Hyper-susceptibility of a fusidic acid-resistant mutant of *Salmonella* to different classes of antibiotics

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Abstract

Fusidic acid resistance (Fus[R]) in *Salmonella enterica* serovar Typhimurium is caused by mutations in *fusA*, encoding elongation factor G (EF-G). Pleiotropic phenotypes are observed in Fus[R] mutants. Thus, the *fusA*1 allele (EF-G P413L) is associated with slow growth rate, reduced ppGpp and RpoS levels, reduced heme levels, and increased sensitivity to oxidative stress. The *fusA*1–15 allele, (EF-G P413L and T423I) derived from *fusA*1 in a selection for growth rate compensation, is partially compensated in each of these phenotypic defects but maintains its resistance to fusidic acid. We show here that the *fusA*1 allele is associated with sensitivity to ultraviolet light and increased susceptibility to the inhibitory action of several unrelated antibiotic classes (β-lactam, fluoroquinolone, aminoglycoside, rifampicin, and chloramphenicol). The *fusA*1–15 allele, in contrast, is less susceptible to UV and to other antibiotics than *fusA*1. The hyper-susceptibility to multiple antibiotics associated with *fusA*1 and *fusA*1–15 is revealed in a novel growth competition assay at sub-MIC concentrations, but not in a standard MIC assay.
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1. Introduction

Fusidic acid is a steroid-like antibiotic which stabilizes EF-G · GDP on the ribosome after translocation has occurred, blocking further protein synthesis [1,2]. Resistance to fusidic acid in *Salmonella enterica* serovar Typhimurium (common name *S. typhimurium*) is caused by mutations in the *fusA*1 gene [3] which encodes elongation factor G (EF-G). Many fusidic acid resistance mutations have pleiotropic phenotypes that affect the fitness of Fus[R] mutants in vitro and in vivo [4–6]. Reduced fitness is associated with slow translation and growth rates, and perturbed levels of the transcriptional regulators ppGpp and RpoS. In addition, some Fus[R] mutants are defective in heme production, are sensitive to oxidative stress, and have a low aerobic respiration rate [6].

In the light of the pleiotropy associated with Fus[R] mutants, in particular their sensitivity to oxidative stress, we considered the possibility that they might be sensitive to other sources of DNA damage. We tested the response of two well characterised Fus[R] mutants to UV light, to fluoroquinolone antibiotics that are associated with DNA damage, and to other unrelated classes of antibiotics.

2. Materials and methods

2.1. Bacterial strains, and growth conditions

The strains used in this study are listed in Table 1. The wild-type *Salmonella enterica* serovar Typhimurium

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Table 1
Strain list

Strain	Genotype	EF-G
TH4527	LT2, Wild-type *S. typhimurium*	Wild-type
MM142	LT2, *fusA*1 *zhb*-736::*Tn*10	P413L
JB2110	LT2, *fusA*1–15 *zhb*-736::*Tn*10	P413L, T423I

strain TH4527 (LT2) is originally TT10000 from the strain collection of John Roth, University of California, Davis. The *fusA*1 mutation was selected as a spontaneous fusidic acid-resistance mutation in EF-G, Pro413Leu [3], and was moved into TH4527 by co-transduction using a linked *Tn*10(*zhb*-736::*Tn*10) to make the isogenic strain MM142. The *fusA*1–15 mutation, EF-G Pro413-Leu and Thr423Ile, was selected as a mutation in *fusA* that compensated for the slow growth phenotype associated with the *fusA*1 allele without causing any loss of fusidic acid resistance [7]. This mutation was also moved in TH4527 by co-transduction using the linked *Tn*10, *zhb*-736::*Tn*10, to make the isogenic strain JB2110. Strains were grown in LB broth at 37 °C with shaking, and on solid Luria agar (LA).

2.2. Antibiotics and MIC tests

The antibiotics penicillin G, ampicillin, norfloxacin, streptomycin, rifampicin and chloramphenicol were purchased from Sigma Aldrich (Stockholm, Sweden). Fusidic acid Na-salt, used to confirm the phenotype of the Fus^R mutants, was a gift from Leo Pharma (Ballerup, Denmark). MIC's were determined using Etest (AB BIODISK, Solna, Sweden) by spreading $\sim10^8$ cells (0.5 McFarland) on LA plates and reading the intercept after 16–18 h incubation at 37 °C.

