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Research Paper

tmRNA Decreases the Bactericidal Activity of Aminoglycosides and the Susceptibility to Inhibitors of Cell Wall Synthesis

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KEY WORDS

tmRNA, ribosome, cell wall, aminoglycosides, ampicillin, fosfomycin

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NOTE

Supplementary Figure 1 can be found at: http://www.landesbioscience.com/rnabiology/luidaleppRNA2-2-sup.pdf.

ABSTRACT

Trans-translation is a process that recycles ribosomes stalled on problematic mRNAs. tmRNA, coded by the Δ*ssrA* gene, is a major component of trans-translation. Bacteria lacking tmRNA are more sensitive to several inhibitors of protein synthesis when compared to a wild type strain. We measured bacterial growth of the Δ*ssrA* and wild type strains in *Escherichia coli* in the presence of 14 antibiotics including some that do not target protein synthesis. Both the optical density of the bacterial cultures and the number of viable cells were monitored. For the ribosome-targeted antibiotics, sensitization was observed on erythromycin, chloramphenicol, kanamycin, puromycin and streptomycin. Minor or no effects were observed with clindamycin, tetracycline and spectinomycin. Surprisingly, the Δ*ssrA* strain is more sensitive than wild type to inhibitors of cell wall synthesis: fosfomycin and ampicillin. No growth difference was observed on drugs with other target sites: ofloxacin, norfloxacin, rifampicin and trimethoprim. Sensitization to antibiotics having target sites other than the ribosome suggests that trans-translation could influence antibiotic-induced stress responses. In trans-translation-deficient bacteria, cell death is significantly enhanced by the two aminoglycosides that induce translational misreading, streptomycin and kanamycin.

INTRODUCTION

Trans-translation is a process that recycles ribosomes stalled on truncated mRNAs lacking a termination codon. Trans-translation is highly conserved among eubacteria.[1] A central component of trans-translation is transfer-messenger RNA (tmRNA),[2] a small, 260 to 430 nucleotides-long ribonucleic acid expressed from the *ssrA* gene.[3] tmRNA has both tRNA- and mRNA-like functions.[3] The tRNA-like domain resembles tRNA^{Ala} and its 3'-end is aminoacylatable by alanyl-tRNA synthase.[3] Trans-translation starts when the alanylated tRNA-like domain enters, with the help of elongation factor Tu, to the A site of a stalled ribosome. The truncated mRNA is then replaced by the mRNA-like domain of tmRNA. Translation resumes at the tmRNA-encoded reading frame. During this process, the truncated protein acquires an 11 amino acid (in *E. coli*) C-terminal extension that is an efficient degradation signal for specific cellular proteases.

The deletion of *ssrA* modifies the bacterial physiology. Cells lacking *ssrA* are hypersensitive to various stresses, including elevated temperatures.[4] In *Salmonella enterica*, tmRNA affects virulence by modifying the expression of several genes.[5] Trans-translation also affects the regulation of the *lac* operon[6] and tmRNA may regulate the metabolism of other sugars, since some mRNAs encoding proteins involved in the sugars metabolisms are tagged by tmRNA.[7]

Salmonella thyphimurium,[8] *E. coli*[9] and *Synechocystis*[10] cells lacking *ssrA* are more sensitive to several antibiotics when compared to wild-type strains. It has been reported that this hypersensitivity is restricted to drugs that inhibit protein synthesis. Therefore, it has been suggested that ribosomes stalled by some protein synthesis inhibitors can be recycled by tmRNA.[8,10] The fact that trans-translation is involved in various aspects of bacterial physiology suggests that the inactivation of the *ssrA* gene could cause sensitivity to antibiotics with other target sites than the ribosome. Therefore, we measured bacterial growth of *E. coli* Δ*ssrA* and wild-type strains in the presence of 14 antibiotics, eight of which affect ribosome function and six have other target sites.

MATERIALS AND METHODS

Chemicals and strains. Antibiotics were from Amresco (erythromycin), Balkanpharma (ampicillin), FATOL-Arzneimittel (rifampicin) and Sigma (fosfomycin, kanamycin, clindamycin, chloramphenicol, norfloxacin, ofloxacin, puromycin, spectinomycin, streptomycin, tetracycline, trimethoprim).

Construction of the *ΔssrA* strain has been described previously.[11]

Media and growth conditions. Cell were grown aerobicaly at 37°C in M9 minimal medium[12] containing 0.4% of glycose (w/v); or on LB plates.[12] Optical density of bacterial cultures was measured at 600 nm.

