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Detection of Live and Antibiotic-Killed Bacteria by Quantitative Real-Time PCR of Specific Fragments of Ribosomal RNA

THÈSE

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RÉSUMÉ

Le but d'un traitement antimicrobien est d'éradiquer une infection bactérienne. Cependant, il est souvent difficile d'en évaluer rapidement l'efficacité en utilisant les techniques standard. L'estimation de la viabilité bactérienne par marqueurs moléculaires permettrait d'accélérer le processus. Ce travail étudie donc la possibilité d'utiliser le RNA ribosomal (rRNA) à cet effet. Des cultures de *Streptococcus gordonii* sensibles (parent Wt) et tolérants (mutant Tol1) à l'action bactéricide de la pénicilline ont été exposées à différents antibiotiques. La survie bactérienne au cours du temps a été déterminée en comparant deux méthodes. La méthode de référence par compte viable a été comparée à une méthode moléculaire consistant à amplifier par PCR quantitative en temps réel une partie du génome bactérien. La cible choisie devait refléter la viabilité cellulaire et par conséquent être synthétisée de manière constitutive lors de la vie de la bactérie et être détruite rapidement lors de la mort cellulaire. Le choix s'est porté sur un fragment du gène 16S-rRNA.

Ce travail a permis de valider ce choix en corrélant ce marqueur moléculaire à la viabilité bactérienne au cours d'un traitement antibiotique bactéricide. De manière attendue, les *S. gordonii* sensibles à la pénicilline ont perdu ≥ 4 $\log_{10}$ CFU/ml après 48 heures de traitement par pénicilline alors que le mutant tolérant Tol1 en a perdu ≤ 1 $\log_{10}$ CFU/ml. De manière intéressant, la quantité de marqueur a augmenté proportionnellement au compte viable durant la phase de croissance bactérienne. Après administration du traitement antibiotique, l'évolution du marqueur dépendait de la capacité de la bactérie à survivre à l'action de l'antibiotique. Stable lors du traitement des souches tolérantes, la quantité de marqueur détectée diminuait de manière proportionnelle au compte viable lors du traitement des souches sensibles. Cette corrélation s'est confirmée lors de l'utilisation d'autres antibiotiques bactéricides.

En conclusion, l'amplification par PCR du RNA ribosomal 16S permet d'évaluer rapidement la viabilité bactérienne au cours d'un traitement antibiotique en évitant le recours à la mise en culture dont les résultats ne sont obtenus qu'après plus de 24 heures. Cette méthode offre donc au clinicien une évaluation rapide de l'efficacité du traitement, particulièrement dans les situations, comme le choc septique, où l'initiation sans délai d'un traitement efficace est une des conditions essentielles du succès thérapeutique.

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Detection of Live and Antibiotic-Killed Bacteria by Quantitative Real-Time PCR of Specific Fragments of Ribosomal RNA

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INTRODUCTION

Classical techniques to identify bacterial pathogens include phenotyping of live bacteria, specific immunostaining, and indirect measurement of the host's serologic response. However, cultures can remain negative in the case of fastidious organisms or after antibiotic administration. Immunostaining is not always specific and is highly dependent on sampling. And serology provides a delayed diagnostic answer and can also lack specificity.

Recent molecular methods may help circumvent some of these limitations. They are aimed at detecting either proteins or nucleic acids by direct molecular probing, or by amplifying specific determinants such as the ribosomal RNA genes (rDNA) by polymerase-chain reaction (PCR) (1, 5, 12-14, 25-27, 35). They play an important role in the case of culture-negative infections (3, 13, 14). Moreover, they yield results within hours as compared to days or sometimes weeks with conventional phenotypic techniques. Yet, none of these methods provide information on the viability of the infecting microorganisms.

Determination of bacterial viability is critical to follow the bactericidal activity of antibiotics *in vitro* and *in vivo*, as well as to assess the presence of viable pathogens in contaminated food or environmental samples (2, 13, 20, 21, 24). Molecular markers of viability might be useful to follow microbiological cure in a variety of clinical situations, and to rapidly appraise drug efficacy. In this regard, notoriously problematic pathogens include *Mycobacterium tuberculosis* and *Mycobacterium leprae*, for which determining drug-induced killing by standard techniques takes between weeks and months (18, 30, 38).

Several physiological and molecular reporters of viability have been explored. These include measurement of cellular integrity (e.g. by vital staining), measurement of metabolic activities (e.g. active electron transport chain, transport of glucose, esterase activity), measurement of synthetic activities (e.g. polymer synthesis and cell elongation) (37), and molecular probing or amplification of DNA, mRNA and 16S rRNA (20). DNA amplification appeared particularly unreliable because it can remain positive for extensive periods of time,

ABSTRACT

Assessing bacterial viability by molecular markers might help accelerate the measurement of antibiotic-induced killing. This study investigated whether ribosomal RNA (rRNA) could be suitable for this purpose. Cultures of penicillin-susceptible and penicillin-tolerant (Tol1 mutant) *Streptococcus gordonii* were exposed to mechanistically different penicillin and levofloxacin. Bacterial survival was assessed by viable counts, and compared to quantitative real-time PCR amplification of either the 16S-rRNA genes (rDNA) or the 16S rRNA, following reverse transcription. Penicillin-susceptible *S. gordonii* lost ≥ 4 $\log_{10}$ CFU/ml of viability over 48 h of penicillin treatment. In comparison, the Tol1 mutant lost ≤ 1 $\log_{10}$ CFU/ml. Amplification of a 427-base fragment of 16S rDNA yielded amplicons that increased proportionally to viable counts during bacterial growth, but did not decrease during drug-induced killing. In contrast, the same 427-base fragment amplified from 16S rRNA paralleled both bacterial growth and drug-induced killing. It also differentiated between penicillin-induced killing of the parent and the Tol1 mutant (≥ 4 $\log_{10}$ CFU/ml and ≤ 1 $\log_{10}$ CFU/ml, respectively), and detected killing by mechanistically unrelated levofloxacin. Since large fragments of polynucleotides might be degraded faster than smaller fragments the experiments were repeated by amplifying a 119-base region internal to the original 427-base fragment. The amount of 119-base amplicons increased proportionally to viability during growth, but remained stable during drug treatment. Thus, 16S rRNA was a marker of antibiotic-induced killing, but the size of the amplified fragment was critical to differentiate between live and dead bacteria.

in spite of effective bacterial killing (4, 7, 15, 16, 19-21, 28, 29, 34, 36, 37). In addition, most other methods provided conflicting results that varied depending on the experimental conditions, including whether bacteria were killed by antibacterial drugs (15, 20, 36) or by physical means (e.g. heat and UV irradiation) (24, 31, 37). Thus, there are currently no reliable molecular tools for routine determination of cell viability and drug-induced killing in medical microbiology.

The present experiments explored the correlation between bacterial viability and the number of copies of 16S rRNA – as determined by reverse transcription followed by quantitative real-time PCR – during bacterial growth and antibiotic-induced killing of *Streptococcus gordonii*. This organism was used as a model because it is poorly lysed by penicillin and provides a well described isogenic pair of kill-susceptible (parent) and kill-resistant (tolerant mutant Tol1) strains (6, 22). Studied were performed with two mechanistically unrelated compounds, i.e. penicillin G and the quinolone levofloxacin. A specific fragment of 16S rRNA of a specific size appeared suitable to quantify drug-induced killing.