2.3. Growth competition assays in the presence of antibiotics

Overnight cultures were grown in LB. 10^6 cells/ml each of TH4527 and a Fus^R mutant were mixed and inoculated into LB containing various concentrations of antibiotic up to the MIC of TH4527. Master stocks of each antibiotic were made at 20 mg/ml. Norfloxacin stock solution was made in 0.1 M NaOH, while other antibiotics were dissolved in water or methanol as appropriate. Antibiotics were diluted from the master stock into LB at the required final concentrations for growth competition experiments which were run for exactly 18 h at 37 °C. The final antibiotic concentrations were typically in the range 0.1–1 × MIC of the wild-type strain. Competitions with rifampicin were made in tubes protected from light. After competitions in the presence of β-lactam antibiotics tubes were vortexed vigorously to disrupt filaments before spreading on agar plates. As a control on relative growth rate, competition cul-

tures were also grown in the absence of antibiotic. Approximately 10 cell doublings occurred in the absence of antibiotic. In order to determine the number of viable wild-type and mutant cells, aliquots were taken after 18 h incubation, diluted in 0.9% NaCl, and plated onto LA (permits growth of all cells) and on LA Tet (10 μg/ml, permits growth of the Fus^R strains marked with *Tn*10). A normalised competition index (NCI) was calculated as the ratio of mutant/wild-type after 18 h incubation at each antibiotic concentration, normalised to the ratio of mutant/wild-type in the absence of antibiotic in that experiment.

2.4. UV sensitivity

The sensitivity of strains to UV light was measured as described elsewhere [8]. Strains were grown in LB and serial dilutions were plated as 100 μl volumes onto LA. Plates were irradiated with UV light using a transilluminator operating at a wavelength of 314 nm for up to 60 s (corresponding to 40 mJ/cm^2). Survivors were counted as CFU after overnight incubation in the dark at 37 °C.

3. Results

3.1. UV sensitivity of Fus^R mutants

Wild-type *S. typhimurium* LT2 (TH4527) and two isogenic Fus^R mutants, MM142 (*fusA*1) and JB2110 (*fusA*1–15), were exposed to UV light for up to 60 s. A *recA* strain was tested in parallel as a control for sensitivity. The results are shown in Fig. 1. No survivors were found in the *recA* population after 30 s exposure (>6 logs of killing). After 60 s exposure MM142 (*fusA*1) CFU were reduced by 4 logs, while wild-type and
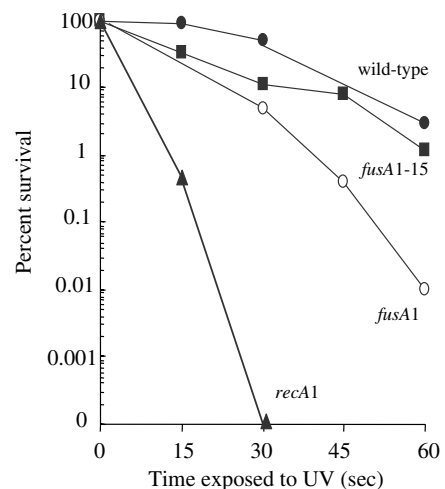

Fig. 1. Percent survival after exposure to UV irradiation. Cell were irradiated on solid LA media and survivors counted as colonies.

JB2110 (*fusA*1–15) were both reduced by 1–2 logs. We conclude that the *fusA*1 allele strongly increases the UV sensitivity of *S. typhimurium*. The sensitivity associated with *fusA*1 is similar to the sensitivity associated with a *recB* knockout mutation (data not shown).

3.2. MIC of wild-type and Fus[R] mutants

The sensitivity of the MM142 Fus[R] mutant to UV suggested the possibility that it might be sensitive to fluoroquinolones, antimicrobials whose action in inhibiting replication increases the frequency of chromosome breaks [9,10]. To extend the scope of this question we asked whether the known pleiotropy of Fus[R] strains might also be reflected in an altered response to other antibiotics with different modes of action. We choose a set of antibiotics representing different classes of structure and activity: β-lactam antibiotics (penicillin G and ampicillin) target cell wall synthesis; norfloxacin targets DNA replication; rifampicin targets RNA transcription; streptomycin targets the ribosomal A-site; chloramphenicol targets peptide bond formation in protein synthesis.

MIC's were measured for TH4527, MM142 and JB2110 (Table 2). The MIC values show that the *fusA*1 allele is associated with a slightly increased susceptibility to some antibiotics (β-lactams, streptomycin and chloramphenicol) but these very small differences (1 step on the Etest scale) would normally be regarded as being on the borderline of significance. MICs were also measured for LT2 *zhb*-736::*Tn*10 (data not shown) and were identical to those for LT2 (except for the tetracycline resistance associated with *Tn*10) showing that the *Tn*10 does not affect the results.