Growth inhibition experiments. Overnight cultures were diluted to an optical density of 0.02; then the antibiotic inhibition experiment was started. Alternatively, cultures were diluted to an optical density of 0.1 and grown to an optical density of 0.8, followed by dilution to an optical density of 0.02; then the antibiotic inhibition experiment was started. Two milliliter cultures were grown with antibiotics for 12 hours and the optical density of the cultures was determined.

Tests of bactericidal activity. Overnight cultures of wild-type and *ΔssrA* strains were diluted into 100 ml to an optical density of 0.02. These cultures were grown to an optical density of 0.2 and then divided into two equal parts. One culture was grown with and the other without antibiotics. At determined time points two dilutions from each culture were made and plated onto LB plates such that on the first plate would grow around 50 and on the second plate around 250 colonies (1 ml of culture with optical density 1 contains approximately 5 x 10^8 colony forming units). Plates were incubated 15 h at 37°C and the colonies counted. Antibiotics were used at following concentrations: 400 μg erythromycin ml^{-1}; 6 μg kanamycin ml^{-1}; 16 μg chloramphenicol ml^{-1}; 160 μg puromycin ml^{-1}; 8 μg streptomycin ml^{-1}; norfloxacin 0.075 μg ml^{-1}, 0.05 μg ml^{-1} and 0.025 μg ml^{-1}; fosfomycin 20 μg ml^{-1}, 15 μg ml^{-1} and 10 μg ml^{-1}; ampicillin 3 μg ml^{-1}, 1.5 μg ml^{-1} and 0.75 μg ml^{-1}.

tmRNA aminoacylation with alanine. Alanylation of tmRNA was performed in 20 μl of 25 mM HEPES pH 7.5, 30 mM NH$_4$Cl, 3.5 mM MgCl$_2$, 2 mM GTP, 2 mM ATP, 6 mM phosphoenol pyruvate, 10 μg ml^{-1} pyruvate kinase and 30 μM L-[^{14}C] alanine. Increasing concentrations of antibiotics, up to 1 mM, were incubated for 10 min at room temperature with 0.5 μM of tmRNA (10 pmoles) in the alanylation buffer before the addition of 2.8 μM AlaRS for 30 min at 37°C. The level of alanylation was determined using a filter binding assay as described previously.[13]

