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 rifampin (Rifr), a rifamicin, occurs due to mutations in the gene rpoB coding for the β-subunit of RNA polymerase, and resistance to streptomycin (Strr), an aminoglycoside, occurs due to mutations in the gene rpsL coding for a ribosomal protein (2). These genetic targets for resistance are common across a wide range of bacterial species, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* (2–4).

Mutations causing antibiotic resistance usually incur a fitness cost in the absence of antibiotics (3–7). 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, rifampin 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-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.

We studied the fitness effects of double resistance mutations (Rifr and Strr), when *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. *Escherichia 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 types of pathogenic *E. coli* evolved from commensal strains (15, 16). *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 that 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 Strr, exhibited a survival advantage over nonresistant *E. coli* in the intracellular niche of Mφs (20). To determine whether such advantage would be altered in the presence of other resistances, we studied doubly resistant clones. We combined Strr mutations—K43N, K43T, K43R, and K88R—with mutations that confer Rifr and measured the competitive fitness of the double-resistance bacteria against a sensitive strain both inside and outside Mφs. The chosen rpoB mutations conferring Rifr—SS12F, SS31F, H526Y, and I572F—exhibited variable effects in competition against sensitive clones (20). Mutations SS12F and I572F showed a survival advantage inside Mφs, SS31F was neutral, and the H526Y phenotype was time dependent, being neutral at 2 h and beneficial at 24 h postinfection (20). Previous work (2, 11, 13, 21–23) has found strong epistatic interactions between alleles that...
confer rifampin and streptomycin resistance in different species and in different environments, a result with important consequences for understanding the possible evolutionary paths toward the acquisition of multiantibiotic resistance. Thus, we sought to answer the following questions. What are the fitness effects of RifR and StrR when bacteria face pressure imposed by Mβs? Does the survival advantage conferred by a single StrR mutation depend on the presence of a RifR allele? Finally, do Mβs show alterations in gene expression when infected with RifR StrR mutants?

MATERIALS 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 (RPMI; Gibco) supplemented with 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), and 50 μM 2-mercaptoethanol solution (Gibco), along with 10% heat-inactivated 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 E. coli K-12 MG1655 ΔlacIZYA galK:CFP/YFP strains and a collection of single StrR and RifR mutants (also ΔlacIZYA galK:CFP/YFP) previously studied (2, 20). To construct the double RifR StrR mutants, RifR and StrR mutants were transferred into a 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-resistance clone was grown from a single colony in Luria-Bertani (LB) medium supplemented with the respective antibiotics and stored in 15% glycerol at −80°C.

Survival assays inside the Mβs. To estimate the effect of double resistance on bacterial survival inside phagocytic cells, Mβs were first seeded in plates for 24 h for acclimatization and then activated with 2 mM L-glutamine (Gibco) and infected independently (not in competition) with the chosen bacterial strain. Mβ/H9004s were treated as described above for the survival assays inside RAW 264.7. Significance was calculated as the change in viable cell counts, of the resistant strain, calculated as follows: ΔX = NFβ/NFb - NFβ/NIb, where NFβ and NFb are the numbers of resistant (β) and susceptible (b) bacteria after competition, and NIb and NIβ are the initial numbers of resistant (β) and susceptible (a) bacteria before the competition. Significance was determined using a 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, Mβs (5 × 10⁵) were seeded per 6-well plate and infected independently (not in competition) with the chosen bacterial strain. Mβs were treated as described above for the survival assays inside the Mβs. At 2 h postinfection, the Mβs were repeatedly washed with warm (37°C) RPMI prior to RNA extraction. RNA extraction was performed using a Direct-Zol RNA miniprep kit (Zymo Research) according to manufacturer’s specifications. RNA was treated with RQ1 DNase (Promega) according to manufacturer’s protocol. A reverse transcriptase reaction was performed with M-MLV RT (Promega) using random primers (Promega) according to manufacturer’s protocol.

qPCR was executed in Bio-Rad CFX 384 with iTaq Universal SYBR green Supermix (Bio-Rad). Mβ 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 s at 95°C and 30 s at 60°C, and finally 30 s at 72°C. The primers used are listed in Table S1 in the supplemental material. Melting-curve analysis was performed to verify product homo- geneity. All reactions included at least three biological replicates for each sample.

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

Competitive fitness in the presence and absence of Mβs. 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 under different conditions in the presence or absence of Mβs. 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 Mβs, 10⁶ Mβs were seeded in the wells. Competitions in the presence or absence of Mβs 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 2.5 × 10⁶ of each bacterial 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 the bacteria on LB agar plates. Competitive fitness outside the Mβs was estimated as the change in relative frequency (ΔX), which was calculated as described above.