3.3. Growth competition in the presence of low levels of antibiotic

The starting inoculum of ~10^6 cells/ml each, of wild-type and Fus[R], allows the total cell population to increase by 3 logs over the course of the growth competition assay in the absence of antibiotic. The antibiotic concentrations present in each culture were generally in the sub-MIC range based on Etests.

Table 2
Antibiotic susceptibility

Antibiotic	MIC[a] (Etest) μg/ml		
	W.T.	*fusA*1	*fusA*1–15
Norfloxacin	0.5	0.5	0.5
Penicillin G	12	8	12
Ampicillin	0.75	0.5	1
Streptomycin	24	16	24
Chloramphenicol	2	1.5	22
Rifampicin	24	24	24

[a] Results are the modal value of 3–6 measurements in each case.

In initial control experiments we competed wild-type LT2 (TH4527) against LT2 *zhb*-736::*Tn*10 showing that this *Tn*10 is neutral (data not shown), in agreement with its neutrality in a variety of other in vivo and in vitro competition assays [6,11]. The results of the competitions between *S. typhimurium* TH4527 (wild-type) and MM142 (*fusA*1) are shown in Fig. 2(a)–(f). In these competition assays MM142 appears to be hypersensitive to several antibiotics. Thus, the MM142 Fus[R] population is killed by norfloxacin and streptomycin at antibiotic concentrations where the wild-type grows fully by 3 logs. Similar assays were made with JB2110 (*fusA*1–15) in competition against the wild-type in the presence of norfloxacin, penicillin G, and streptomycin (Fig. 3). To quantify the relative magnitude of the antibiotic inhibition for MM142 and JB2110 we converted the raw data from the competition assays into normalised competition indices (NCI, see Section 2). An NCI value of 1 is the antibiotic concentration that inhibits growth of, but does not kill, the wild-type strain. NCI values record the difference in population size relative to that which would be expected in the absence of any antibiotic. The NCI values for *fusA*1 and *fusA*1–15 in the presence of norfloxacin, penicillin G, and streptomycin are shown in Fig. 4. These NCI values show that the *fusA*1 allele is associated with a very large drop in survival, relative to the wild-type, at sub-MIC concentrations of each of these three antibiotics. The *fusA*1–15 allele is also associated with strong inhibition by penicillin G, a relatively small inhibition by norfloxacin at close to MIC, and little or no relative inhibition by streptomycin.

