Enhanced survival of Rifampicin and Streptomycin double resistant *E. coli* inside macrophages

Running Title: Double resistance enhances survival in macrophages

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ABSTRACT

Evolution of multiple antibiotic resistant bacteria is an increasing global problem. Even though mutations causing resistance usually incur a fitness cost in the absence of antibiotics, the magnitude of such costs varies across environments and genomic backgrounds. Here we have studied how the combination of mutations that confer resistance to rifampicin (RifR) and streptomycin (StrR) affects the fitness of E. coli when it interacts with cells from the immune system - macrophages (MΦs). We found that 13 RifRStrR double resistant genotypes, out of 16 tested, show a survival advantage inside MΦs indicating that double resistance can be highly beneficial in this environment. Our results suggest that there are multiple paths to acquire multiple-drug resistance in this context, i.e. if a clone carrying RifR alleles H526 or S531 acquires a second mutation conferring StrR, the resulting double mutant has a high probability of showing increased survival inside MΦs. On the other hand, we have found two cases of sign epistasis between mutations leading to a significant decrease in bacteria survival. Remarkably, infection of MΦs with one of these combinations, K88R+H526Y, resulted in an altered pattern of gene expression in the infected MΦs. This indicates that the fitness effects of resistance may depend on the pattern of gene expression of infected host cells. Notwithstanding the benefits of resistance found inside MΦs, the RifRStrR mutants have massive fitness costs when the bacteria divide outside MΦs, indicating that the maintenance of double resistance may depend on the time spent within and outside phagocytic cells.
INTRODUCTION

Antibiotic resistance in many pathogens has become a worldwide problem, incurring both loss of human lives and economic costs (1). Bacteria can acquire antibiotic resistance as a result of transfer and acquisition of new genetic material between individuals of the same or different species but also by chromosomal mutations, which alter existing proteins. For instance, resistance to rifampicin (Rif\textsuperscript{R}, a rifamicin) occurs due to mutations in the gene \textit{rpoB} coding for the β-subunit of RNA polymerase and resistance to streptomycin (Str\textsuperscript{R}, an aminoglycosidase) occurs due to mutations in the gene \textit{rpsL} coding for a ribosomal protein (2). These genetic targets for resistance are common across a wide range of bacteria species including \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa} and \textit{Mycobacterium tuberculosis} (2-4). Mutations causing antibiotic resistance usually incur a fitness cost in the absence of antibiotics (5-7). However the magnitude of such costs is known to vary with the environment (8,9). Even though most resistances are deleterious in the absence of antibiotics, some can be beneficial. Remarkably, rifampicin resistance can even be selected for in populations evolving without antibiotics (10). Furthermore, evidence is mounting that epistasis is widespread among resistance mutations (2,11,12) and the level of epistasis is also dependent on the environment (13). Given the strong effect of Genotype by Environment interactions on the fitness of both single and double resistances, it is important to determine the effects of resistance in environments that are relevant in the context of infection.

In here, we have studied the fitness effects of double resistance mutations conferring Rif\textsuperscript{R} and Str\textsuperscript{R}, when \textit{E. coli} encounters macrophages (MΦs), as will happen in an infection. MΦs are key players of the host’s innate immune system by recognizing, engulfing and killing microorganisms, and thus, an important selective pressure in the context of infection. \textit{E. coli} is both a commensal and a versatile pathogen, acting as a major cause of morbidity and mortality worldwide (14) and there is evidence that some pathogenic \textit{E. coli} evolved from commensal strains (15,16). \textit{E. coli} colonizes the infant gastrointestinal tract within hours after
birth and typically builds a mutualistic relation with its host. However, it can become pathogenic when the gastrointestinal barrier is disrupted as well as in immunosuppressed hosts (17-19). Non-pathogenic *E. coli* does not replicate inside MΦs but different mutants may have different abilities to persist inside these phagocytic cells (20). In a previous study we found that *E. coli* clones with single point mutations in the *rpsL* gene, conferring Str$^R$, exhibited a survival advantage over non-resistant *E. coli* in the intracellular niche of MΦs (20). To determine if such advantage would be altered in the presence of other resistances, we studied double resistant clones. We combined Str$^R$ mutations - K43N, K43T, K43R and K88R - with mutations that confer resistance to Rif$^R$, and measured the competitive fitness of the double resistance bacteria, against a sensitive strain, inside and outside MΦs. The chosen *rpoB* mutations conferring Rif$^R$ - S512F, S531F, H526Y and I572F – were shown to exhibit variable effects in competition against sensitive clones (20). S512F and I572F showed a survival advantage inside the MΦs, S531F was neutral and H526Y phenotype was time-dependent being neutral at 2h and beneficial at 24h post-infection (20). Previous work (2,11,13,21-23) has found strong epistatic interactions between alleles that confer rifampicin and streptomycin resistance in different species and in different environments, a result with important consequences for understanding the possible evolutionary paths towards the acquisition of multi-antibiotic resistance. Thus, we ask the following questions: What are the fitness effects of double RifStr resistance when bacteria face the pressure imposed by MΦs? Does the survival advantage conferred by a single Str$^R$ mutation depend on the presence of a Rif$^R$ allele? And, do MΦs show alterations in gene expression when infected with Rif$^R$Str$^R$ mutants?