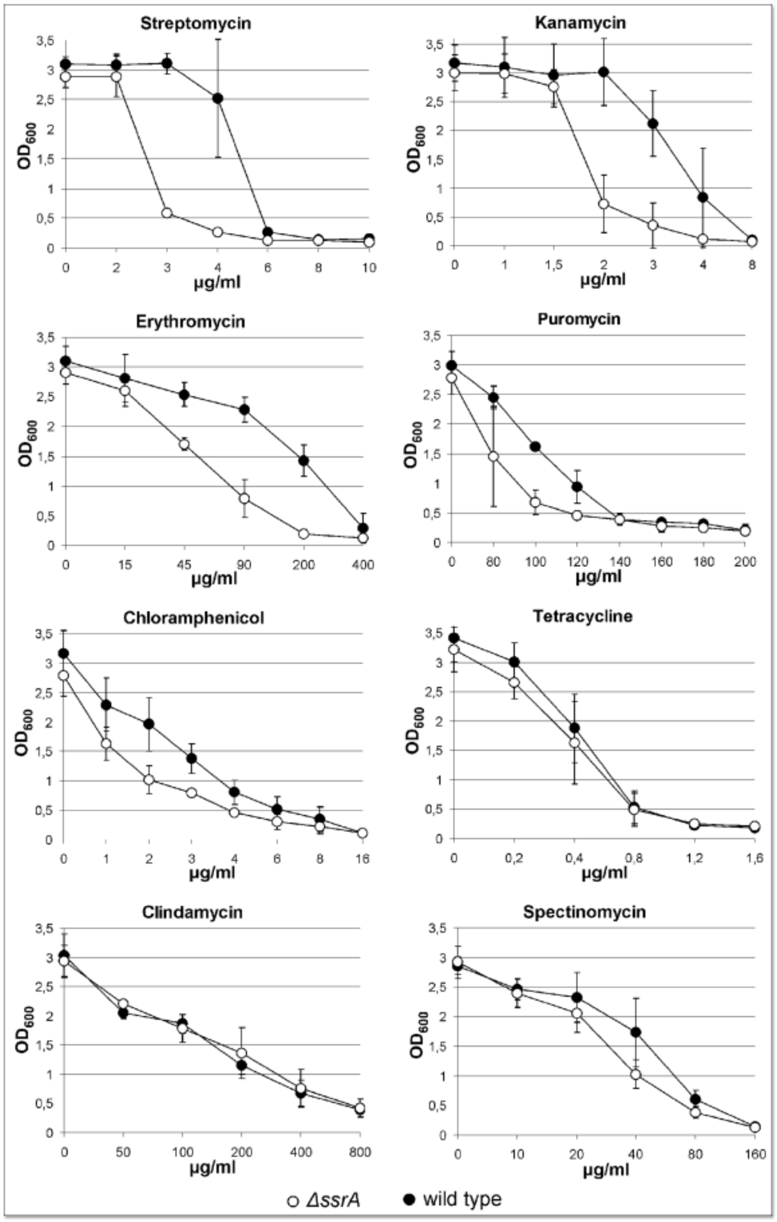

Figure 1. Growth inhibition of *ΔssrA* and wild type strains with inhibitors of protein syhthesis. Overnight wild type (closed symbols) and *ΔssrA* (open symbols) cultures were diluted to an optical density of 0.02 with antibiotics added at the concentrations indicated on the X-axis. After 12 hours of growth the optical densities were measured.

RESULTS AND DISCUSSION

To methods were used to test differences in the antibiotic sensitivity between *ΔssrA* and wild type strains. Firstly, growth inhibition was measured by following the optical density of bacterial cultures at 600 nm. Secondly, to test possible differences in the bactericidal activity of antibiotics against the two strains, the viability of bacteria in the antibiotic treated cultures was measured by plating aliquots and counting colony-forming units (CFU).

Growth inhibition. Growth of wild-type and *ΔssrA* bacteria was measured in liquid culture in the presence of one of 14 antibiotics. Antibiotic concentrations were selected to cover a range from causing little or no inhibition to those causing maximal inhibition. Special effort was made to measure datapoints where partial inhibition is observed as here the differences in sensitivity are the largest. Experiments were repeated at least three times. Both overnight cultures and exponentially growing bacteria were used for the dilutions to start experiments. No differences in the antibiotic sensitivity paterns were observed for the starting cultures in different growth phases. Therefore only the results of the experiments started from overnight cultures are shown in Figures 1 and 2.

In agreement with previous studies,[8,9,10] we observed that the *ΔssrA* is more sensitive than the wild-type strain to several protein synthesis inhibitors (Fig. 1). The effect was more pronounced with erythromycin, kanamycin and streptomycin. Slightly smaller differences between the wild-type and *ΔssrA* strains were observed when grown on puromycin and chloramphenicol (Fig. 1). Very weak or no differences in antibiotic sensitivity between wild type and *ΔssrA* strains (Fig. 1) were observed when grown on tetracycline, clindamycin or spectinomycin.

Is it possible to correlate the mode of action of the ribosome-targeted antibiotics and the sensitization of the *ΔssrA* strain? Antibiotics causing large differences between the sensitivity profiles of wild type and *ΔssrA* strain inhibit protein synthesis by the following mechanisms: erythromycin, a macrolide, binds to the peptide exit tunnel and blocks sterically the extension of the nascent peptide causing dissociation of peptidyl-tRNA from the ribosome.[14,15] Both streptomycin and kanamycin induce misreading of mRNAs during protein synthesis.[16-18] Puromycin is a structural analog of

