduction**

To estimate the fitness cost of resistance mutations we performed competition assays (as
commonly done to estimate fitness effects of mutations (16)) in three different
environments: LB, RPMI and in RPMI with the MΦs. The resistant mutants constructed
in MG1655-CFP (or in the MG1655-YFP) strain were competed against a susceptible
MG1655-YFP (or susceptible MG1655-CFP) strain in an antibiotic-free environment, at
a ratio 1:1. For competitions in LB, both resistant and susceptible strains were grown
separately for 48 hours for acclimatization (bacteria were diluted at 1:10^3 after 24 hours
for passage) at 37°C with aeration, then mixed and 10 µl of 10^{-2} dilution was inoculated
to the final volume of 150 µl of LB media in 96-well microtiter plates (Costar #3595)
for 24 hour competition. Plates were arranged in a checkerboard configuration where half of the wells were without cells to control for well-to-well and external contamination. For competitions in RPMI, resistant and susceptible strains were grown in antibiotic-free RPMI media for 48 hours (bacteria were diluted at $1:10^3$ after 24 hours for acclimatization) at 37°C with 5% CO2. Competitions were performed in a 24-well cell culture tissue plates (containing 1 ml of culture media in each well), by inoculating 10 µl of $10^{-1}$ dilution (approximately $5\times10^4$ bacteria). For competitions in the presence of the MΦs, strains were competed in the same conditions as used for competitions in the RPMI, except that MΦs were present. In the infection with $10^6$ E. coli with $10^6$ MΦs (RAW 264.7), after 3 hours the number of CFUs inside Mfs is approximately $10^4$ and the CFUs outside are of $10^5$. MΦs were seeded in a 24-well tissue culture plate at approximately 2 to $3\times10^5$ cells per well and allowed to attach overnight. Cells were then washed, re-suspended in fresh antibiotic-free RPMI media and activated with 2 µg/ml CpG-ODN 1826 (5’TCCATGACGTTCCAGCTGTGTT 3’ - Sigma) for 24 hours. After 24 hours, cells were washed from remaining CpG-ODN, fresh antibiotic-free RPMI media was added and macrophages were infected with bacteria in the manner described before. The initial and final ratios of resistant and susceptible strains were determined by Flow Cytometry. The fitness cost of each of the resistance mutation was measured four times (twice in YFP and twice in CFP background). The selection coefficient, a measure of competitive fitness, was estimated as:

$$S_{\text{coeff}} = \frac{\ln\left(\frac{N_{fb}/N_{fa}}{N_{fb}/N_{fa}}\right)}{\ln\left(\frac{N_{fa}/N_{ia}}{N_{fa}/N_{ia}}\right)}$$

(36)

Where $S_{\text{coeff}}$ is a selection coefficient of the resistant strain $b$ against the susceptible strain $a$, $N_{fa}$ and $N_{fb}$ are the numbers of resistant ($b$) and susceptible ($a$) bacteria after
competition and $N_{ia}$ and $N_{ib}$ are the initial numbers of resistant ($b$) and susceptible ($a$) bacteria before the competition (results are shown in Figure 1B, D and F).

**Competitive fitness inside the MΦs: test of the effect on survival**

Non-pathogenic *E. coli* does not replicate inside the MΦs and thus in this niche survival is the most important fitness component (23). To estimate fitness effect of the resistant mutations on survival inside phagocyte cells, MΦs were prepared in the manner described above, infected with $5 \times 10^6$ bacteria (1:1 of resistant to susceptible strain) and centrifuged at $203 \times g$ (1000 rpm) for 5 min to enhance bacterial internalization. After 2 hours of infection, MΦs were washed from extracellular bacteria and fresh cell culture medium containing 100 µg of gentamicin/ml was added to kill the remaining extracellular bacteria. After incubation for an additional hour, the medium was removed, monolayers of macrophages were washed and RPMI medium containing 20 µg of gentamicin/ml was added (0 h post-infection time point). To determine the number of intracellular bacteria after 5 h and 24 h of incubation, infected MΦs were washed 3 times with phosphate-buffered saline (PBS), 0.1% Triton-X was added for 30 min at 37°C in order to lyse the MΦs, centrifuged at $10600 \times g$ (10000 rpm) for 5 min, washed in PBS and overall number of bacteria was counted by plating on LB agar plates. To measure intracellular survival after 48 h post-infection, fresh culture medium containing gentamicin (20 µg/ml) was added after 24 h post-infection period to the infected cells.