**MATERIAL AND METHODS**

**Strains and Media**
The RAW 264.7 murine macrophage cell line was maintained in an atmosphere containing 5% CO2 at 37°C in RPMI 1640 (Gibco) supplemented with 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 10mM HEPES (Gibco), 50µM 2-mercaptoethanol solution (Gibco), with 10% heat-inactivated FBS (fetal bovine serum, Gibco). Bacterial strains were grown and competed in antibiotic-free RPMI medium in an atmosphere containing 5% CO2 at 37°C.

**Construction of strains**

We used susceptible strains *E. coli* K-12 MG1655 ∆lacIZYA galK::CFP/YFP, and a collection of single StrR and RifR mutants (also ∆lacIZYA galK::CFP/YFP) previously studied (2,20). To construct the double RifRStrR mutants, RifR and StrR mutants were transferred into the background of each of the single StrR and RifR mutants (ΔlacIZYA galK::CFP/YFP) by general transduction using P1 bacteriophage (24). To confirm the double mutations, each antibiotic resistance target gene was amplified by PCR and then sequenced. Each confirmed double resistant clone was grown from a single colony in LB medium supplemented with the respective antibiotics and stored in 15% glycerol at -80°C.

**Survival assays inside the MΦs**

To estimate the effect of double resistance on bacterial survival inside phagocytic cells, MΦs were first seeded in plates for 24h for acclimatization and then activated with 2 µg of CpG-ODN 1826 (5’-TCCATGACGTTCCTGACGTT- 3’)/ml for 24 h (see Figure 1). Afterwards, the cells were washed from the remaining CpG-ODN, fresh antibiotic-free RPMI medium was added, and MΦs were infected with 5x10⁶ bacteria (at a 1:1 ratio of double resistant to susceptible strain), and centrifuged at 203 x g (1,000 rpm) for 5 min to enhance bacterial internalization. The initial ratios of resistant and susceptible strains were determined by flow cytometry (see below). At 1 h of infection, the MΦs were washed from the extracellular bacteria, and fresh cell culture medium containing 100 µg of gentamicin/ml was added to kill
the remaining extracellular bacteria. To determine the number of intracellular bacteria after 2 and 24 h of incubation, infected MΦs were washed with phosphate-buffered saline (PBS), and 0.1% Triton-X was added for 10 min at 37°C in order to lyse the MΦs. The MΦs were then centrifuged at 10,600 x g (10,000 rpm) for 5 min and washed in PBS, and the overall number of bacteria was counted by plating them on LB agar plates. Survival inside the MΦs was estimated as the change in frequency (ΔX), measured as differences in viable cell counts, of the resistant strain, calculated as follows: ΔX = Nf_b/(Nf_a + Nf_b) - Ni_b/(Ni_a + Ni_b), where Nf_a and Nf_b are the numbers of resistant (b) and susceptible (a) bacteria after competition, and Ni_a and Ni_b are the initial numbers of resistant (b) and susceptible (a) bacteria before the competition. Significance was determined using the Wilcoxon signed rank test.