4. Discussion

The Fus[R] mutation *fusA*1 is associated with multiple phenotypes including, as shown here, sensitivity to UV irradiation and hyper-susceptibility to different classes of antibiotic. The *fusA*1–15 mutation, partially compensated with regard to all previously assayed phenotypes, is also intermediate between the wild-type and *fusA*1 in terms of susceptibility to different classes of antibiotic. Some or all of these phenotypes may be related to the down-stream effects of altered gene regulation associated with the Fus[R] mutants having altered ppGpp and RpoS levels. In the case of norfloxacin one possibility is that increased susceptibility parallels increased sensitivity to DNA damaging agents as seen with UV light. Thus one of the pathways of DNA repair may be under-expressed in the *fusA*1 strain. It has also been shown that many Fus[R] mutations, including *fusA*1, are associated with altered cell shapes and phage sensitivity patterns [5]. These differences affecting the bacterial cell wall might thus be associated with the increased susceptibility to β-lactam antibiotics noted in the growth competition assays. There remains controversy over exactly
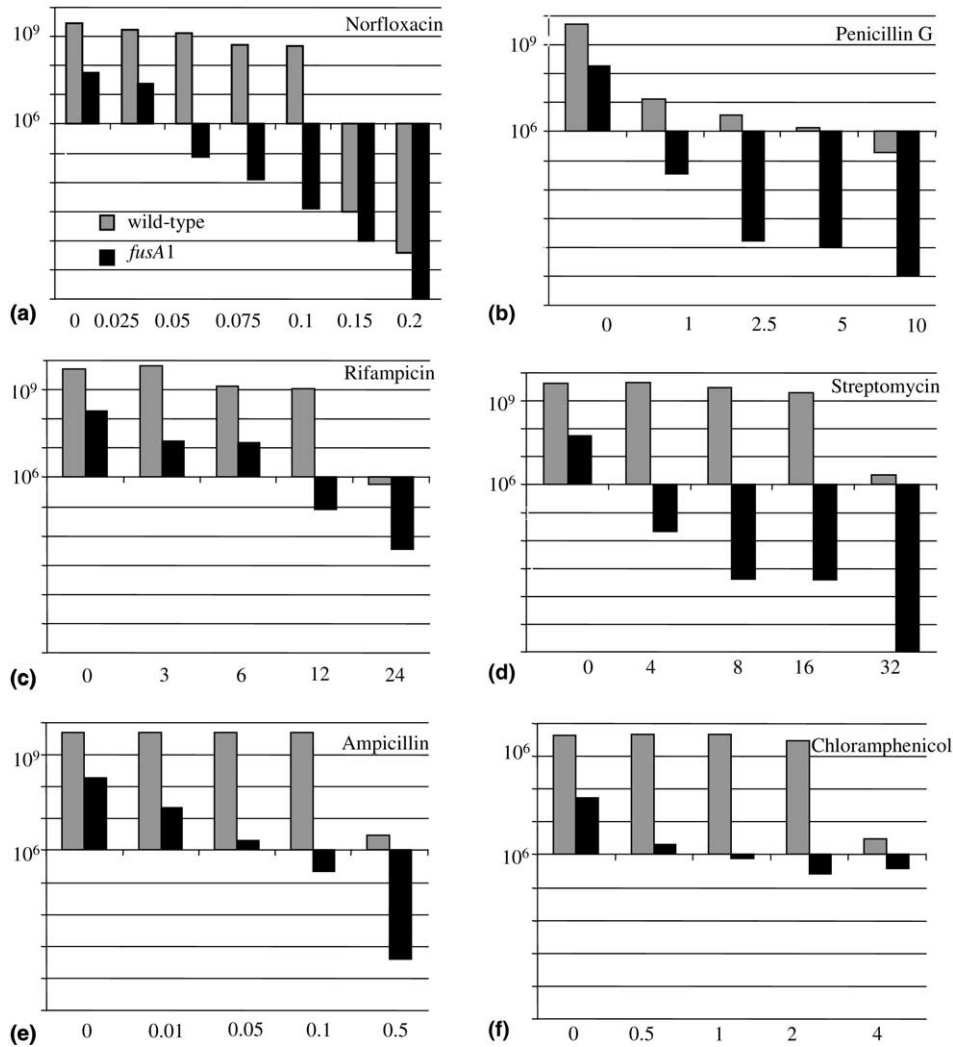
Fig. 2. Survival of wild-type and isogenic *fusA*1 strains in competition in liquid LB in the presence of various amounts of antibiotic. At the beginning of each competition each culture contains a mixture of 10^6 cells/ml of each strain. The *Y*-axis shows on a log scale the total number of viable cells of each type after 18 h incubation. The *X*-axis shows the concentration of antibiotic in each competition, as μg/ml. In each case the highest concentration is close to the MIC of the wild-type (see Table 2). The relevant antibiotic is indicated on each panel. The result shown is a representative experiment.

how streptomycin kills bacteria [12] but resistance to streptomycin caused by mutations is very strongly associated with reduced affinity for its binding site on the ribosome [13,14]. Thus, the hyper-susceptibility to streptomycin associated with the *fusA*1 might be due to mutant EF-G influencing access of the antibiotic to its ribosomal binding site. In terms of streptomycin susceptibility the growth-rate compensated Fus[R] mutant (*fusA*1–15) clearly differs from *fusA*1 and is no more sensitive than the wild-type (Fig. 4). It might be possible to test whether the cause of this difference in susceptibility is ribosome-associated by using an in vitro translation system with purified components [15]. However, because the Fus[R] mutants alter global gene regulators such as ppGpp [5] and RpoS [11], the effects on streptomycin susceptibility might be indirect.