Survival inside the macrophages was estimated as the change in relative frequency:

$$\Delta X = \frac{N_{fa}}{N_{fa} + N_{fb}} - \frac{N_{ia}}{N_{ia} + N_{ib}}$$

where $N_{fa}$ and $N_{fb}$ are the numbers of resistant ($b$) and susceptible ($a$) bacteria after competition and $N_{ia}$ and $N_{ib}$ are the initial numbers.
Survival to oxidative stress of streptomycin resistant mutants

Given that all STR resistant mutants showed a survival advantage inside MΦs, we sought to determine if the mutants would also show an advantage during nutrient limitation in the stationary growth phase and under oxidative stress, which are characteristics of environment inside the MΦs.

To determine if STR resistant clones have differential fitness advantage in exponential (4 h), early stationary (24 h) and late stationary (48 h) phases, competition assays between STR resistant and susceptible strains were done. Briefly, STR resistant and susceptible strains were grown in antibiotic-free RPMI media separately for 48 hours at 37°C with 5% CO₂ (bacteria were diluted at 1:10³ after 24 hours for acclimatization), then mixed at a ratio of 1:1 (1 resistant to 1 susceptible strain) and 10 µl of 10⁻¹ dilution inoculated to 1 ml of culture media. At 4, 24 and 48 h, a sample of bacterial suspension was plated onto LB plates to estimate the frequencies of STR resistant to susceptible strain at different growth phases (before exposure to H₂O₂ (Sigma), results are shown in Figure 2A).

To determine if STR resistant clones would show an advantage to survive oxidative stress during different growth phases, a mixture of STR resistant and susceptible strains (see description above – before exposure to H₂O₂), was treated with different concentrations of H₂O₂ (10 mM at 4 hours, 20 mM at 24 hours and 40 mM at 48 hours) for 30 min at 37°C. Appropriate dilutions were immediately plated onto LB to determine the frequencies of STR resistant to susceptible strain after exposure to H₂O₂.
Different concentrations of H$_2$O$_2$ were chosen because of higher cell mortality at exponential phase in comparison to stationary phase to the same concentration of H$_2$O$_2$ (31). Four independent replicate experiments were done for each strain (two in YFP and two in CFP background). Survival to oxidative stress was calculated dividing relative frequencies of STR mutant after and before exposure:

$$\Delta X(H_2O_2) = \frac{(N_{fb} / (N_{fa} + N_{fb}))}{(N_{nb} / (N_{na} + N_{nb}))}$$

Where $N_{fa}$ and $N_{fb}$ are the numbers of resistant (b) and susceptible (a) bacteria after exposure to H$_2$O$_2$ and $N_{na}$ and $N_{nb}$ are the numbers of resistant (b) and susceptible (a) bacteria, before exposure (results are shown in Figure 2B).

**Statistical analysis**

Wilcoxon signed rank test and Wilcoxon sum rank test with Bonferroni correction (when multiple comparisons across mutants were made) were performed. Kruskal-Wallis rank sum test was done for comparisons across post-infection times. All statistical analysis was done in the R software: [http://www.r-project.org/](http://www.r-project.org/). Analysis of linear regression between survival and reproduction of antibiotic resistant mutants in the presence of the MΦs (Figure 3) was performed using SigmaPlot 9.0 software (Systat Software Inc., Chicago, USA).