MATERIALS AND METHODS

Microorganisms and growth conditions. A described streptomycin-resistant *S. gordonii* Challis and its penicillin-tolerant mutant Tol1 were used as model organisms (6, 22). Streptococci were grown at 37°C without aeration either in brain heart infusion (BHI; Difco laboratories, Detroit, MI) supplemented with 200 mg/liter of streptomycin (in order to respect the experimental conditions in which these isolates were described)(6, 22), or on Columbia agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 3% blood. *Escherichia coli* XL-1 blue were grown at 37°C in Luria Bertani broth (LB, Difco) or on LB agar (Difco), supplemented with 50 mg/liter of ampicillin (Sigma Chemicals). Stocks were stored at -70°C in culture medium supplemented with 10% (vol/vol) glycerol. Bacterial growth was followed by optical density at a wavelength of 620 nm (OD_{620}) with a spectrophotometer (Sequoia-Turner, Montainville, CA), and by colony count on agar plates. When appropriate, penicillin G (Hoechst-Pharma, Zurich, Switzerland) and levofloxacin (Aventis Pharma Ltd., Romainville, France) were added to the medium at final concentrations of 2 mg/liter and 12.5 mg/liter, respectively, mimicking high-dose treatment in human. The minimal inhibitory concentrations of these antibacterials for the test bacteria were 0.004 and 0.5 mg/liter (6, 11).

Antibiotics and chemicals. Streptomycin was purchased from Sigma AG (Buchs, Switzerland), penicillin G from Hoechst-Pharma AG (Zurich, Switzerland) and levofloxacin from Aventis Pharma Ltd. (Romainville, France). The restriction enzymes (Boehringer Mannheim, Germany), *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD) and T4 DNA ligase (Gibco) were used according to the manufacturer's recommendations. Nucleic acid sequencing and synthesis was performed by Microsynth GmbH (Balgach, Switzerland). All other chemicals were reagent-grade, commercially available products.

Antibiotic susceptibility and time-kill curves. The MICs were determined by standard macrodilution methods (32). Time-kill curves were determined by adding appropriate concentrations of antibiotics to bacterial cultures in the exponential phase of growth at an OD_{620} of 0.2 (6, 22). At various time points before and after drug addition, samples were removed and processed (i) for viable count, (ii) for DNA extraction and (iii) for RNA extraction. For viable count, antibiotic carryover on the agar plates was avoided as described previously (10, 11). Colonies were counted after 48 h of incubation at 37°C. DNA was extracted from frozen culture samples kept at -70°C, whereas RNA was isolated directly from fresh samples.

DNA extraction and purification. Total DNA from 3 ml of culture samples was extracted and purified using the DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Lysis of streptococci was performed as follows. Samples were centrifuged at 14'000 rpm for 15 min at 4°C. Pellets were washed two times with NaCl 0.9% and resuspended in 220 µl buffer [Tris-Hcl 20 mM pH 8.0, EDTA 5 mM, 25% sucrose and 17 µg/ml of lysozyme (Sigma)]. The samples were then incubated for 30 min at 37°C, before the addition of 200 µl of lysis buffer (Buffer AL, Qiagen) supplemented with final concentrations of 0.2% of SDS (Sigma) and 1 mg/ml of proteinase K (Sigma). After an additional 30 min incubation at 70°C, DNA was purified according to the standard protocol (Qiagen), resuspended in a 200 µl final volume and kept frozen at -20°C°. Input volumes for further amplification consisted in 1 µl aliquots of the stored samples.

RNA extraction and purification. For total RNA purification, 9 ml of fresh culture samples were centrifuged at 10'000 rpm for 8 min at 4°C and processed according to the FastRNA BLUE Protocol of BIO 101 (Bio 101 Inc., La Jolla, CA) modified as follows. Bacterial pellets were resuspended in 500 µl of CRSR-BLUE reagent and transferred into tubes containing

ceramic beads, supplemented with 500 µl of phenol acid reagent (PAR) and 100 µl of a solution of 24:1 chloroform-isoamyl alcohol (CIA). The samples were further processed at 4°C using a FastPrep FP120 apparatus (Bio 101, Savant Instruments, Inc. Holbrook, NY) for 25 sec at a speed of 6.5 m/s, before being centrifuged at 14'000 rpm for 10 min. The aqueous phase was collected and added to a 500 µl of the CIA solution. The samples were then centrifuged at 14'000 rpm for 5 min. The aqueous phase was collected and mixed with 350 µl of RLT Buffer (RNeasy Mini Kit, Qiagen) supplemented with 1% β-Mercaptoethanol and 250 µl RNase-free Ethanol (96-100%). Total RNA was further purified according to the standard RNase-Free DNase Set Protocol of Qiagen, resuspended in a 50 µl final volume and kept frozen at -80 C^0. Input volumes for further amplification consisted in 5 µl aliquots of the stored samples.

The yield of RNA was quantified in each experiment by absorbance at 260 nm (Biophotometer ThermoStat Plus, Eppendorf AG, Germany). As an additional control, the presence of rRNA in the extracts was qualitatively assessed by electrophoresis and ethidium bromide staining on a 1.2% agarose-0.66 M formaldehyde gel in morpholinepropanesulfonic acid running buffer (Sigma), next to a molecular weight ladder.

Construction of molecular standards for quantitative real-time PCR. To build standard curves for both real-time PCR and RT-PCR it was important to first generate control molecules with known molar concentrations. First, a DNA standard was generated by cloning the PCR product corresponding to the 427-base fragment of the 16S rDNA of *S. gordonii* into the pGemT-Easy vector system (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions and transformed in *Escherichia coli* by the standard technique. Effective amplicon ligation by T4 DNA ligase (Gibco BRL, Gaithersburg, Md.) was verified by standard restriction enzymes, amplification was performed by using the Wizard Midiprep

UI of Taq DNA Polymerase (Life Technologies AG). Amplification was performed for 25 cycles at the following conditions: 94°C for 30 sec, 50°C for 30 sec and 72°C for 20 sec. Quantities of polynucleotides were assessed on ethidium bromide containing 1% agarose gel using standard techniques scanning densitometry at a wavelength of 280 using the EagleEye II Still Video System and the EagleSight software (Stratagene, La Jolla, CA).

Reverse-transcriptase amplification. Prior to quantification, reverse-transcriptase (RT) PCR was performed on all RNA samples using the Omniscript RT Kit (Qiagen) in a total volume-reaction of 20 µl according to the manufacturer's instructions. Different solutions containing between 10^2-10^8 RNA molecules were prepared as above. RT-PCR of test and standard samples were run in parallel. Test samples consisted in 20 µl aliquots of the stored materials. RT-PCR efficiency was 30% and was calculated as follows: number of molecules of cDNA/number of molecules of RNA x 100%. The number of RNA molecules of each unknown sample was calculated on the base of both the standard curve (quantitative real-time PCR) and the RT-PCR efficiency. Each standard and test samples were run in quintuplicate and processed in parallel for further quantification, in order to appraise the intra-experiment relative variation. This variation was $\leq$ 20%. The results of the experiments are expressed as the mean of values of these quintuplicates.