RNA Extraction, Reverse Transcription and Quantitative Real Time PCR (RT-qPCR)

To determine changes in macrophage gene expression after infection with bacteria, MΦs (5x10^6) were seeded per 6-well plate and infected independently (not in competition) with the chosen bacterial strain. MΦs were treated as described above for the survival assays inside the MΦs. At 2h post-infection MΦs were repeatedly washed with warm (37°C) RPMI prior to RNA extraction. RNA extraction was performed using the Direct-Zol RNA MiniPrep Kit (Zymo research) according to manufactures specifications. RNA was treated with RQ1 DNase (Promega) according to manufactures protocol. Reverse transcriptase reaction was performed with M-MLV RT (Promega) using random primers (Promega) according to manufactures protocol.

qPCR was executed in BioRad CFX 384 with iTaq Universal SYBR Green Supermix (BioRad). MΦ cDNA was diluted 10-fold before being used for qPCR. The cycling conditions were as follows: one step of 5 min at 95°C and then 40 cycles of 30 sec at 95°C, 30 sec at 60°C and finally 30 sec at 72°C, primers used are listed in Supplemental Table 1.
Melting curve analysis was performed to verify product homogeneity. All reactions included at least three biological replicates for each sample.

For analysis, data was normalized by the Pfaffl method (25) using actinB housekeeping gene as reference for murine cDNA. When comparing the antibiotic resistance strains against the susceptible strain, significance in differences of expression levels were determined by a t-test on the fold change values. Multiple t-tests were performed when comparing directly the double mutants K88R+H526Y and K88R+I572F.

**Competitive fitness in the presence and absence of MΦs**

The double resistant mutants constructed in the MG1655-CFP strain were competed against a susceptible MG1655-YFP strain in an antibiotic-free environment at a ratio of 1:1 in two different conditions - in the presence or absence of MΦs. Before the competitions, resistant and susceptible strains were grown separately in antibiotic-free RPMI medium for 48 h (with a dilution of 1:100 after 24 h) for acclimatization at 37°C with 5% CO₂. For competitions in the presence of the MΦs, 10⁶ MΦs were seeded in the wells. Competitions in presence or absence of MΦs were then performed in 24-well cell culture tissue plates (containing 500 µl of RPMI culture medium in each well), by inoculating a mix of 2.5x10⁴ of each bacteria strain. The initial ratios of resistant and susceptible strains were determined by flow cytometry (see below). To determine the number of extracellular bacteria after 24 h of incubation, supernatant RPMI was diluted in PBS, and the overall number of bacteria was counted by plating them on LB agar plates. Competitive fitness outside the MΦs was estimated as the change in relative frequency (ΔX), calculated as described above.

Significance for the competitive assays was determined using the Wilcoxon signed rank test. Wilcoxon sum rank test was performed to analyse the behaviour of the mutants in the presence and absence of MΦs during the competitive fitness. To test for a possible trade-off between competitive fitness in RPMI and survival inside MΦs a sign-test was used.
Flow Cytometry

To determine the initial ratios of resistant and susceptible strains in the survival and competition assays, bacteria were quantified prior to infection with a LSR Fortessa Flow Cytometer using a 96 well plate auto sampler. Samples were always run in the presence of SPHERO (AccuCount 2.0µm blank particles) in order to accurately quantify bacterial numbers in the cultures. Briefly, flow cytometry samples consisted of 180µl of PBS, 10µl of SPHERO beads and 10µl of a 100-fold dilution of the bacteria culture in PBS. Bacterial concentration was calculated based on the known number of beads added. CFP was excited with a 442 nm laser and measured with a 470/20nm pass filter. YFP was excited using a 488nm laser and measured using a 530/30nm pass filter.