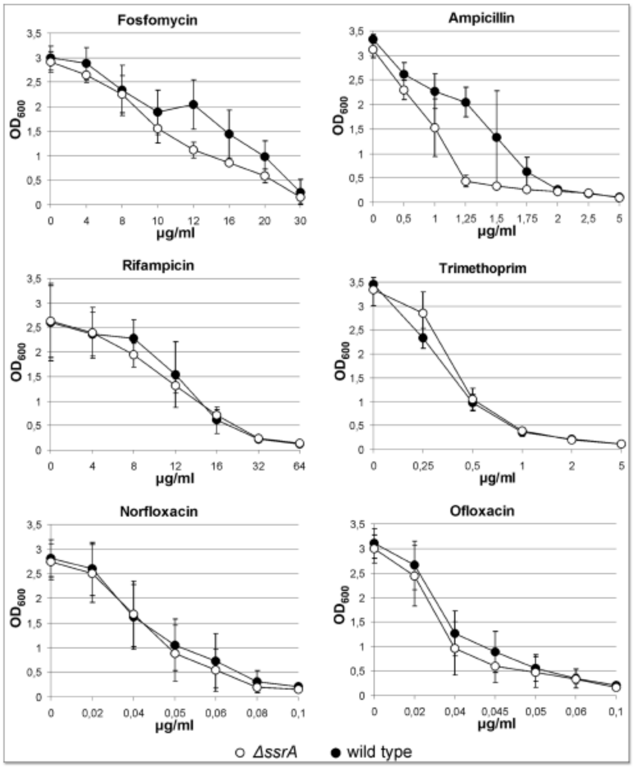

Figure 2. Growth inhibition of *ΔssrA* and wild type strains with drugs that do not affect protein synthesis. Overnight wild type (closed symbols) and *ΔssrA* (open symbols) cultures were diluted to an optical density of 0.02 with antibiotics added at the concentrations indicated on the X-axis. After 12 hours of growth the optical densities were measured.

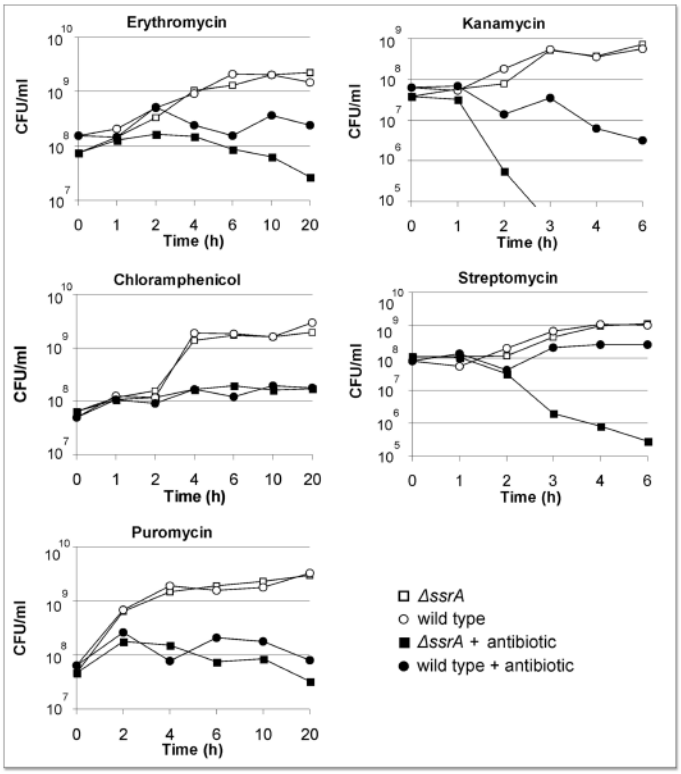

Figure 3. Viability of bacteria in cultures treated with inhibitors of protein synthesis. The antibiotics were added (closed symbols) or not (open symbols) at the 0 time point and the changes in CFUs were followed.

the tyrosyl-tRNA 3'-end and therefore acts as an acceptor substrate for the peptidyl transferase reaction.[17] Chloramphenicol inhibits peptidyl transfer by disturbing the binding of the aminoacyl-tRNA to the A site of the ribosome.[17]

In the presence of spectinomycin, a drug that blocks translocation[18] there is no measurable difference in the antibiotic sensitivity profiles of the two strains. Similarly, no difference is detected in the presence of tetracycline, which hinders tRNA binding to the ribosomal A site[19] or clindamycin, which inhibits peptidyl transfer and induces peptidyl-tRNA dissociation.[14]

In conclusion, the mechanism of action of a given antibiotic does not predict the sensitvity of the *ΔssrA* strain to this antibiotic. For example, chloramphenicol (*ΔssrA* strain is sensitized) and tetracyclin (activity is similar against both strains) both decrease the A site binding of the substrate; chloramphenicol (*ΔssrA* strain is sensitized) and clindamycin (the activity is similar in both strains) inhibit the peptidyl transferase reaction. Both clindamycin (the activity is similar in both strains) and erythromycin (*ΔssrA* strain is sensitized) induce peptidyl-tRNA drop-off. Therefore, there are no clear correlations between how the protein synthesis inhibitors act at the molecular level and the sensitization effects induced by a strain that cannot express tmRNA.