Quantitative evaluation by real-time PCR. Determination of 16S rDNA copy numbers was performed using the following probe FAM-5'-TTG CAC CAC TAC CAG ATG GAC CTGC-3'-TAMRA (nucleotides 220-244) according to the manufacturer's instructions (Perkin-Elmer) on a Sequence Detection System 5700 (Perkin-Elmer). The fragment 88-514 (427-base) of 16S rDNA was amplified using the above described primer pair. In certain experiments a shorter 119-base internal portion of the 427-base fragments was amplified

(corresponding to nucleotides 158-276 of the *S. gordonii* 16S rDNA), using the primers 5'-GGA AAC GAT AGC TAA TAC CGC ATAA-3' and 5'-AAT CGA TCA TCC ACT CCA TTG CCG AG-3'. Reactions were started on a Sequence Detection System 5700 (Perkin-Elmer) in a total volume of 50 µl 1x PCR buffer (Gibco) containing 0.5 µM of each primer, 120 nM of fluorescent probe, 0.3 mM of each dNTP, 2.5 mM $MgCl_2$ and 1.5 UI of Taq DNA polymerase (Life Technologies). The following program was applied during 40 cycles: 94°C for 15 sec, 52°C for 30 sec, and 72°C for 30 sec. Each experiment was done in triplicate.

The yield of real-time PCR was assessed by plotting the Ct values versus the $\log_{10}$ numbers of input DNA copies. In a typical experiment (6 parallel runs for each sample), the mean slopes of the plots were (Mean $\pm$ SD) -3.56 $\pm$ 0.1 for the 119 bp fragment and 3.9 $\pm$ 0.04 for the 427 bp fragment. This translates into yields of 98.6% and 80.2% for the two fragments, respectively. Although these yields were different, they did not affect the interpretation of the results because amplicons resulting from the different fragments were not compared between them.

DNA content of each sample was determined using a standard curve obtained by processing, in parallel, samples containing fixed DNA concentrations. Determination of DNA concentrations was performed by spectrophotometry, and molar concentration determined using the following formula: 1 µg of a 1000 bp DNA fragments = 1.52 pmol = 1.52 10^{-12} moles x N molecules, where N stands for the Avogadro number (6.023 x 10^{23} molecules/mole). Different solutions were then accordingly prepared (10^8-10^2 molecules/µl) and used as standard solutions in the further experiments.

Assessment of drug-induced killing by vital staining. The parent *S. gordonii* and its Tol1 mutant were treated with penicillin as described. Just before and 24 h after drug addition samples (100 µl) were removed from the cultures, washed twice with PBS buffer (by

centrifugation), and processed both for viable counts and for vital staining according to the manufacturer's instructions (Live/Dead® *BacLight*™, Molecular Probes, Eugene, Oregon, USA). In brief, bacterial samples (100 µl) were mixed with 0.3 µl of a mixture of equal parts of SYTO 9 and propidium iodide and incubated in the dark for 20 min. 2 µl of the stained suspension was deposited on a glass slide and covered with a cover slip. Image acquisition was performed with a confocal scanning laser microscope (model TCS SL; Leica Lasertechnik GmbH, Heidelberg, Germany). Confocal illumination was provided by Ar and HeNe lasers (488-nm and 543-nm laser excitation, respectively). The SYTO 9 signal was collected in the range 503–523 nm and the propidium iodide signal between 575 and 630 nm. The sensitivities of the photomultipliers and the laser intensity were adjusted and thereafter kept constant throughout the experiments. Randomly selected regions of each sample were imaged using an x20 oil immersion objective. The area of each section was transformed into a digital image of 512 by 512 pixels.

RESULTS

Absence of correlation between viable counts, optical density and non-quantitative PCR of 16S rDNA. Physically intact bacteria are considered alive if they are able to give rise to a progeny. Optical density is a correlate of bacterial mass, but bacteria forming this mass are not necessarily alive. Likewise, non-quantitative PCR amplification reveals the presence of specific polynucleotides fragments, but non-quantitative detection of polynucleotides fragments bears no linear correlation with bacterial mass or viability. Hence, these two methods are not likely to be good indicators of bacterial viability. This was confirmed in the present experimental setting, as a control (Figure 1). Optical density increased proportionally to viable counts during bacterial growth (Figure 1A). Yet, it barely decreased during penicillin treatment, in spite of a viability loss of ≥ 4 $\log_{10}$ CFU/ml within 48 h of drug exposure (Figure 1B). Non-quantitative PCR amplification was even less representative of viability. Amplification of a 427-base fragment of the 16S rDNA yielded quantities of amplicons that had no correlation with either bacterial mass or penicillin-induced killing (Figure 1). Thus, more precise techniques were needed.

Limited correlation between viable counts and quantitative real-time PCR of 16S rDNA. To improve quantitative assessment we repeated the experiments using real-time PCR. Amplification of the same 427-base fragment of the 16 rDNA yielded quantities of amplicons that increased proportionally to bacterial mass during logarithmic growth, and stabilized after entry into stationary phase (Figure 2). This correlated well with bacterial viability in the non-treated cultures (Figure 2A), but not with penicillin-induced killing (Figure 2B), as the quantities of real-time PCR products remained stable in spite of an extensive loss of viability of $\geq$ 4 Log_{10} CFU/ml/48 h. Thus, the real-time PCR products were a good correlate of bacterial mass, but not of bacterial viability *senso strictu*. This confirms the good stability of

DNA during non-lytic killing of bacteria by antibiotics (4, 7, 15, 16, 19-21, 28, 29, 34, 36, 37).

Good correlation between viable counts and quantitative real-time PCR of 16S rRNA. We next tested whether RNA would be more sensitive than DNA to monitor drug-induced killing. Both messenger RNA (mRNA) and rRNA were potential targets. However, mRNA has a very short half life in bacteria. Hence, drugs inhibiting DNA transcription (e.g. rifampin) may result in a rapid drop in mRNA, irrespective to their bacteriostatic or bactericidal effect (15).

rRNA might more appropriate. Therefore we tested the stability of 16S rRNA as a potential marker of drug-induced killing. Total RNA was reverse-transcribed and the quantities of 16S rRNA were assessed by real-time PCR amplification of the 427-base fragment from cDNA. This yielded quantities of amplicons that correlated very closely with viable counts during logarithmic growth and stationary phase, and also correlated with drug-induced killing during penicillin treatment (Figure 3). Moreover, the system could readily differentiate between the extensive drug-induced killing of the *S. gordonii* parent (Figure 3A), and the kill-resistance phenotype of mutant Tol1 (Figure 3B).

Good correlation between real-time PCR of 16S rRNA and quinolone-induced killing. To assess whether degradation of 16S rRNA might also apply to killing by a mechanistically different antibiotic we repeated the experiments with the quinolone levofloxacin. Quinolones generate breaks in the chromosomal ladder (17, 33), but are not expected to perturb rRNA. The stability of both the 16S rDNA (Figure 4 A) and the 16S rRNA (Figure 4B) was tested by quantitative amplification of the 427-base fragment described above. Killing by levofloxacin was fast, as previously reported (11). Amplification of both rDNA and rRNA paralleled bacterial viability during the logarithmic and stationary growth phases. After addition of

levofloxacin, the quantities of rDNA amplicons remained remarkably stable, in spite of rapid drug-induced killing (> 3 $\log_{10}$ CFU in 6 h)(Figure 4A). In contrast, the quantities of 16S rRNA copies dropped by > 3 $\log_{10}$, thus reflecting the bactericidal effect of the drug (Figure 4B). Therefore, quantitative amplification of the 427-base fragment of 16S rRNA correlated with killing by both cell wall inhibitors and DNA gyrase inhibitors.