RESULTS

Survival advantage of double resistance strains when competing inside MΦs

Non-pathogenic E. coli K12 does not replicate inside MΦs, so survival is an important fitness component in this niche (20,26). Survival inside the MΦs was estimated as the change in frequency (ΔX), measured as differences in viable cell counts. We measured the relative survival ability of 16 E. coli K-12 strains carrying resistance to two antibiotics inside RAW 264.7 murine MΦs. After growing double resistant and susceptible strains separately, we infected activated MΦs in antibiotic-free medium with a co-culture of bacteria. This co-culture was obtained by mixing the appropriate volumes of resistant and susceptible strains so that they start competing at equal densities (1 double resistant cell to 1 susceptible cell) in the co-culture (see Figure 1). After 1 h of infection, gentamicin was added to kill the remaining extracellular bacteria, which is sensitive to this drug. To control for the efficacy of gentamicin treatment, we plated the supernatant with bacteria which were exposed 1h to gentamicin and detected a residual number of colonies < 10³ CFUs/mL, which corresponds to < 1% of the
total numbers of bacteria found inside the MΦs at the same time point ($>10^5$ CFUs/mL). To determine the relative numbers of resistant versus susceptible intracellular bacteria, infection was halted after 2 and 24 h of incubation and the content of MΦs was plated on LB plates. We found that 13 out of 16 double mutants showed a survival advantage inside MΦs either at 2 or at 24 h post-infection (Figure 2). At 2 h post-infection, 62.5% of the double mutants displayed a significant increase in survival inside MΦs and this percentage increased to 81.3% at 24 h post-infection. These results indicate that the combination of Str$^R$Rif$^R$ double resistance is generally beneficial inside MΦs, in the absence of antibiotics. All but one of the Rif$^R$Str$^R$ double mutants resulting from combining any single (beneficial) Str$^R$ mutation with beneficial Rif$^R$ (S512F or I572F) showed increased survival inside the MΦs when compared to a susceptible strain. Thus, the combination of two resistances which individually are beneficial often results in an overall benefit for the double mutant. Two interesting cases of the opposite scenario were found. In the K43R+H526Y and K88R+H526Y combinations of double resistance decreased survival was observed even though each mutation alone does not confer a survival cost – examples of sign epistasis. By combining these results of the fitness effects of double resistance with the previously measured for single resistances (20), it follows as an outcome that single Rif$^R$ mutations can acquire increased survival inside the macrophages by acquiring a Str$^R$ mutation in 50% of the cases (see Supplemental Figure 1). For instance, the clinically common Rif$^R$ S531F mutation, which is neutral when alone, may hitchhike with beneficial Str$^R$ mutations suggesting a path towards acquired double antibiotic resistance in the context of infection in the absence of antibiotics. To further corroborate this hypothesis, we performed competitions between Rif$^R$Str$^R$ double mutant K43T+S531F against the single S531F (Rif$^R$) and found that the double mutant outcompeted the single inside the MΦs ($\Delta X = 0.02 \pm 0.01$, p-value < 0.05). On the other hand, single Str$^R$ mutations can acquire increased survival inside the macrophages by acquiring a Rif$^R$ mutation in 4 out 16 (25%) of the cases.
(Supplemental Figure 1). Those 4 combinations are K43N+S512F, K43T+S531F, K43R+S531F and K88R+I572F.

Double resistance showing sign epistasis prompts an altered inflammatory response

Macrophages undergo changes in gene expression after the phagocytosis of bacteria (27). Given the differential survival of the double resistant strains, we hypothesized that MΦs gene expression could differ between the Rif$^R$Str$^R$ mutants and the susceptible strain. We selected 7 macrophage transcripts (ccl5, ifit1, ifnβ, il1a, il10, nlrp3, and stx11) previously identified as important in the context of bacterial infection (27) and tested their expression by RT-qPCR.

In a previous work, we have adapted E. coli to MΦs by propagating bacterial populations for 30 days when facing MΦs, while inhabiting both the intracellular and extracellular environments (28). Infection of MΦs with these E. coli previously adapted to MΦs also lead to the alteration in the expression of the tested genes (unpublished data from our lab). To confirm that all macrophage genes tested were significantly upregulated when bacterial infection occurs, we infected MΦs with a susceptible strain and compared the transcription levels of the above mentioned genes to a mock (uninfected MΦs) experiment (Figure 3A).