**RESULTS**

We studied twelve different antibiotic resistance mutations in *rpsL*, *rpoB* and *gyrA*, conferring resistance to streptomycin (STR), rifampicin (RIF) and nalidixic acid (NAL) antibiotics, respectively (see Table 1). These mutations had been previously...
studied for fitness costs in Luria Bertani (LB) medium, when present in another genetic background (50). Because fitness of antibiotic resistant clones can depend on the genetic background (19), we measured competitive fitness of these twelve mutants in LB media and found that all showed a cost in LB. The costs of antibiotic resistant mutations were not significantly different in the new genetic background in the LB media (Wilcoxon sum rank test with Bonferroni corrections; four out of twelve mutations were significantly different without Bonferroni corrections, Fig. S1).

To determine the fitness effects of antibiotic resistant clones in the presence of macrophages (MΦs), competition experiments between the susceptible and the resistant mutants were performed. Two main fitness traits are important during the infection process: reproduction, which occurs outside MΦs, and survival, which is the main fitness component inside MΦs. The effects on both traits, reproduction outside the MΦs and survival inside the MΦs, were measured (see Materials and Methods). In order to estimate fitness effects of resistance in bacterial reproduction, competition assays in the RPMI cell culture media in the presence (+MΦs) and absence (−MΦs) of the MΦs were performed.

Global survival advantage of STR resistant mutants inside the MΦs. Figure 1A shows the effects on survival of E. coli strains carrying mutations K43N, K43T, K43R or K88R, which confer resistance to streptomycin. Surprisingly, all STR resistant mutants showed a survival advantage inside MΦs during 5, 24 and 48 hours post-infection. There was no significant difference for survival effects of STR resistant mutants between post-infection periods (Kruskal-Wallis rank sum test, p>0.05), except for K43R mutant, that increased survival inside the MΦs at later time points (Kruskal-Wallis rank sum test, p=0.04). Contrary to the global fitness survival advantage inside
MΦs, two mutants showed a cost and two other mutants were neutral when bacteria are allowed to reproduce, which was measured in competitive fitness assays against the susceptible strain in the presence and absence of the MΦs (Fig. 1B). The cost of one mutation (K43N) differed significantly due to the presence of the MΦs (Wilcoxon sum rank test, p=0.04), while the costs of other three mutations were not different. In summary, single point mutations in rpsL gene, provide a survival fitness advantage in commensal E. coli in the intracellular niche of MΦs, leading to an increased risk of treatment failure during an infection process.

Variable fitness effects in RIF resistant mutants. Half of the RIF mutants (S512F, I572F and H526Y) showed a survival advantage inside the MΦs (Fig. 1C). These were neutral or only slightly advantageous in competitive fitness assays where growth can occur outside MΦs (Fig. 1D – white bars). Two RIF resistant mutants showed impaired survival inside the MΦs (R529H and H526D) and also showed the highest fitness costs for reproduction (Fig. 1C and 1D). For the S531F mutation no effect was detected on survival, but a deleterious effect was measured on reproduction. There was no overall difference for effects on survival of RIF resistant mutants between different post-infection periods (Kruskal-Wallis rank sum test, p>0.05), except for one mutant (H526Y), that ceased to be advantageous for survival inside the MΦs at later time points (Kruskal-Wallis rank sum test, p=0.01). Fitness effects on reproduction of three E. coli RIF resistant mutants (Wilcoxon sum rank test, p=0.01 for H526Y, p=0.009 for I572F, p=0.005 for H526D) were significantly different due to the presence of the MΦs, while effects for reproduction of other three mutants did not differ between presence and absence of MΦs in the environment (Fig. 1D).
NAL resistant mutants are advantageous or neutral. The fitness of S83L mutant was higher than the susceptible, for both, reproduction in the culture media and survival inside MΦs, while fitness of D87Y mutant remained neutral (Fig. 1E and 1F). There was no effect on survival between different post-infection time points (Kruskal-Wallis rank sum test, p>0.05). We did not observe significant differences in fitness effects for reproduction for the two studied NAL resistant mutants (S83L and D87Y) due to the presence of the MΦs (Wilcoxon sum rank test, p>0.05 for both mutations) (Fig. 1F).