Large fragments of rRNA are better markers of drug-induced killing than smaller fragments. Since large polynucleotides fragments might be degraded faster than smaller ones (23), we repeated the 16S rRNA experiment by amplifying a shorter (119-base) segment internal to the 427-base fragment used above (Figure 5). The quantities of 119-base amplicons correlated with viable counts during logarithmic growth and stationary phase, but decreased only by one order of magnitude (instead of $\geq$ 3 orders of magnitude) during penicillin-induced killing (Figure 5A). Nevertheless, they still revealed a difference between the kill-susceptible parent and the Tol1 mutant (Figure 5B). Thus, the shorter 119-base fragment was less optimal than the longer 427-base fragment to assess penicillin-induced killing and tolerance.

Poor correlation between penicillin-induced killing and vital staining. The parent *S. gordonii* and its Tol1 mutant were exposed to penicillin G as above. Samples were removed juts prior and 24 h after drug addition, and processes for both viable counts and vital staining. For each sample, 20 random area continent 100 - 200 bacterial bodies were visually assessed by phase contrast microscopy and counted for green (alive) and red (dead) staining. Before penicillin addition all bacteria on the slides appeared in short chains of 2-10 cells at phase contrast. Accordingly, the number of green/red bacteria counted were 2524/2544 (98%) for the parent and 2537/2553 (99%) for the Tol1 mutant. After 24 h of penicillin treatment, the normally-looking organisms decreased by up to 300x in the penicillin-killed parent, as opposed to only 20 in the kill-tolerant mutant. Most bacteria appeared as single or double

bulged cells, condensed ghost, or intermediate forms. These forms were not stained by the *Backlight*^{TM} system (dead bacteria should have appeared in red) and thus escaped the quantification by vital staining. Among the remaining forms, red/green counts were 670/1165 (58%) for the parent (in spite of a killing of ≥ 3 $\log_{10}$ CFU/ml), and 2166/2924 (74%) for the Tol1 mutant, which had lost < 1 $\log_{10}$ CFU/ml. As a control, heat-killed *S. gordonii* and *Bacillus subtilis* killed by membrane-active agents turned entirely red (data not presented). Thus, although combining phase contrast microscopy and vital staining revealed a difference between live and antibiotic-killed bacteria, it was less accurate and more difficult to interpret than determining the number of copies of rRNA.

DISCUSSION

The present results disclosed a reproducibly good correlation between the loss of viable counts and the drop in the quantities of copies yielded by real-time PCR amplification of a 427-base fragment of the 16S rRNA. Coherent results were obtained with both the parent and the tolerant mutant Tol1, as well as with mechanistically unrelated penicillin and levofloxacin, provided that a minimal (427-base) fragment length of rRNA was used. A shorter (119-base) template was much less reliable in differentiating between live and dead bacteria.

The experiments support the work by van der Vliet et al. (36), who observed that rRNA may be degraded during antibiotic-induced killing of *Mycobacterium smegmatis* treated with rifampin and ofloxacin. However, this pioneer report disclosed only a modest correlation between the viability loss and decay in rRNA, which underestimated drug-induced killing by > 100x (36). The present molecular system was much more sensitive and predicted cell death in the same order of magnitude as actual killing. The principal difference between these two observations may rely on technical issues, one using nucleic acid sequence-based amplification (NASBA)(36) and one (presented herein) quantitative real-time PCR.

On the other hand, both the van der Vliet et al. report (36) and the present experiments are different from the results by Hellyer et al. (15), who studied the decrease in mRNA and 16S rRNA during treatment of *M. tuberculosis* with isoniazid and rifampin. These authors observed that cell death was accompanied by a sharp decrease in mRNA, but barely found any decrease in 16S rRNA. However, rRNA was detected by sequential reverse transcription and non-quantitative PCR amplification of cDNA from a short (160-base) rRNA fragment. This approach may have missed degradation of rRNA for at least two reasons. First, amplification was not quantitative, and second the length of the rRNA fragment might have been suboptimal.

Both of these issues were critical in the present work. In particular, the inverse relation between polynucleotide length and the lack of polynucleotide amplification is supported by

earlier reports, for instance during chlorine-induced killing of *Legionella pneumophila* (23). Long strands of nucleic acids have a greater chance than shorter strands to carry cleavage sites for endonucleases. In turn, cleaved strands cannot be amplified by PCR anymore. While DNA is very stable in spite of bacterial death (4, 7, 15, 16, 19-21, 28, 29, 34, 36, 37), rRNA may behave differently. In the present case, it is possible that antibiotic-induced killing allowed non-specific deregulation of bacterial RNases (8, 9), leading to progressive RNA decay. Moreover, the kinetics of RNA decay were somewhat delayed as compared to viability loss, thus complying with the assumption that RNA degradation was the consequence rather than the cause of cell death.

RNA degradation was not formally demonstrated herein. However, other causes of impairment of amplification seem less likely. Secondary structures impeding amplification is one alternative, but such structure should not have sustained the PCR conditions. Another alternative could involve inhibitors of amplification present only in antibiotic-killed cells. However, such inhibitors should also affect amplification of the smaller 119-base internal fragment, which was not the case in these observations. Eventually, the precise limit of RNA size useful to differentiate between live and dead bacteria was not determined. This size could indeed vary between different RNA stretches, and between different bacteria. Nevertheless, the experiments described herein provide clear evidence that size must be taken into account.

Killing of *Staphylococcus aureus* and *E. coli* by physical or chemical means indicated that 16S rRNA was unstable after autoclaving (24), but was more stable after killing by less drastic conditions such as lower temperatures (60 or 80°C), ethanol, or UV irradiation (24, 31). In one study (24) the authors used both Northern blotting and reverse-transcription followed by non-quantitative PCR to detect rRNA. Fragments length (ca 400-base or larger) was not an issue. However, since cDNA was amplified non-quantitatively, the PCR data are difficult to interpret. On the other hand, Northern blots clearly indicated a hierarchy of rRNA degradation, with total disappearance of rRNA at harsh (120°C) conditions, partial

disappearance at less harsh (80°C) conditions, and minimal disappearance after UV treatment. It was suggested that the less drastic conditions could respect the physical integrity of the ribosomes, thus preventing them from the attack by RNases or other enzymes (24). If so, then penicillin and levofloxacin treatments appear closer to harsh than milder conditions. Indeed, real-time PCR detected precisely both drug-induced killing and survival of the parent *S. gordonii* and its Tol1 mutant.

Eventually, quantitative assessment of rRNA was a better marker of drug-induced killing than vital staining. In this particular case, the poor performance of vital stain was due to physical alterations of dying bacteria, which became unstainable by the coloration system. Although the effect might be more pronounced with cell wall active drugs than other compounds, this kind of limit was also reported in other systems, especially when the plasma membrane is not physically altered (37).

Taken together, the present results revive the perspective of using molecular markers to detect antibiotic-induced killing of bacteria. They clarify certain differences between various reports, and help delineate a molecular system that might detect drug-induced killing. An interesting observation was the inverse relationship between rRNA amplification and the length of the amplicons. This raises the possibility of combining both short and long amplicons in the same assay. Short amplicons would detect both the presence and the nature of the organism, while long amplicons would assess its viability. Calculating a ratio between the short and long fragments could provide information on the total-over-killed cells both *in vitro* and in clinical samples.