A standard assay of antibiotic susceptibility, the MIC test, revealed only very small differences in susceptibility to various antibiotics associated with these mutations in *fusA* (Table 2). The new method we have introduced in this study, growth competition at sub-MIC antibiotic concentrations, shows that under the right conditions it is possible to reveal large differences in the relative antibiotic susceptibilities of strains with apparently similar MICs. This method might have some application in evaluating alternative antibiotic therapies. Thus, when dealing with a significant resistance problem an important decision is which alternative antibiotic treatment to recommend. This decision is based in part on epidemiological information, and on the pharmacodynamic and pharmacokinetic properties of the available antibiotics. MIC plays a large part
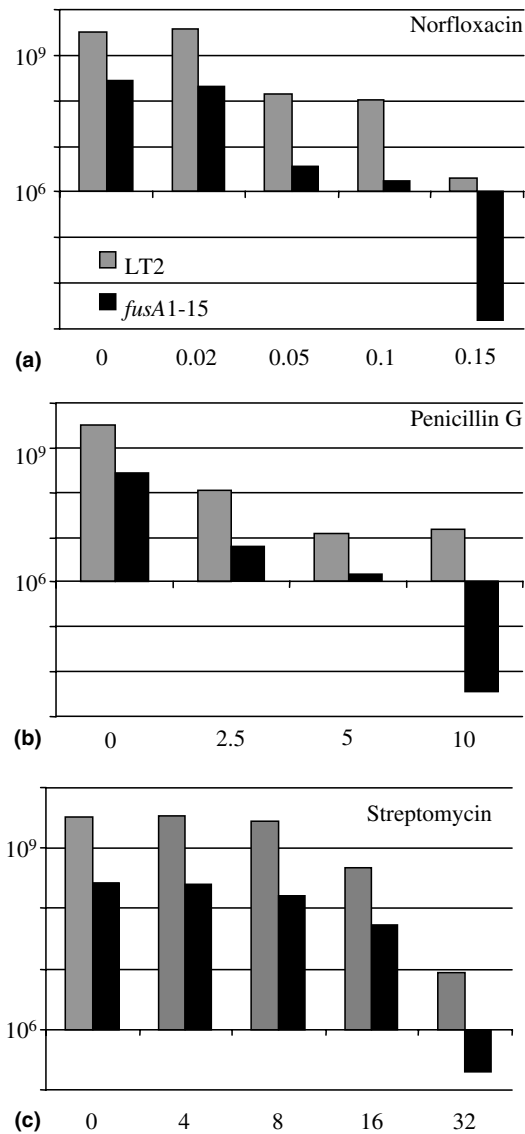
Fig. 3. Survival of wild-type and isogenic *fusA*1–15 strains in competition in liquid LB in the presence of various amounts of antibiotic. At the beginning of each competition each culture contains a mixture of 10^6 cells/ml of each strain. The *Y*-axis shows on a log scale the total number of viable cells of each type after 18 h incubation. The *X*-axis shows the concentration of antibiotic in each competition, as µg/ml. In each case the highest concentration is close to the MIC of the wild-type (see Table 2). The relevant antibiotic is indicated on each panel. The result shown is a representative experiment.

Fig. 4. Comparison of the susceptibility of *fusA*1 (black diamonds) and *fusA*1–15 (white squares) relative to the wild-type, in the presence of three antibiotics. The *Y*-axis shows the normalised competition index (NCI) on a log scale. NCI is defined in Section 2. The *X*-axis shows the antibiotic concentration relative to MIC for the wild-type. This MIC was calculated based on the response of the wild-type strain in these growth assays (not based on the Etest). MIC 1 is defined as the antibiotic concentration that results in a wild-type cell concentration of 10^6 cells/ml after 18 h incubation.

in this decision and it is generally agreed that an antibiotic with a low MIC is to be preferred. Our data suggest that a standard MIC test would not detect some significant differences in bacterial susceptibility at sub-MIC concentrations. Although antibiotics are typically administered so that they achieve serum concentrations well over MIC, for some infections this can be difficult to achieve at the site of infection. In those cases it can be important, both for effective clearance of the infection and to minimise sele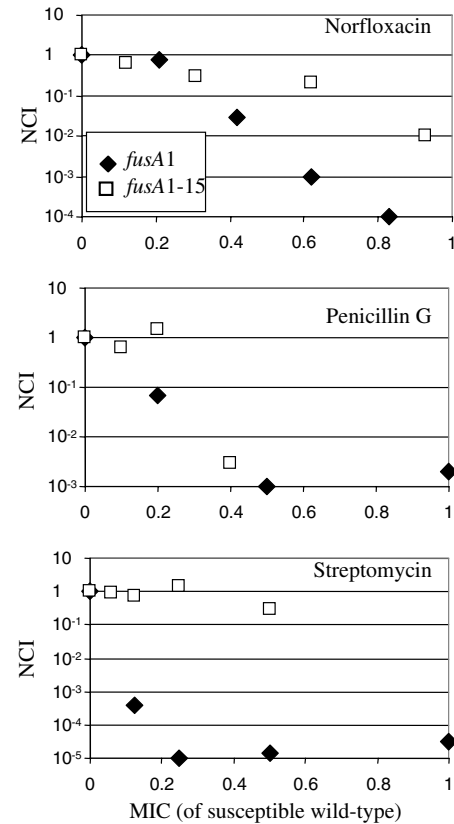ction for resistance, to use an antibiotic that is effective even at low concentrations relative to MIC. The competition assay we have described here could be one tool to help make the optimal choice of antibiotic in treating problematic infections.