Having found that these genes were induced upon infection with the susceptible strain, we used the same set of genes to compare the transcriptional response by RT-qPCR of MΦs infected by a susceptible strain or by several resistance strains. The MΦs were infected independently but in parallel with a similar number of bacteria of: a) the double Rif$^R$Str$^R$ mutants K88R+H526Y (which showed sign epistasis which resulted in decreased survival inside the MΦs) or K88R+I572F (increased survival inside the MΦs); b) the susceptible strain; c) a single resistant RpsL$^{K88R}$ mutant - Str$^R$; d) a RpoB$^{H526Y}$ and a RpoB$^{I572F}$ mutant, each conferring Rif$^R$. Figure 3B shows that, at 2h post-infection, the expression of tested genes was altered in all but one of the resistance strains. Interestingly, for the infection with the K88R+H526Y mutant, which showed a decreased survival, three transcripts were
significantly upregulated (Figure 3B) whereas for the other mutants less changes were detected. The infection with mutant K88R+H526Y resulted in a significant increase in ifit1 expression (p = 0.026, one sample t-test), il-10 (p = 0.0005) and nlrp3 (p = 0.009) relative to the infection with a susceptible strain. Comparing the transcript expression levels between K88R+H562Y and K88R+I572F infections, we found significant differences for ifit1 (p = 0.022, multiple t-test), il1-α (p = 0.014) and il-10 (p = 0.012). Differences in the level of ifn-β transcripts (p = 0.062) and stx11 (p = 0.056) between the double mutants were marginally significant (0.05 < p < 0.1).

Trade-off between survival and competitive fitness outside the MΦs

To determine the fitness effects of double resistance mutations when bacteria can grow outside macrophages, we performed competition assays (29) in two different environments: in RPMI medium alone (absence of MΦs) or in RPMI medium with the presence of MΦs (to which we did not apply gentamicin to allow for bacterial growth). Figure 4 shows that in most cases double resistance incurs a strong decrease in competitive fitness in both environments. Remarkable exceptions are detected for the K43R+S512F, K43R+H526Y and K43R+S531F double mutants which show no competitive disadvantage when grown in the presence of MΦs. The K43R+S512F is a particular worrisome combination of alleles, given that it results in a double resistant clone with no fitness costs for survival inside MΦs and a competitive growth advantage in the presence of MΦs. However, a clear cost is measured when MΦs where absent (p < 0.0001, Wilcoxon sum rank test) which suggests that MΦs are altering the medium to a more beneficial environment for this mutant. We have also found that K43R+H526Y is the only mutant that did not show a decreased competitive fitness when growing in RPMI, irrespective of the presence or absence of MΦs (Figure 4). This double mutant was actually one of the three exceptions that did not show increased survival inside
the MΦs at any of the time points measured. We noticed that the massive fitness costs observed for the Str\(^R\)Rif\(^R\) double mutants when bacteria are allowed to divide seemed to correlate with the substantial fitness benefits when bacteria are inside the MΦs. Thus, we used our data for the Str\(^R\)Rif\(^R\) double mutants plus the available data from previous results for the single Str\(^R\) and Rif\(^R\) mutants (20) to test this hypothesis. We found a trade-off between survival inside the MΦs and competitive fitness in RPMI both in the presence and absence of MΦs (p < 0.01 in both cases, sign-test).

The observed loss in competitive ability of the double resistance bacteria could be associated with a reduced nutritional competence (30,31). To test for this, we have analysed the growth rates of the double Rif\(^R\)Str\(^R\) mutants by performing growth curves in RPMI in microaerobic conditions (without shaking). For all the mutants, the growth curves displayed a biphasic behaviour with two distinct growth rates separated by a short plateau (at OD\(_{600\text{nm}}\) of ≈0.4): an initial, faster growth rate (\(\varepsilon_{r1}\)) presumably due to the presence of oxygen in low amounts in the RPMI medium, followed by a second slower growth rate (\(\varepsilon_{r2}\)), presumably in the absence of oxygen (Table I).