Significance for the competitive assays was determined using the Wilcoxon signed-rank test. A Wilcoxon rank-sum test was performed to analyze the behavior of the mutants in the presence or absence of Mβs during the competitive fitness assessment. To test for a possible trade-off between competitive fitness in RPMI and survival inside Mβs, 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 an LSR Fortessa flow cytometer using a 96-well plate autosampler. Samples were always run in the presence of SPHERO (AccuCount 2.0-μm blank particles) in order to accurately quantify bacterial numbers in the cultures. Briefly, flow cytometry samples consisted of 180 μl of PBS, 10 μl of SPHERO beads, and 10 μl of a 100-fold dilution of the bacterial culture in PBS. The bacterial concentration was calculated based on the known number of beads added. Cyan fluorescent protein (CFP) was excited with a 442-nm laser and measured with a 470/20-nm pass filter. Yellow fluorescent protein (YFP) was excited using a 488-nm laser and measured using a 530/30-nm pass filter.

RESULTS

Survival advantage of double resistance strains when competing inside Mβs. Nonpathogenic E. coli K-12 does not replicate inside Mβs, so survival is an important fitness component in this niche (20, 26). Survival inside the Mβs 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 Mβs. After growing double resistant and susceptible strains separately, we infected activated Mβs in antibiotic-free medium with a coculture of bacteria. This coculture was obtained by mixing the appropriate volumes of resistant and susceptible strains so that
they start competing at equal densities (one double resistant cell to one susceptible cell) in the coculture (Fig. 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 the gentamicin treatment, we plated the supernatant with bacteria, which were exposed 1 h to gentamicin, and detected a residual number of colonies of $<10^5$ CFU/ml, which corresponds to $<1\%$ of the total numbers of bacteria found inside the Ms at the same time point ($>10^5$ CFU/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 Ms was plated onto LB plates. We found that 13 of 16 double mutants showed a survival advantage inside Ms at either 2 or 24 h postinfection (Fig. 2). At 2 h postinfection, 62.5% of the double mutants displayed a significant increase in survival inside Ms, and this percentage increased to 81.3% at 24 h postinfection. These results indicate that the combination of Strt Rifr double resistance is generally beneficial inside Ms in the absence of antibiotics. All but one of the Rifr Strt double mutants resulting from combining any single (beneficial) Strt mutation with beneficial Rifr (S512F or I572F) showed increased survival inside the Ms 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+S531F and K88R+S531F combinations of double resistance, a decreased survival was observed even though each mutation alone does not confer a survival cost; these are examples of sign epistasis. By combining the results of the fitness effects of double resistance with the previously measured for single resistances (20), it follows as an outcome that single Rifr mutations can acquire increased survival inside the macrophages by acquiring an Strt mutation in 50% of the cases (see Fig. S1 in the supplemental material). For instance, the clinically common Rifr S531F mutation, which is neutral when alone, may hitchhike with beneficial Strt mutations, suggesting a path toward acquired double antibiotic resistance in the context of infection in the absence of antibiotics. To further corroborate this hypothesis, we performed competitions between the Rifr Strt double-mutant K43T+S531F against the single-mutant S531F (Rifr) and found that the double mutant outcompeted the single mutant inside the Ms (DA = 0.02 ± 0.01, P < 0.05). On the other hand, single Strt mutations acquired increased survival inside the macrophages by acquiring a Rifr mutation in 4 of 16 (25%) of the cases (see Fig. S1 in the supplemental material). The four 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 Ms gene expression could differ between the Rifr Strt mutants and the susceptible strain. We selected seven macrophage transcripts (ccl2, ifit1, ifit3, 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 adapted *E. coli* to Ms by propagating bacterial populations for 30 days when facing Ms, while inhabiting both the intracellular and the extracellular environments (28). Infection of Ms with these *E. coli* strains previously adapted to Ms also led to an alteration in the expression of the tested genes (unpublished data). To confirm that all macrophage genes tested were significantly upregulated when bacterial infection occurs, we infected Ms with a susceptible strain and compared the transcription levels of the above-mentioned genes to those in a mock-infection experiment (i.e., uninfected Ms) (Fig. 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 Ms infected by a susceptible strain or by several resistance strains. The Ms were infected independently but in parallel with a similar number of various bacterial strains: (i) the double Rifr Strt mutant strain K88R+H526Y (which showed sign epista-
sis that resulted in decreased survival inside the Mφs) or K88R+I572F (which showed increased survival inside the Mφs), (ii) the susceptible strain, (iii) a single resistant RpsL<sup>K88R</sup> Str<sup>+</sup> mutant, and (iv) a RpoB<sup>H526Y</sup> and a RpoB<sup>I572F</sup> mutant, each conferring Rif<sup>+</sup>. Figure 3B shows that, at 2 h postinfection, the expression of tested genes was altered in all but one of the resistance strains. Interestingly, for the infection with the K88R<sup>H526Y</sup> mutant, which showed a decreased survival, three transcripts were significantly upregulated (Fig. 3B), whereas for the other mutants fewer changes were detected. The infection with mutant K88R<sup>H526Y</sup> 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 infection with a susceptible strain. Upon comparing the transcript expression levels between the 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 levels of ifnβ 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 results in a strong decrease in competitive fitness in both environments. Remarkable exceptions were detected for the K43R+S512F, K43R+H526Y, and K43R+S531F double mutants, which show no competitive disadvantage when grown in 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 results in a strong decrease in competitive fitness in both environments. Remarkable exceptions were detected for the K43R+S512F, K43R+H526Y, and K43R+S531F double mutants, which show no competitive disadvantage when grown in the presence of Mφs (to which we did not apply gentamicin to allow for bacterial growth). The K43R+S512F mutant is a particularly worrisome combination of alleles, given that it results in a double-