This study has shown that the pleiotropic phenotypes of an antibiotic resistant mutant can include hyper-susceptibility to other classes of antibiotic, i.e., that antibiotic-resistant bacteria may have an Achilles heel [6,16].

Acknowledgements

References

[1] Rodnina, M.V., Savelsbergh, A., Katunin, V.I. and Wintermeyer, W. (1997) Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. Nature 385, 37–41.

[2] Willie, G.R., Richman, N., Godtfredsen, W.P. and Bodley, J.W. (1975) Some characteristics of and structural requirements for the interaction of 24,25-dihydrofusidic acid with ribosome – elongation factor g Complexes. Biochemistry 14, 1713–1718.

[3] Johanson, U. and Hughes, D. (1994) Fusidic acid-resistant mutants define three regions in elongation factor G of *Salmonella typhimurium*. Gene 143, 55–59.

[4] Macvanin, M., Björkman, J., Eriksson, S., Rhen, M., Andersson, D.I. and Hughes, D. (2003) Fusidic acid-resistant mutants of *Salmonella enterica* serovar Typhimurium with low fitness in vivo are defective in RpoS induction. Antimicrob. Agents Chemother. 47.

[5] Macvanin, M., Johanson, U., Ehrenberg, M. and Hughes, D. (2000) Fusidic acid-resistant EF-G perturbs the accumulation of ppGpp. Mol. Microbiol. 37, 98–107.

[6] Macvanin, M., Ballagi, A. and Hughes, D. (2004) Fusidic acid-resistant mutants of *Salmonella enterica* serovar typhimurium have low levels of heme and a reduced rate of respiration and are sensitive to oxidative stress. Antimicrob. Agents Chemother. 48, 3877–3883.

[7] Johanson, U., Aevarsson, A., Liljas, A. and Hughes, D. (1996) The dynamic structure of EF-G studied by fusidic acid resistance and internal revertants. J. Mol. Biol. 258, 420–432.

[8] Michel, B., Recchia, G.D., Penel-Colin, M., Ehrlich, S.D. and Sherratt, D.J. (2000) Resolution of holliday junctions by RuvABC prevents dimer formation in rep mutants and UV-irradiated cells. Mol. Microbiol. 37, 180–191.

[9] Marians, K.J. and Hiasa, H. (1997) Mechanism of quinolone action. A drug-induced structural perturbation of the DNA precedes strand cleavage by topoisomerase IV. J. Biol. Chem. 272, 9401–9409.

[10] Drlica, K. and Malik, M. (2003) Fluoroquinolones: action and resistance. Curr. Top. Med. Chem. 3, 249–282.

[11] Macvanin, M., Bjorkman, J., Eriksson, S., Rhen, M., Andersson, D.I. and Hughes, D. (2003) Fusidic acid-resistant mutants of *Salmonella enterica* serovar typhimurium with low fitness in vivo are defective in RpoS induction. Antimicrob. Agents Chemother. 47, 3743–3749.

[12] Fast, R., Eberhard, T.H., Ruusala, T. and Kurland, C.G. (1987) Does streptomycin cause an error catastrophe. Biochimie 69, 131–136.

[13] Bilgin, N., Claesens, F., Pahverk, H. and Ehrenberg, M. (1992) Kinetic properties of *Escherichia coli* ribosomes with altered forms of S12. J. Mol. Biol. 224, 1011–1027.

[14] Bohman, K., Ruusala, T., Jelenc, P.C. and Kurland, C.G. (1984) Kinetic impairment of restrictive streptomycin-resistant ribosomes. Mol. Gen. Genet. 198, 90–99.

[15] Pavlov, M.Y. and Ehrenberg, M. (1996) Rate of translation of natural mRNAs in an optimized in vitro system. Arch. Biochem. Biophys. 328, 9–16.

[16] Macvanin, M. and Hughes, D. (2003) The physiological cost of antibiotic resistance. Recent Res. Devel. Microbiol. 7, 607–629.