Advantage of STR mutants to oxidative stress in the stationary phase. Given the striking survival advantage of all STR resistant mutants, we tried to determine if such results could be caused by the specific stress that bacteria face upon internalization, namely nutrient starvation and/or oxidative stress. To test this hypothesis, competition assays were performed during exponential phase where bacteria are growing and stationary phase where growth is resumed. Possible advantage to oxidative stress was tested during those phases, by addition of H₂O₂. While fitness cost for reproduction was the highest after 24 hours of bacterial growth, it was relieved after 48 hours for the two most costly STR mutants – K43N and K43T (Wilcoxon sum rank test, p=0.03 for K43N, p=0.03 for K43T, Fig. 2A), indicating that STR resistant mutations could bear an advantage during stationary phase induced by nutrient limitation. Interestingly, all STR resistant mutants showed an increased survival to oxidative stress after 24 hours, but not during exponential growth phase (Fig. 2B). The results therefore indicate that nutrient deprivation and oxidative stress are key factors on the survival advantage that these mutants exhibit inside MΦs.

Trade-off between survival and growth. It has been proposed that resistance to stress is associated with reduced resource uptake (18). This trade-off between self-
preservation and nutritional competence, so called SPANC balance, has been observed in several studies (14, 18). Recently, SPANC trade-off has been directly linked to the growth rate, stress resistance, outer membrane permeability, morphotype characteristics and virulence properties of antibiotic resistant *E. coli* isolates from deep and visceral infections in humans (30). In our study we tested for a trade-off between survival inside the MΦs and growth rate without MΦs (Fig. 3). We did not find evidence for a trade-off, but, on the contrary, antibiotic resistant clones which survived better inside the MΦs also had growth rate (Fig. 3).

**DISCUSSION**

Drug-resistant bacteria pose a significant threat to human health, and it is important to understand how the fitness of such bacteria can be impaired during infection. Here, we studied how antibiotic resistance affects two important fitness traits: the ability to survive and reproduce in the presence of MΦs. It is known that during the entry into MΦs, bacteria experience a set of environmental stresses, such as host-induced nutrient limitation, acidification, toxic peptides, osmotic stress and reactive oxygen species (ROS), of which the latter is believed to be the major cause of bacterial killing (44). To our knowledge, this is the first study that measures fitness effects for survival of several antibiotic resistant mutants in the intracellular environment of the MΦs. Surprisingly, we found that all STR resistant mutants had increased survival inside MΦs. RIF mutants were highly variable and NAL mutants showed survival advantage of small effect. Importantly streptomycin resistance, although carrying substantial fitness costs for growth rate, shows a global advantage for survival.