Molecular tools are increasingly applied for rapid diagnosis, detection of non-cultivable pathogens and detection of antibiotic-resistance genes. In the future they will also include virulence genes such as toxins. Yet, an important limit of current molecular methods is that they do not differentiate between live bacteria and dead remnants. The present observations indicate that analyzing rRNA fragments could help solve the issue. At this stage

the technique using RT-PCR followed by real-time PCR is still too complicated for clinical or field application. However, the results identify at least one of the molecular approaches that might be worth pursuing into engineering development.

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FIGURE LEGENDS

Figure 1. Growth (A) and penicillin-induced killing (B) of the parent *S. gordonii* (squares) and its tolerant mutant Tol1 (circles) followed by optical density, viable counts and non-quantitative PCR of 16S rDNA. (A) During growth both optical densities and viable counts increased in parallel, while non-quantitative rDNA amplification remained stable. After addition of 500x MIC of penicillin G (B) the viable counts decreased sharply in the parent strain while remaining stable in the Tol1 mutant. In contrast, optical densities and rDNA densitometries remained steady in both organisms. The experiments were performed in triplicate on 3 independent occasions. Inter-experimental variation was ≤ 20 %.

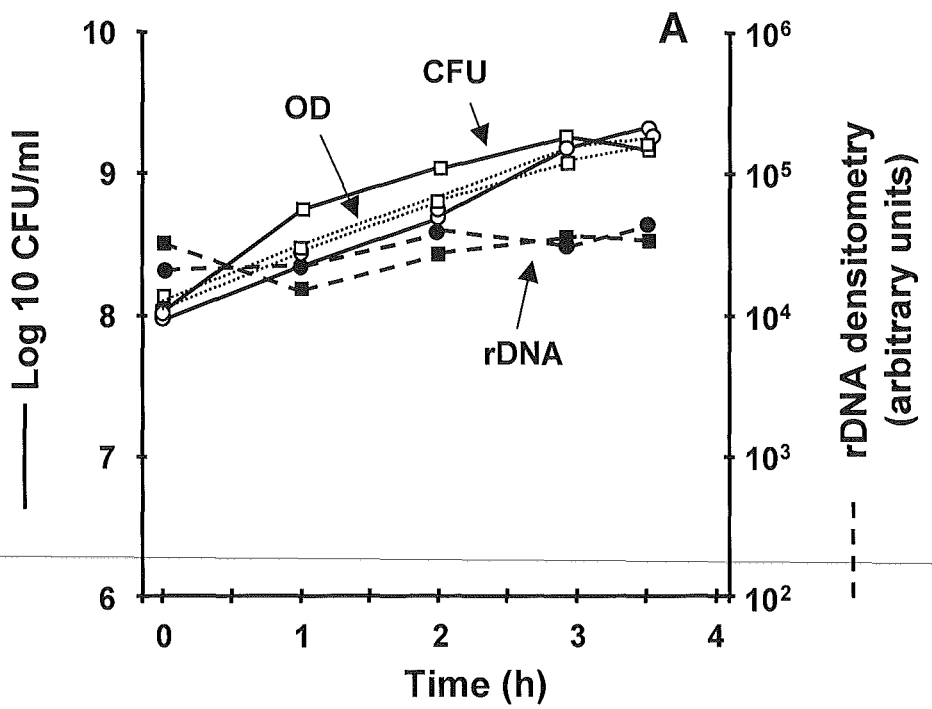
Figure 2. Bacterial growth (A) and penicillin-induced killing (B) followed by viable counts and quantitative-PCR 16S rDNA. (A) Both viable counts and numbers of rDNA copies increased in parallel and stabilized in the stationary phase (data in panel A are for the parent *S. gordonii*). (B) After penicillin addition viable counts decreased in the parent strain while remaining stable in the Tol1 mutant. Yet, quantitative-PCR of rDNA remained steady in both organisms. Details and experimental reproducibility are as in Figure 1.

Figure 3. Growth and penicillin-induced killing of the *S. gordonii* parent (A) and penicillin-induced killing of the Tol1 mutant (B) followed by viable counts and quantification of a 427-base fragment of 16S rRNA. In both cases the number of copies of rRNA was proportional to the viable counts. The insets at the bottom of the graphs depict crude 23S and 16S rRNA prepared from culture aliquots removed at various times during penicillin treatment and separated by agarose gel electrophoresis. In the kill-susceptible parent (A) the bands disappeared after 24 h of drug treatment. In the kill-resistant Tol1 mutant (panel B) the bands remained visible. Details and experimental reproducibility are as in Figure 1.

Figure 4. Bacterial growth and levofloxacin (levo)-induced killing followed by viable counts and quantification of either rDNA (A) or rRNA (B). (A) The number of copies of rDNA paralleled viable counts during bacterial growth, but remained stable during levofloxacin treatment in spite of extensive killing. (B) In contrast, the number of copies of rRNA paralleled viable counts both during growth and drug-induced killing. Details and experimental reproducibility are as in Figure 1.

Figure 5. Bacterial growth and penicillin-induced killing of the *S. gordonii* parent (A) and Tol1 mutant (B) followed by viable counts and quantification of a short (119-base) fragment of 16S rRNA. The number of amplified 119-base fragments increased proportionally to viable counts during bacterial growth, but did not decrease proportionally do bacterial killing during penicillin treatment. Details and experimental reproducibility are as in Figure 1.

Fig. 1


A
CFU
OD
rDNA
Log 10 CFU/ml
rDNA densitometry (arbitrary units)
Time (h)
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9
8
7
6
0
1
2
3
4
10^6
10^5
10^4
10^3
10^2