Surprisingly, differences between the antibiotic sensitivity profiles of the two strains were also observed for ampicillin and fosfomycin, inhibitors of cell wall synthesis (Fig. 2).[20] It is important to note that the increased ampicillin sensitivity of the *ΔssrA* strain was observed only when a fresh solution of the antibiotic was used. After several freeze-thaw cycles, while the ampicillin solution retained its ability to inhibit bacterial growth, this solution inhibited both strains to a similar extent. The probable explanation for this changed inhibition pattern is the different mechanims of action of the ampicillin degradation products accumulating during the freeze-thaw cycles.

Very weak or no differences in the antibiotic sensitivity between the wild type and *ΔssrA* strains (Fig. 2) were observed when grown in the presence of rifampicin (inhibits RNA polymerase[21]), norfloxacin, ofloxacin (inhibitors of DNA topoisomerase[20]) or trimethoprim (inhibits dihydrofolate reductase[20]). The experiments with norfloxacin and ofloxacin were less reproducible than those using other compounds, showing sometimes considerably increased sensitivity of the *ΔssrA* strain compared to the wild type. We were not able to find a reason for this variablity. The average of more than 20 experiments is shown in Figure 2 with the variation between experiments indicated by the error bars.

It is not clear how the deletion of *ssrA* influences antibiotic sensitivity. In addition to specific actions, antibiotics can affect the overall bacterial physiology. The previously reported specificity of the *ΔssrA* sensitization effect to inhibitors of protein synthesis suggested that the interplay between tmRNA and the antibiotics occurs on the ribosome.[8-10] Our data demonstrate that this is not always the case, since the *ΔssrA* strain has an increased sensitivity to inhibitors of cell wall synthesis. Several cellular stresses are induced by antibiotics.[22-26] Trans-translation is implicated in the regulation of the expression of selected genes.[6] Therefore, in the absence of tmRNA, the cell may not react efficiently to a stress induced by an antibiotic and therefore sensitization may occur. It is known that different groups of antibiotics trigger different stress responses, although the details of antibiotic induced stresses remain to be elucidated. We propose that the common feature of antibiotics for which the sensitization effect occurs is the similarity of stress responses that these drugs trigger.

An interesting link that might connect *ssrA* to cell wall synthesis is the observation that tmRNA tags SecM,[27] a regulator of SecA expression. As SecA is an ATPase that targets protein precursors to the SecYEG core translocon for secretion, lack of trans-translation might influence the ability of the cell to respond to extracellular stresses.

Aminoacylation of tmRNA. Both erythromycin and clindamycin induce peptidyl-tRNA drop-off by blocking the egress of the nascent polypeptide down the tunnel.[14,15] On the other hand, inactivation of tmRNA