![FIG 2](http://aac.asm.org/)
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 rank-sum 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 (Fig. 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+ Rif+ 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+ Rif+ double mutants plus the available data from previous results for the single Str+ and Rif+ 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 in the 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 analyzed the growth rates of the double Rif+ Str+ mutants by determining growth curves in RPMI under microaerobic conditions (without shaking). For all of the mutants, the growth curves displayed a biphasic behavior with two distinct growth rates separated by a short plateau (at an optical density at 600 nm of ω = 0.4): an initial, higher growth rate (ε1), presumably due to the presence of oxygen in small amounts in the RPMI medium, followed by a second lower growth rate (ε2), presumably in the absence of oxygen (Table 1).

**DISCUSSION**

Multidrug-resistant bacteria pose a significant threat to human health, and it is important to determine the fitness effects of such bacteria during infection. Both single Str+ and Rif+ isolates have been identified in many important pathogens, such as *Mycobacterium tuberculosis*, *Shigella flexneri*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and even in commensal *Escherichia coli* sampled from healthy individuals (32–35). In the present study, we tested 16 Rif+ Str+ double mutants of *E. 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. tuberculosis* owes its success as a pathogen to its ability to interfere with the normally effective antimicrobial properties of the macrophage and is frequently both Str+ and Rif+ (36–39). We found that most Rif+ Str+ mutants in *E. coli* had increased survival inside MΦs after 24 h postinfection, and a similar effect was also observed at 2 h postinfection. It would be important to determine whether similar effects are true for the combinations of the highly frequent *rpoB*(H526Y) and *rpoB*(S531L) mutations in natural pathogens, such as *M. tuberculosis* (4, 38, 39). In fact, our *E. coli* results suggest that such pathogens could benefit from the combination of these Rif alleles with certain Str 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 Rif+ mutants.

Our findings regarding the fitness benefits of Rif+ Str+ mutations in the absence of antibiotics add to the cases recently found for other resistances. For instance, it has been shown that knockouts of the oprD and glpT genes, resulting in antibiotic resistance to carbapenem and fosfomycin, also provided an *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 *ampC* (encoding a cephalosporinase conferring resistance to amoxicillin-clavulanic acid), *aph* (encoding an aminoglycoside phosphotransferase conferring resistance to kanamycin), and the *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 *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 decrease in
(multi)drug-resistant strains, might produce an unfortunate outcome, a finding that contrasts with 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 M/H9278s, where each single resistance is either beneficial or neutral, but the combination is deleterious. When we compared the expression level of genes in M/H9278s infected with a double-resistant mutant exhibiting sign epistasis (K88R/H11001H526Y), we found that several genes were upregulated. The significant upregulation of NLRP3 and IFIT1 compared directly with the results obtained for the K88R/H11001I572F point to an exacerbated proinflammatory response from the M/H9278s when in the presence of K88R/H11001H526Y. Indeed, NLRP3 is activated in response to a vari-