The experience of the early single use of streptomycin in 1946, for treating *Mycobacterium tuberculosis* infections indicated that resistance to this drug could be
acquired very rapidly (12). To this day STR resistant isolates have been identified in many other important pathogens, such as *Shigella flexneri*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and even in commensal *E. coli* sampled from healthy individuals (5, 25, 42, 51). High resistance incidence to this drug is frequently due to point mutations in *rpsL* gene, with the most common mutations at the codon K43 and K88 (46) that were included in this study. These mutations were shown here to be beneficial in the intracellular environment of MΦs in *E. coli*. One possible explanation for the fitness advantage of STR resistant mutations could be the approximately 7-fold improvement in the accuracy of ribosomes in *rpsL* mutants (54). It was shown that streptomycin resistance mutations in *rpsL* gene often lead to hyper-accurate, but slower ribosomes (7). Indeed, all STR resistance mutations that were tested in our study are responsible for the increased fidelity of ribosomes (38). Though fast ribosomes are required in actively dividing cells, hyper-accurate ribosomes are advantageous in non-dividing cells during starvation, because they exhibit attenuated protein oxidation during growth arrest (4) and oxidized proteins are known to be more susceptible to proteolytic degradation (10). This should be extremely relevant upon entry to the MΦs, where *E. coli* not only undergoes growth arrest and nutrient starvation, but also has to deal with ROS generated by the MΦs (44). Consistent with this hypothesis, we found that STR resistant mutants have reduced fitness costs when nutrients are deprived and survive better than the susceptible under oxidative stress in stationary phase (Fig. 2). Certainly, finding that most commonly identified mutations, conferring resistance to STR, enhanced the survival capacity of *E. coli* inside the MΦs, begs the question if that advantage could exist in other bacterial species, such as *M. tuberculosis* and other pathogenic bacteria.
Many bacterial pathogens (32, 45, 53) acquired resistance to RIF in the last decade. It is known that in 96% of RIF resistant clinical isolates of TB, resistance is due to the mutations in \textit{rpoB} gene, with the most common mutations at the codon 531 and 526 in distinct geographical locations (11, 47). In this study, besides prevalent mutations in codons 531 and 526 (S531F, H526Y, H526D), other mutations in codons 512, 529 and 572 (S512F, R529H, I572F) were also included. The fitness effects on survival of RIF mutants varied in our study. Interestingly, different base substitutions leading to different amino acids even at the same codon position (see Fig. 1C – H526D and H526Y) gave differential outcomes for \textit{E. coli} survival inside MΦs. The mutation at the codon 526 has been shown to be responsible for oxidative stress sensitivity in \textit{E. coli} and \textit{Staphylococcus aureus}. However, the molecular mechanism for this remains unknown (28). Several observations suggest that single point mutations in the \textit{rpoB} gene encoding \(\beta\) subunit of RNA polymerase, can have an effect on RNA polymerase interaction with several promoters and transcriptional regulators, leading to different phenotypes (37, 40, 55). For example, in \textit{Bacillus subtilis} the RNA polymerase complex interacts with every promoter in bacterial genome, so the mutations in RNA polymerase lead to global changes in gene transcription and, hence, affect several physiological processes, such as growth and metabolism, chemotaxis, competence, spore resistance and many others (37). Since RIF mutations have been found to affect physiological processes to different extents, it may not be surprising that we found a great variation in their fitness effects of RIF mutants inside MΦs.

The emergence of NAL resistant isolates during treatment of \textit{Shigella}, \textit{Campylobacter} or \textit{Salmonella} infections has been of a great concern (20, 29, 52). Single point mutations in the quinolone resistance-determining region of the DNA subunit...
gene gyrA, at a codon 83 (42% frequency) and 87 (35% frequency) have been attributed to the high levels of resistance to this antibiotic (26). Although the fitness costs of those mutations appear to be low in laboratory medium (50), it is not known how resistance to this drug may affect survival and replication of these bacteria in the context of infection.

In *E.coli* we found no fitness costs (for D87Y mutation) or even slightly enhanced fitness (for S83L mutation) of NAL resistant clones for survival inside the MΦs, which is compatible with those of the previous reports showing that NAL resistance is usually associated with very small fitness costs (9).

It was previously demonstrated that fitness effects for reproduction of antibiotic resistant bacteria generally increases under stressful conditions (41, 49). Effects on reproduction of more than half (58%) antibiotic resistant mutants was either neutral or slightly advantageous in the presence of the MΦs, however this effect was mainly attributed to the growth in the RPMI cell culture medium we used for maintenance of eukaryotic cells. Still, this is altogether relevant, because RPMI cell culture medium is supposed to mimic abiotic conditions in the human host. Moreover, fitness effects for reproduction differed in 33% of antibiotic resistant cases, due to the presence of the MΦs (compare black and white bars in Fig. 1B, D and F). It is however not surprising, given that MΦs not only inflict several different stresses on bacteria, but can also modify composition of the extracellular media. This is consistent with earlier findings suggesting that fitness costs of antibiotic resistant mutants may vary in the different environmental conditions (22, 49).