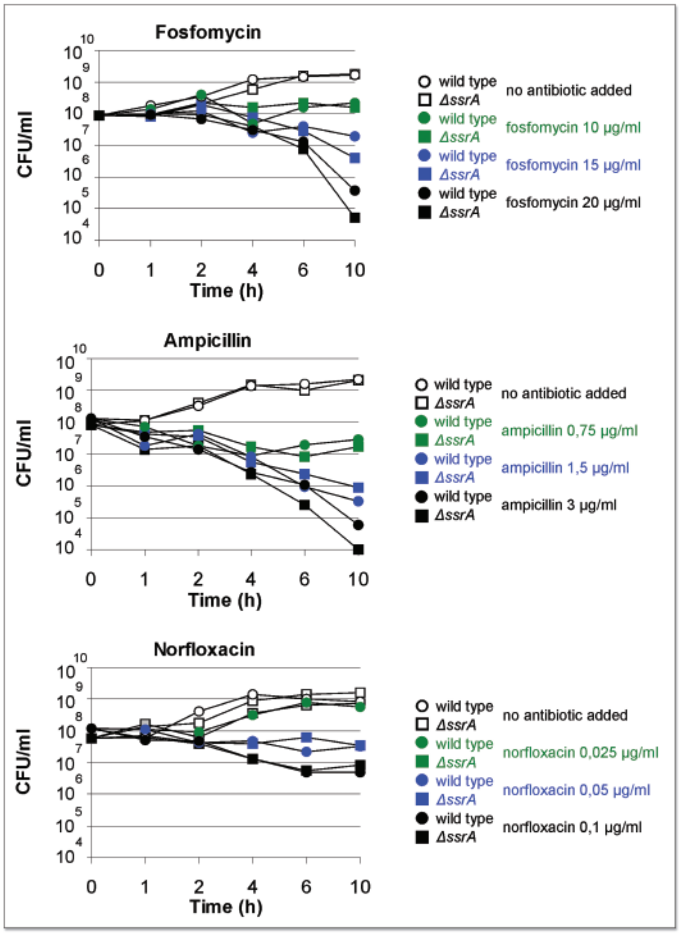

Figure 4. Viability of bacteria in cultures treated with drugs that do not affect protein synthesis. The antibiotics were added (closed symbols) or not (open symbols) at the 0 time point and the changes in CFUs were followed

makes the cells more sensitive to erythromycin but not to clindamycin. In addition, both tetracyclin and chloramphenicol decrease the A site binding of aminoacylated tRNAs. Again, the effect of tmRNA inactivation is different when tested on these two antibiotics: sensitization to chloramphenicol but not to tetracyclin. The absence of sensitization effects of the addition of either tetracyclin or clindamycin on growth of cells lacking tmRNA could be caused by inhibition of trans-translation in wild-type cells. Indeed, it has been reported previously that some aminoglycosides can impair tmRNA aminoacylation, and therefore trans-translation in vitro.[13,28] Therefore we tested the aminoacylation of tmRNA, catalysed by purified alanyl-tRNA syntethase in the presence of either chloramphenicol, tetracyclin, clindamycin or erythromycin. In all cases no inhibition was observed, up to a 1 mM concentration of each drug (Supplementary Fig. 1). Thus, the absence of growth differences is not due to a direct detrimental effect of these drugs on tmRNA aminoacylation.

Bactericidal activity. The ability to kill bacteria (bactericidal activity) is an important parameter when assessing the potency of antibiotics. Therefore, we measured the viability of bacteria in the antibiotic-treated cultures by plating aliquots and counting the CFUs. For the test of bacteriocidal activity, we used antibiotics causing growth differences between the wild type and *ΔssrA* strains. As there was a relatively large variation in the results of growth inhibition tests for the fluoroquinolones (norfloxacin and ofloxacin), norfloxacin was also used in the bactericidal test. Antibiotics were used at concentrations which decrease the optical density of the *ΔssrA* culture about ten fold (Figs. 1 and 2). The experiments were repeated at least three times. The shapes of the curves were reproducible.

Large differences between the number of CFUs per ml of the wild-type and *ΔssrA* strains were observed when treated with kanamycin or streptomycin (Fig. 3). Both drugs inhibit protein synthesis by inducing misreading of codons by the ribosome.[16-18] Kanamycin and streptomycin induce the miscoding of termination codons from model mRNAs, even at sublethal concentrations, increasing the overall amount of proteins tagged by tmRNA.[9] The pool of ribosomes stuck at the 3'-end of mRNAs significantly increases in the presence of aminoglycosides, and trans-translation might become an essential process to recycle these ribosomes.

In the presence of erythromycin, a reproducible CFU decrease in the *ΔssrA* culture was observed. The erythromycin concentration used in the experiment is bacteriostatic for the wild type culture, no decrease in the viability counts was detected. In the presence of other inhibitors of protein synthesis, chloramphenicol and puromycin, there are no reproducible differences in the viability between the two strains (Fig. 3).

The original concentrations of the drugs that do not affect protein synthesis, ampicillin (3 μg ml^{-1}), fosfomycin (20 μg ml^{-1}) and norfloxacin (0,1 μg ml^{-1}), used in our experiments may kill the bacteria too rapidly to observe differences between the two strains. Therefore, lower concentrations of these drugs were tested (Fig. 4). In the presence of ampicillin, fosfomycin or norfloxacin, there were no reproducible differences in the viability between the two strains; although when ampicillin or fosfomycin was used, in some experiments the viability count of the ΔssrA strain decreased faster than the viability count of the wild type strain. Therefore we conclude that the decreased viability of the ΔssrA strain is specific for aminoglycosides as it was in the presence of kanamycin or streptomycin that the largest effects were observed.

In recents years there has been increasing interest in searching for novel compounds that would potentiate the effects of the drugs in current medical use.[29,30] Our results suggest that trans-translation might be an interesting target for new drugs that could potentiate the action of many inhibitors of protein and cell wall synthesis.

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