DISCUSSION

Multidrug-resistant bacteria pose a significant threat to human health, and it is important to understand what are the fitness effects of such bacteria during infection. Both single Str\(^R\) and Rif\(^R\) isolates have been identified in many important pathogens, such as \(M.\) \(\text{tuberculosis}\), \(S.\) \(\text{flexneri}\), \(V.\) \(\text{cholerae}\), \(P.\) \(\text{aeruginosa}\), and even in commensal \(E.\) \(\text{coli}\) sampled from healthy individuals (32-35). In this study, we have tested 16 Rif\(^R\)Str\(^R\) double mutants of \(E.\) \(\text{coli}\) for their ability to survive in the presence of MΦs. This viability is an important fitness trait because numerous pathogens, which have evolved different mechanisms to survive inside the MΦs, are rapidly acquiring multidrug resistance to these drugs. For instance, \(M.\) \(\text{tuberculosis}\) owes its success as pathogen to its ability to interfere with the normally effective
antimicrobial properties of the macrophage and is frequently found to be resistant both to $\text{Str}^R$ and $\text{Rif}^R$ (36-39). We found that most $\text{Rif}^R\text{Str}^R$ mutants in $E.\ coli$ had increased survival inside $\text{M}\Phi$s after 24h post-infection and a similar effect was also observed at 2h post-infection. It would be important to test if similar effects are found for the combinations of the highly frequent $\text{rpoB}^{H526Y}$ and $\text{rpoB}^{S531L}$ mutations in natural pathogens, such as $M.\ tuberculosi$sis (4,38,39). In fact our $E.\ coli$ results suggest that such pathogens could benefit from the combination of these $\text{Rif}^R$ alleles with certain $\text{Str}^R$ alleles and suggests a possible path to acquire multidrug resistance in the context of infection and in the absence of antibiotics. This finding suggests that streptomycin treatment should be avoided in patients infected with rifampicin resistant mutants.

Our results of fitness benefits of $\text{Rif}^R\text{Str}^R$ resistance mutations in the absence of antibiotics add to the cases recently found for other resistances. For instance, it has been shown that knock-outs of the $\text{oprD}$ and $\text{glpT}$ genes, resulting in antibiotic resistance to carbapenem and fosfomycin, provided an $\text{in vivo}$ fitness advantage during infection of $P.\ aeruginosa$ in the absence of drugs (40,41). In this same organism the loss of genes such as $\text{ampC}$ (encoding a cephalosporinase conferring resistance to amoxicillin-clavulanic acid), $\text{aph}$ (encoding an aminoglycoside phosphotransferase conferring resistance to kanamycin) and the $\text{mexAB-oprM}$ operon (encoding an efflux pump conferring resistance to both nalidixic acid and trimethoprim-sulfonamide) bears a fitness cost in the absence of antibiotics, indicating that these genes are important fitness determinants for both gastrointestinal colonization and lung infection (40) in the absence of antibiotics. Another study has shown that $Staphylococcus\ aureus$ can acquire intermediate levels of resistance to vancomycin in the absence of antibiotic and during $\text{in vivo}$ infection in a mouse model, solely due to competition between coevolving bacterial strains (42). Overall, our results add to a growing body of evidence suggesting that a reduction in antibiotic use, which a priori should lead to a drop of
(multi)drug-resistant strains, might result in an unfortunate outcome, a finding which contrasts to the currently prevailing view that increased antibiotic resistance has a negative fitness cost.