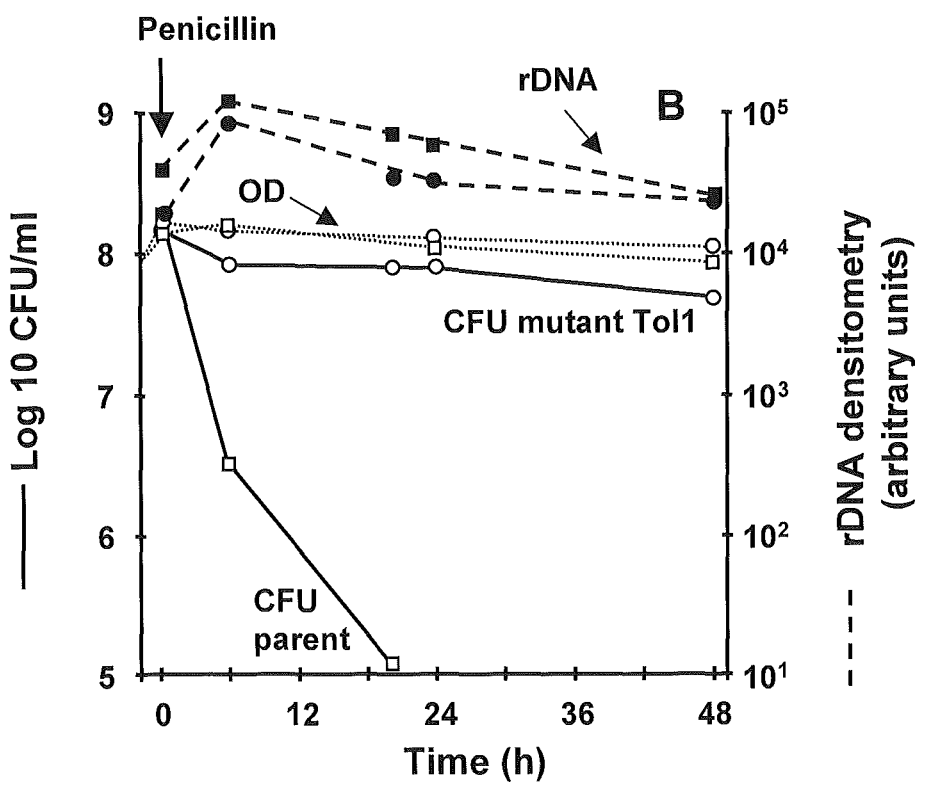
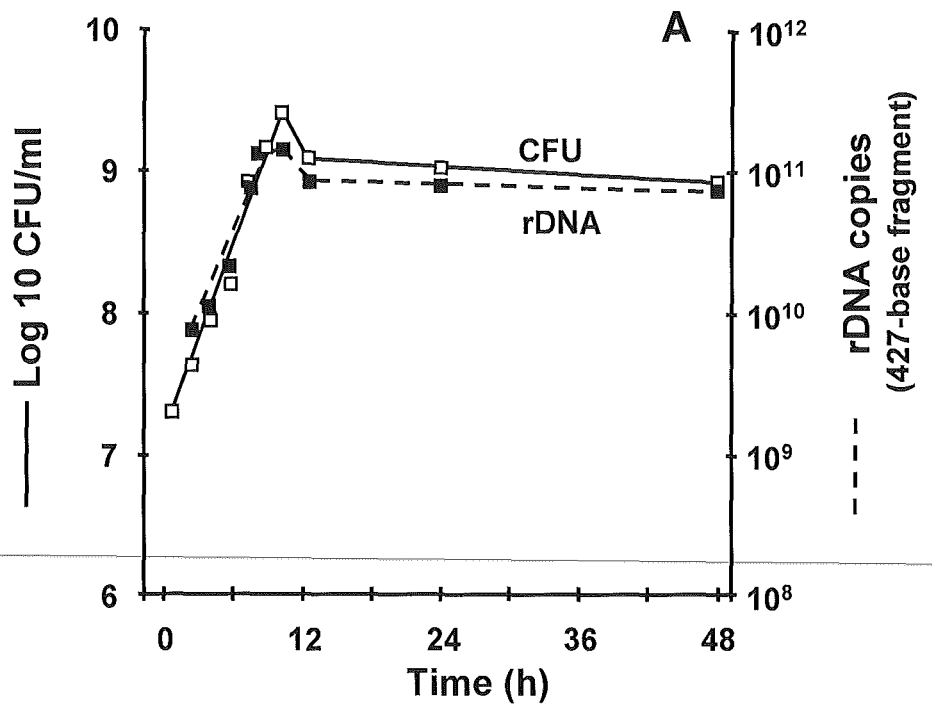

Penicillin
rDNA
B
OD
CFU mutant Tol1
CFU parent
Log 10 CFU/ml
rDNA densitometry (arbitrary units)
Time (h)
9
8
7
6
5
0
12
24
36
48
10^5
10^4
10^3
10^2
10^1

Fig. 2


A
Log 10 CFU/ml
rDNA copies
(427-base fragment)
CFU
rDNA
10
9
8
7
6
10^{12}
10^{11}
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10^{8}
0
12
24
36
48
Time (h)

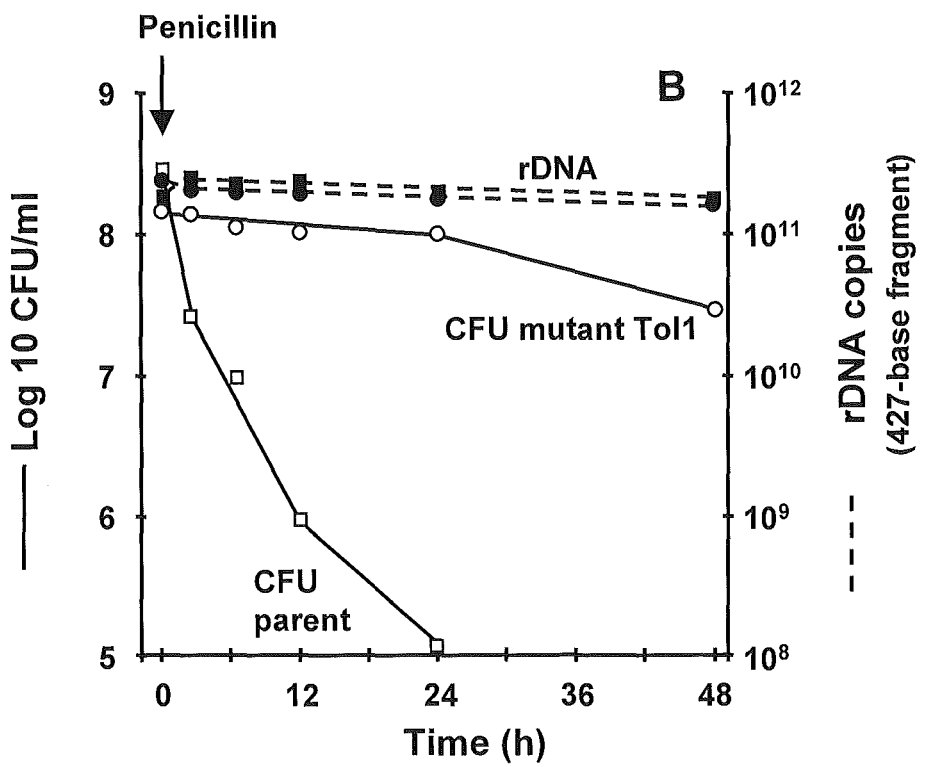
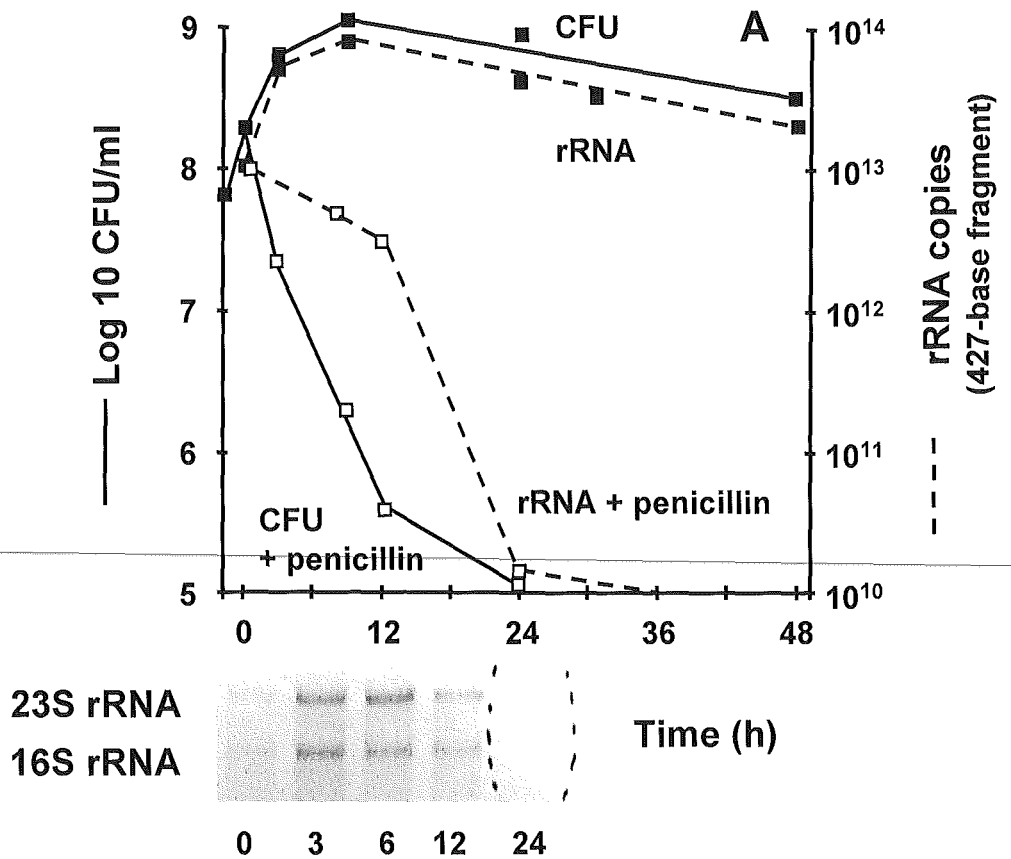