The findings made here have several medically relevant implications. First, this work shows that the presence of macrophages can have drastic consequences for the biological fitness of antibiotic resistant *E. coli*. This conclusion points towards
measuring fitness costs in such environment in other bacterial species as well as studying mutational targets of widely used antibiotics in clinics. Second, this study identifies single point mutations that are advantageous for bacterial survival in macrophages because of the environmental stresses imposed by MΦs, such as exposure to H$_2$O$_2$. Taken all together, our main finding that stressful intracellular environment of MΦs can select for antibiotic resistance has important consequences for predictions of the spread of drug resistance.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the European Research Council under the European Community’s Seventh Framework Programme (FP7/2007-2013) /ERC grant agreement no 260421 – ECOADAPT. I.G. acknowledges the salary support of LAO/ITQB & FCT.

REFERENCES


Table 1. Genotypes of single-point mutations used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aa change ; nt change</td>
<td></td>
</tr>
<tr>
<td>rpsL</td>
<td>K 43 N; AAA to AAC</td>
<td>STR</td>
</tr>
<tr>
<td>rpsL</td>
<td>K 43 T; AAA to ACA</td>
<td>STR</td>
</tr>
<tr>
<td>rpsL</td>
<td>K 43 R; AAA to AGA</td>
<td>STR</td>
</tr>
<tr>
<td>rpsL</td>
<td>K 88 R; AAA to AGA</td>
<td>STR</td>
</tr>
<tr>
<td>rpoB</td>
<td>S 531 F; TCC to TTC</td>
<td>RIF</td>
</tr>
<tr>
<td>rpoB</td>
<td>H 526 Y; CAC to TAC</td>
<td>RIF</td>
</tr>
<tr>
<td>rpoB</td>
<td>I 572 F; ATC to TTC</td>
<td>RIF</td>
</tr>
<tr>
<td>rpoB</td>
<td>R 529 H; CGT to CAT</td>
<td>RIF</td>
</tr>
<tr>
<td>rpoB</td>
<td>S 512 F; TCT to TTT</td>
<td>RIF</td>
</tr>
<tr>
<td>rpoB</td>
<td>H 526 D; CAC to GAC</td>
<td>RIF</td>
</tr>
<tr>
<td>gyrA</td>
<td>S 83 L; TCG to TTG</td>
<td>NAL</td>
</tr>
<tr>
<td>gyrA</td>
<td>D 87 Y; GAC to TAC</td>
<td>NAL</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Effects of resistance on survival (left panel) and reproduction (right panel) of mutations in \textit{rpsL} (A and B), \textit{rpoB} (C and D) and \textit{gyrA} (E and F) in \textit{E. coli}. A, C and E show fitness effects on survival inside the MΦs after 5 h (black dashed bars), 24 h (light grey dashed bars) and 48 h (dark grey dashed bars) post-infection. B, D and F show the effects of the mutations when bacteria can reproduce in the presence (white bars) and absence (black bars) of MΦs. All fitness effects were estimated using competition assays against a susceptible strain. The asterisk (*) represents significant differences (p<0.05) using Wilcoxon sum rank test.

Figure 2. Starvation and oxidative stress diminish the fitness cost of STR resistant mutations. (A) Effects on reproduction of STR resistant mutations during 4, 24 and 48 hours competition assays against a susceptible strain in RPMI medium. (B) Advantage of STR resistant mutants against a susceptible strain after exposure to H$_2$O$_2$ at different phases of bacterial growth in RPMI medium. Bars above the dashed line represent an increased survival of the STR mutant against a susceptible strain. The asterisk (*) represents statistical significant difference (p<0.05) using Wilcoxon sum rank test.

Figure 3. Test for correlation between survival inside MΦs and growth rate. Intracellular survival of resistant mutants against a susceptible strain was measured at 5 h after bacterial internalization. Reproduction in the RPMI media without MΦs was measured after 24 h competition assay. The slope of the regression line (solid line) is 0.9 ± 0.34 (Std. Err) and p=0.02, with R$^2$=0.41. The graph is divided into quarters where R+ and R- (or S+ and S-) means advantage and disadvantage for reproduction (or survival), respectively. Triangles represent STR, circles RIF, and squares NAL mutants.