In our sample of double resistance, we found two cases of sign epistasis for survival of the bacteria inside the MΦs, where each single resistance is either beneficial or neutral but the combination is deleterious. When we compared the expression level of genes in MΦs infected with a double resistant mutant exhibiting sign epistasis, K88R+H526Y, we found several genes to be upregulated. The significant upregulation of NLRP3 and IFIT1 (and IL1-α when compared directly with the results obtained for the K88R+I572F) point to an exacerbated pro-inflammatory response from the MΦs when in presence of K88R+H526Y. Indeed, NLRP3 is activated in response to a variety of pathogen-associated and danger-associated molecular patterns and the active NLRP3 inflammasome leads to the secretion of potent pro-inflammatory cytokines. *E. coli* has previously been shown to induce NLRP3 activation in MΦs (43,44) and enterohemorrhagic *E. coli* (EHEC) are able to target NLRP3 inflamasome activation and block IL-1β cytokine production (45). It would be interesting to study the fitness effects of these resistances in this pathogenic strain. IFIT1 is induced upon treatment with interferon (in particular, by IFN-α/β) and is better characterized in the context of a viral infection (46). IFN-β is also involved in the regulation of NLRP3 inflammasome (47,48). The observed upregulation of IL-1α, a protein involved in various immune responses and inflammatory processes, is also in agreement with a pro-inflammatory response from the MΦs. These cytokines are produced by MΦs in response to cell injury and are involved in the inflammatory response with many interactions with other cytokines, ultimately inducing apoptosis (49). On the other hand, we also see a significant upregulation of *il-10* (0.55 log2 fold change) in the presence of this double mutant. The protein encoded by *il-10* is a cytokine produced primarily by monocytes with pleiotropic effects involved in limiting the
inflammatory response (50). Together, our results suggest that K88R+H526Y mutant may be
able to modify the inflammatory response by the MΦs when compared to the susceptible
strain, in the specific experimental conditions that we tested. In a real infection both bacterial
numbers and macrophage number are likely to be variable, so this effect may be dependent on
the context. It is noteworthy to compare our results with those from a previous study by
Mavromatis and colleagues (51) where a co-transcriptomics analysis was performed in MΦs
infected with two phenotypically different uropathogenic *E. coli* strains, one able to survive
and another unable to survive within MΦs. Mavromatis and colleagues did not detect
significant host gene expression differences following infection with the different bacteria
strains at 2 and 4 hours post infection. Only one gene (*Slc7a11*) coding for a
cysteine/glutamate exchanger was found to be upregulated at 24 h post-infection for the strain
that was able to survive inside the MΦs (51). In our bacterial strains, which only differ in the
mutations conferring resistance to antibiotics, several MΦs genes were found to be differently
upregulated, especially in the double mutant that displayed sign epistasis.

Our results also suggest that the increased survival inside the MΦs conferred by the double
resistance is associated to a substantial loss of competitive fitness in RPMI. The results
displayed in Table I also show that Rif\(^R\)Str\(^R\) double resistance incurs a strong cost in the
initial growth rate (\(\varepsilon_{r1}\)) but this cost is reduced along growth. This is in agreement with the
notion that the Rif\(^R\)Str\(^R\) mutants have a decreased ability in competing for the resources
present in RPMI and is consistent with the observed decreased competitive fitness (*Figure 4*).
Slower growth rates and increased survival suggest that antibiotic resistance mutations might
be tilting the so-called SPANC balance (standing for *self-preservation* and *nutritional
competence*) to an increased general stress response and starvation survival at the expense of
a decreased nutritional ability (30,31). Mutations in the *rpsL* gene, conferring Str\(^R\), have been
described to improve the accuracy of ribosomes but also to slow down the translation process
(52,53) and slower ribosomes could explain the observed slower growth rates in RPMI.

Concurrently, while fast ribosomes are required in actively dividing cells, hyper-accurate ribosomes are advantageous in non-dividing cells, because they lower the fraction of misfolded proteins which are known to be more prone to protein oxidation during growth arrest (54). This should be extremely relevant upon entry to the MΦs, where *E. coli* undergoes growth arrest and nutrient starvation. Importantly, the trade-off between survival and competitive fitness seems to be strong enough to prevent the dissemination of multi-antibiotic resistance. However, while the *E. coli* K-12 strain used for this study is not able to replicate in the phagolysosome, many intracellular pathogens can replicate inside the macrophages (55).

For pathogens which are mainly intracellular, it remains an open question how strong the described trade-off will be.

**FUNDING INFORMATION**

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REFERENCES


Table I – Relative growth rates ($\varepsilon_r$) normalized to the susceptible strain.

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<td>K43N</td>
<td>0.240 ± 0.026</td>
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<td>0.187 ± 0.007</td>
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<table>
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<tr>
<td>K88R</td>
<td>0.643 ± 0.054</td>
<td>0.836 ± 0.146</td>
<td>0.579 ± 0.101</td>
<td>0.634 ± 0.118</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

**Fig. 1 – The experimental setup.** Bacteria and macrophages were acclimatized independently for a total of 48h. Macrophages were activated with CpG for 24h during the period of acclimatization. After the period of acclimatization, 1x10^6 macrophages were infected with 5x10^6 bacteria (in a ratio of 1:1, resistant versus susceptible strain) labelled either with YFP or CFP. After 1 h of infection, the MΦs were washed from the extracellular bacteria, and fresh RPMI cell culture medium containing 100 µg of gentamicin/ml was added to kill the remaining extracellular bacteria. To determine the number of intracellular bacteria after 2 and 24 h of incubation, infected MΦs were washed with phosphate-buffered saline (PBS) with 0.1% Triton-X in order to lyse the MΦs. The overall number of bacteria was counted by plating on LB agar plates. Survival inside the MΦs was estimated as the change in relative frequency (ΔX), calculated as described in the Methods section.