Penicillin
B
Log 10 CFU/ml
rDNA copies
(427-base fragment)
rDNA
CFU mutant Tol1
CFU
parent
9
8
7
6
5
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
0
12
24
36
48
Time (h)

Fig. 3


A
CFU
rRNA
rRNA + penicillin
CFU
+ penicillin
Log 10 CFU/ml
rRNA copies
(427-base fragment)
9
8
7
6
5
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}
0
12
24
36
48
23S rRNA
16S rRNA
Time (h)
0
3
6
12
24

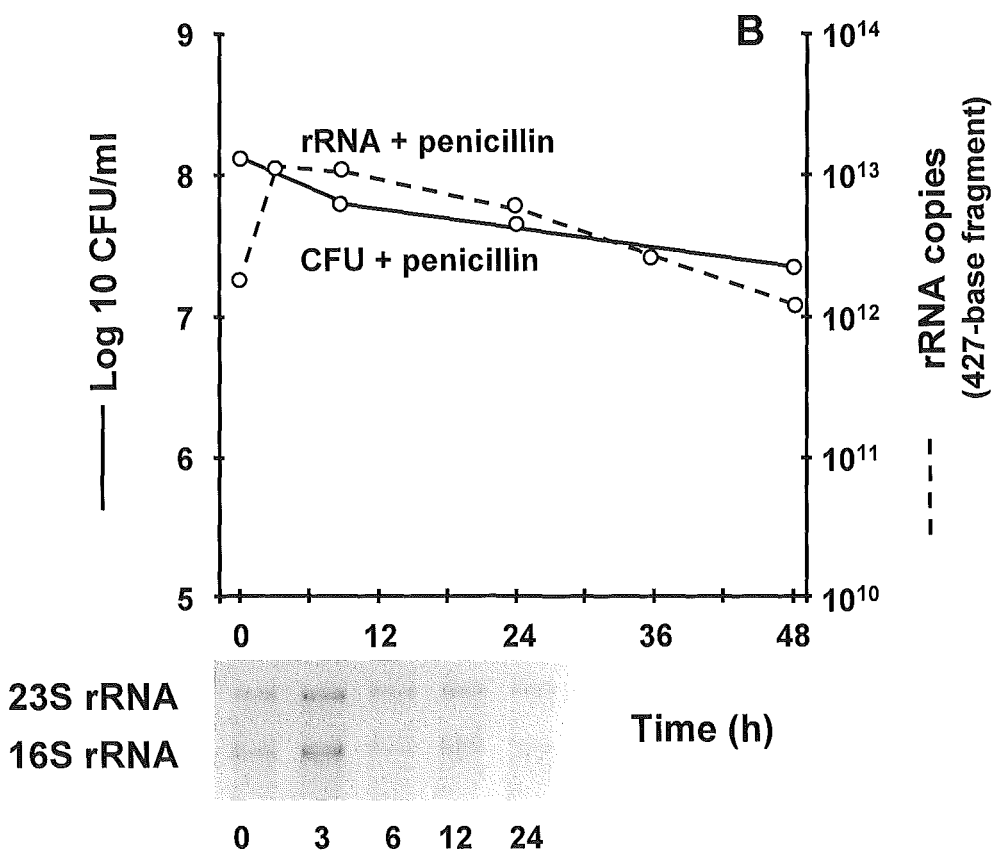
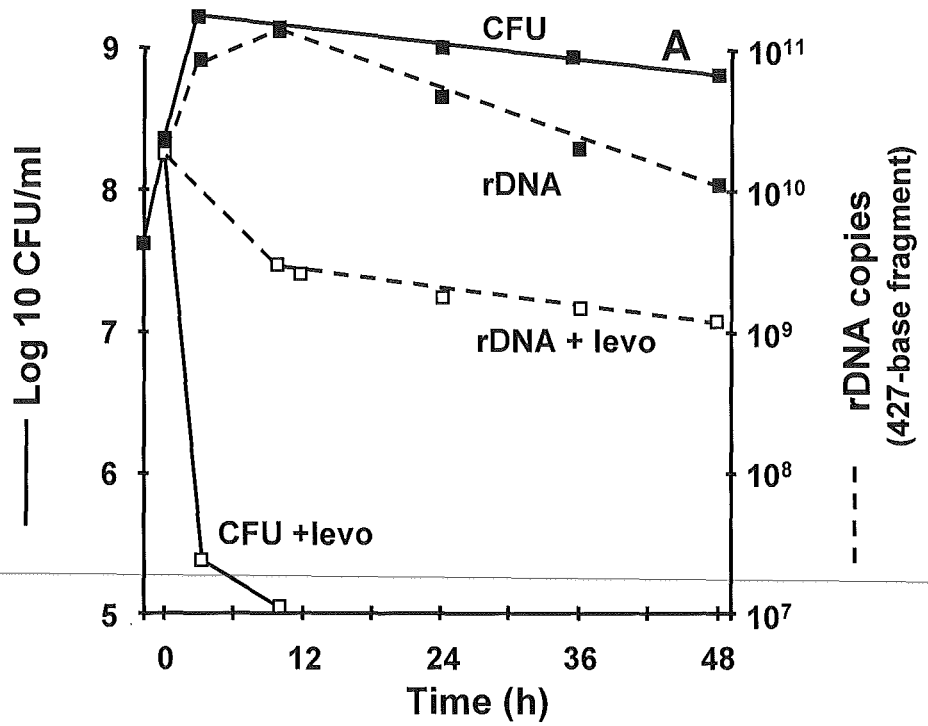

B
rRNA + penicillin
CFU + penicillin
Log 10 CFU/ml
rRNA copies
(427-base fragment)
9
8
7
6
5
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}
0
12
24
36
48
23S rRNA
16S rRNA
Time (h)
0
3
6
12
24