![FIG 4 Trade-off between survival and competitive fitness outside M/H9278s. The competitive fitness levels of Rif' Str' double mutants were measured in RPMI medium both in the absence (■) and in the presence (□) of M/H9278s. All fitness effects were estimated after 24 h using competition assays against a susceptible strain. At least three biological replicates were performed for each measurement. All mutants showed a statistically significant decrease in frequency (P < 0.05, Wilcoxon signed-rank test) compared to the susceptible strain except for K43R+S512F (in the presence of M/H9278s), K43R+H526Y (in both the presence and the absence of M/H9278s), and K43R+S531F (in the presence of M/H9278s).](http://aac.asm.org/)

| Rate and mutation | Mean relative growth rate ($\epsilon_r$) ± SEM |
|-------------------|---------------------------------------------|
|                   | SS12F                                      | H526Y                                      | S531F                                      | I572F                                      |
| $\epsilon_{r1}$   |                                             |                                             |                                             |                                             |
| K43N              | 0.240 ± 0.026                               | 0.201 ± 0.008                              | 0.114 ± 0.010                              | 0.528 ± 0.221                              |
| K43T              | 0.187 ± 0.007                               | 0.191 ± 0.002                              | 0.241 ± 0.047                              | 0.202 ± 0.005                              |
| K43R              | 0.203 ± 0.007                               | 0.194 ± 0.003                              | 0.203 ± 0.006                              | 0.227 ± 0.005                              |
| K88R              | 0.194 ± 0.003                               | 0.204 ± 0.021                              | 0.157 ± 0.009                              | 0.202 ± 0.009                              |
| $\epsilon_{r2}$   |                                             |                                             |                                             |                                             |
| K43N              | 0.772 ± 0.441                               | 0.770 ± 0.053                              | 0.666 ± 0.090                              | 0.432 ± 0.027                              |
| K43T              | 0.420 ± 0.052                               | 1.046 ± 0.232                              | 1.008 ± 0.626                              | 0.355 ± 0.020                              |
| K43R              | 0.843 ± 0.138                               | 0.803 ± 0.133                              | 0.758 ± 0.139                              | 0.589 ± 0.039                              |
| K88R              | 0.643 ± 0.054                               | 0.836 ± 0.146                              | 0.579 ± 0.101                              | 0.634 ± 0.118                              |
eity of pathogen-associated and danger-associated molecular patterns, and the active NLRP3 inflammasome leads to the secretion of potent proinflammatory cytokines. Escherichia coli has previously been shown to induce NLRP3 activation in Møs (43, 44), and enterohemorrhagic E. coli is able to target NLRP3 inflammasome activation and block IL-1β cytokine production (45). It would be interesting to study the fitness effects of these resistances in this pathogenic strain. IFTT1 is induced upon treatment with interferon (IFN), in particular 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 proinflammatory response from the Møs. These cytokines are produced by Møs 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 saw a significant upregulation of il10 (a 0.55-log-fold change) in the presence of this double mutant. The protein encoded by il10 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 Møs compared to the susceptible strain in the specific experimental conditions that we tested. In a real infection, both bacterial numbers and macrophage numbers 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 et al. (51), who performed a cotranscriptomics analysis in Møs infected with two phenotypically different uropathogenic E. coli strains, one able to survive and another unable to survive within Møs. Mavromatis et al. did not detect significant host gene expression differences after infection with the different bacterial strains at 2 and 4 h postinfection. Only one gene (Slc7a11) encoding a cysteine/glutamate exchanger was found to be upregulated at 24 h postinfection for the strain that was able to survive inside the Møs (51). In our bacterial strains, which only differ in the mutations conferring resistance to antibiotics, several Mø 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 Møs conferred by the double resistance is associated with a substantial loss of competitive fitness in RPMI. The results displayed in Table 1 also show that Rif\(^\text{R}\)/Str\(^{\text{s}}\) double resistance incurs a strong cost in the initial growth rate (ɛ\(_\text{int}\)), but this cost is reduced along with growth. This finding is in agreement with the notion that the Rif\(^\text{R}\)/Str\(^{\text{s}}\) mutants are less able to compete for the resources present in RPMI and is consistent with the observed decreased competitive fitness (Fig. 4).

Lower growth rates and increased survival suggest that antibiotic resistance mutations might be tilting the so-called SPANC balance (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 rhs\(_1\) gene, conferring Str\(^{\text{s}}\), improve the accuracy of ribosomes but also slow down the translation process (52, 53), and slower ribosomes could explain the observed lower growth rates in RPMI. Concurrently, although fast ribosomes are required in actively dividing cells, hyperaccurate 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 Møs, 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 multiantibiotic 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 that are mainly intracellular, it remains an open question how strong the described trade-off will be.

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