**Fig. 2 – Rif^R Str^R double mutants have an increased survival inside the MΦs.** The panel shows the fitness effects of double antibiotic resistance on survival inside MΦs at 2h (black bars) and 24 h (grey bars) post-infection. All fitness effects were estimated using competition assays against a susceptible strain. At least 5 biological replicates were made for each measurement. All mutants showed statistical significance increases in frequency (p < 0.05, Wilcoxon signed rank test) when compared to the susceptible strain except for K43R+S512F (both 2 and 24h post-infection), K88R+S512F (at 24h post-infection), K43N+H526Y (at 2h post-infection). Results show that most Rif^R Str^R double mutants have an increased survival inside the MΦs. The opposite scenario occurs for two combinations which display sign epistasis – K43R+H526Y and K88R+H526Y.

**Fig. 3 – Double resistance with sign epistasis is associated with an enhanced pro-inflammatory response.** A) Relative amount of murine transcripts of macrophages infected...
with *E. coli* MG1655 susceptible to antibiotics relative to transcript levels of uninfected macrophages (mock). The significant higher transcript levels of all the tested genes after infection evidence their role in this context (p < 0.01, one sample t-test). B) Overall analysis by RT-qPCR of macrophages transcripts infected with different *E. coli* antibiotic resistant mutants. The coloured boxes show the survival effect (ΔX) of the mutants at 24h post-infection. Data was normalized against a susceptible strain and is shown in log 2 fold change. At least 3 biological replicas were made for each measurement.

**Fig. 4 – Trade-off between survival and competitive fitness outside the MΦs.** Competitive fitness of Rif\(^R\)Str\(^R\) double mutants were measured in RPMI medium both in the absence (black bars) and in the presence (grey bars) of MΦs. All fitness effects were estimated after 24h using competition assays against a susceptible strain. At least 3 biological replicas were made for each measurement. All mutants showed statistical significance decrease in frequency (p < 0.05, Wilcoxon signed rank test) when compared to the susceptible strain except for K43R+S512F (in the presence of MΦs), K43R+H526Y (both in the presence and absence of MΦs) and K43R+S531F (in the presence of MΦs).
**Figure 1**

**Bacteria acclimatization**

- Inoculate bacteria from frozen stocks
- Dilute $10^2$ in fresh RPMI medium
- Infect MΦs with bacteria $2.5 \times 10^9$ cells of each strain

**Macrophages acclimatization**

- Inoculate MΦs $1.5 \times 10^6$ cells
- Activate MΦs with CpG
- Activated MΦs $1 \times 10^9$ cells

**Infection procedure**

- $t = 0h$
- $t = 1h$
- Gentamicin is added to kill extracellular bacteria;
- $1st$ time point to measure the survival inside the MΦs;
- $t = 2h$
- $t = 22h$
- $2nd$ time point to measure the survival inside the MΦs
Figure 2

K43N+S512F
K43N+H526Y
K43N+S531F
K43N+I572F
K43T+S512F
K43T+H526Y
K43T+S531F
K43T+I572F
K43R+S512F
K43R+H526Y
K43R+S531F
K43R+I572F
K88R+S512F
K88R+H526Y
K88R+S531F
K88R+I572F

Change of frequency inside the MΦs (ΔX)
Figure 4

The diagram shows the change in frequency outside the MFDs (ΔX) for various combinations of amino acid substitutions. The x-axis represents the change in frequency, while the y-axis lists different combinations such as K43N+S512F, K43N+H526Y, K43N+S531F, etc. The data points indicate growth in the absence of MFDs and growth in the presence of MFDs.