Fig. 4


CFU
A
rDNA
rDNA + levo
CFU +levo
Log 10 CFU/ml
rDNA copies
(427-base fragment)
Time (h)
9
8
7
6
5
0
12
24
36
48
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}

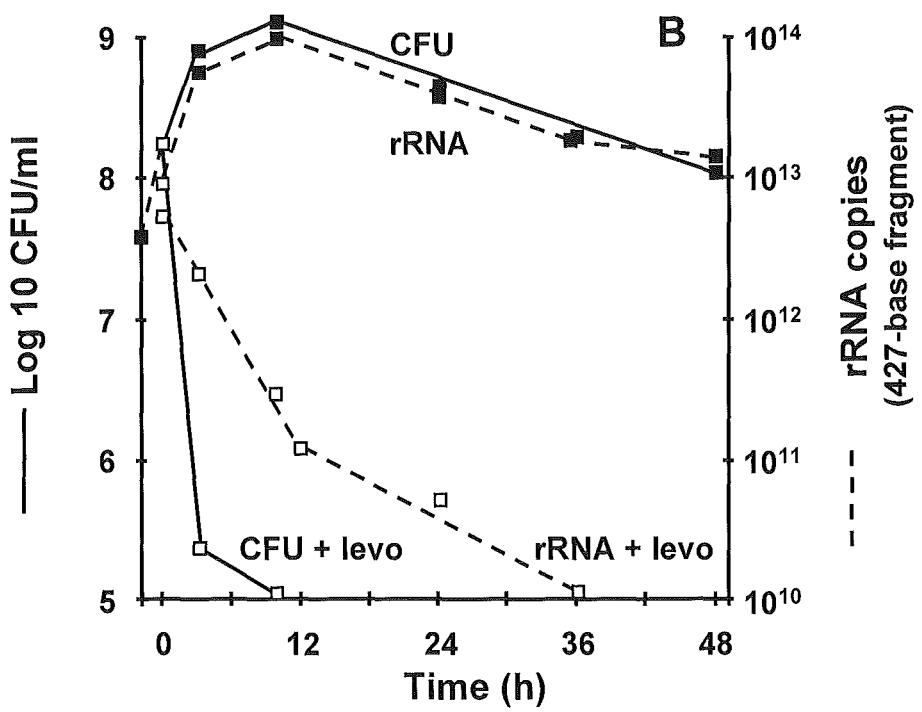
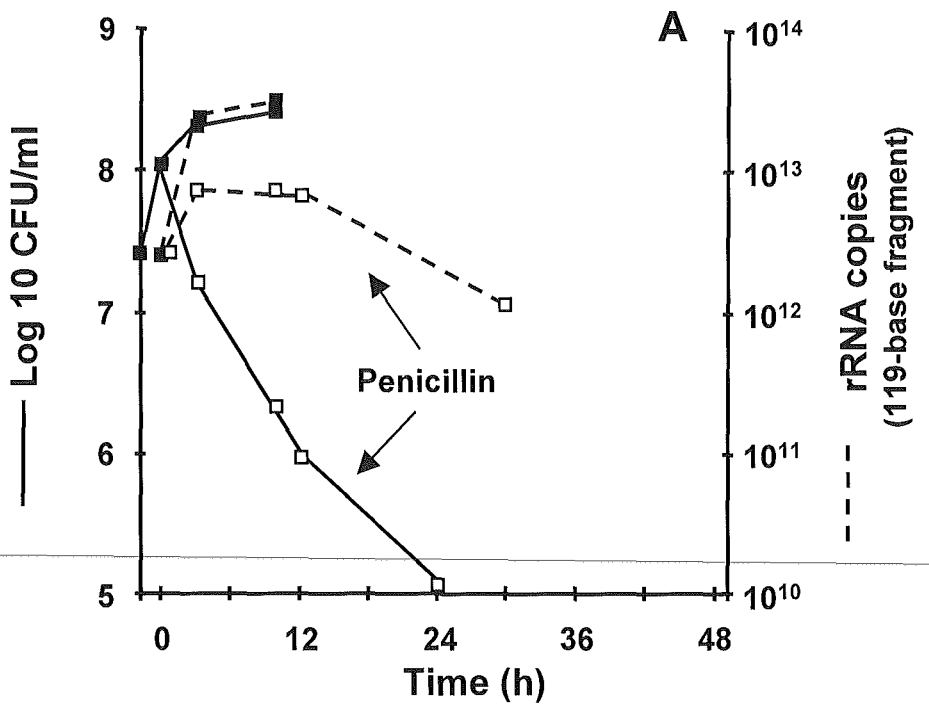
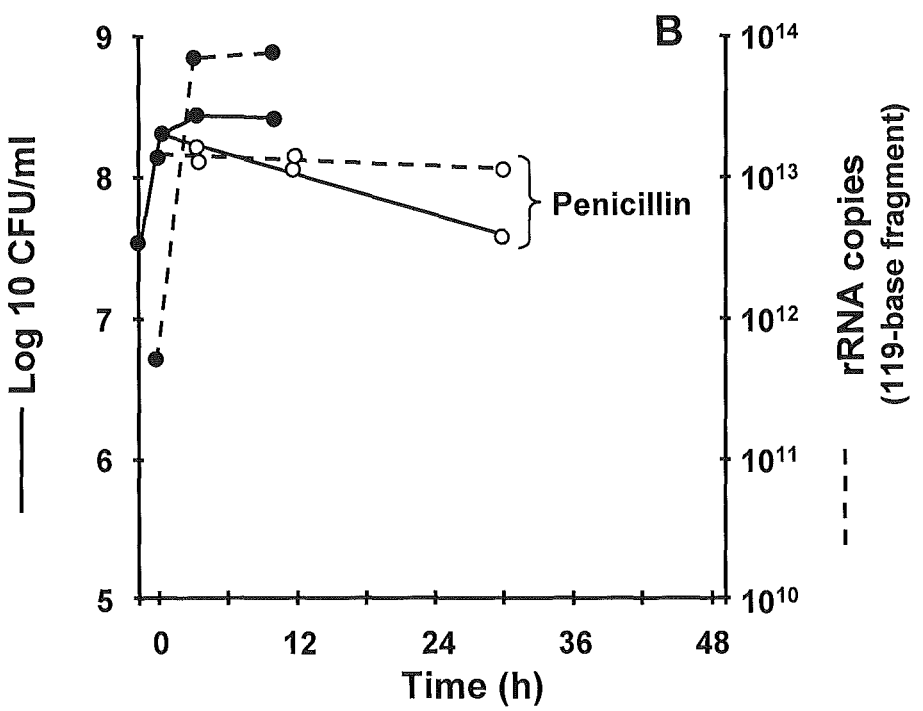

CFU
B
rRNA
CFU + levo
rRNA + levo
Log 10 CFU/ml
rRNA copies
(427-base fragment)
Time (h)
9
8
7
6
5
0
12
24
36
48
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}

Fig. 5


A
Log 10 CFU/ml
rRNA copies
(119-base fragment)
Penicillin
Time (h)
9
8
7
6
5
0
12
24
36
48
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}


B
Log 10 CFU/ml
rRNA copies
(119-base fragment)
Penicillin
Time (h)
9
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10^{14}
10^{13}
10^{12}
10^{11}
10^{10}