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Impairment of ribosomal subunit synthesis in aminoglycoside treated ribonuclease mutants of *Escherichia coli*

Ashley D. Frazier and **W. Scott Champney***
Department of Biochemistry and Molecular Biology, Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614 USA

Abstract

The bacterial ribosome is an important target for many antimicrobial agents. Aminoglycoside antibiotics bind to both 30S and 50S ribosomal subunits, inhibiting translation and subunit formation. During ribosomal subunit biogenesis, ribonucleases (RNases) play an important role in rRNA processing. *E. coli* cells deficient for specific processing RNases are predicted to have an increased sensitivity to neomycin and paromomycin. Four RNase mutant strains showed an increased growth sensitivity to both aminoglycoside antibiotics. *E. coli* strains deficient for the rRNA processing enzymes RNase III, RNase E, RNase G, or RNase PH showed significantly reduced subunit amounts after antibiotic treatment. A substantial increase in a 16S RNA precursor molecule was observed as well. Ribosomal RNA turnover was stimulated and an enhancement of 16S and 23S rRNA fragmentation was detected in *E. coli* cells deficient for these enzymes. This work indicates that bacterial RNases may be novel antimicrobial targets.

Keywords

Escherichia coli; ribonuclease mutants; ribosome assembly; neomycin; paromomycin

Introduction

Bacterial resistance to the most commonly used antimicrobial agents is increasing (Davies and Davies 2010; Rosen 2011). Well examined resistance mechanisms include the acquisition of resistance genes, up-regulation of genes encoding cellular efflux pumps and spontaneous mutations in target genes (Zinner 2007). Development of novel antibacterial agents and the identification of additional bacterial targets have become important research endeavors (Champney 2008).

One significant target of many antibiotics is the bacterial ribosome (Wallis and Schroeder 1997; Champney 2006). The prokaryotic ribosome consists of a large 50S and a small 30S subunit. The 50S subunit is composed of 23S and 5S rRNA and 34 ribosomal proteins. The 30S subunit contains 16S ribosomal RNA and 21 ribosomal proteins. Some antibiotics function by targeting both the 30S and 50S subunits. Neomycin and paromomycin are two aminoglycoside antibiotics that bind to helix 44 of 16S rRNA and stimulate mistranslation of mRNA (Schroeder et al. 2000; Sutcliffe 2005; Arya 2007; Długosz and Trylska 2009). Aminoglycosides also bind to helix 69 of 23S rRNA. Binding to helix 69 diminishes the ability of the ribosome recycling factor to recycle the ribosome (Hirokawa et al. 2005; Borovinskaya et al. 2007; Scheunemann et al. 2010). Both antibiotics have also been shown

*Corresponding author: Tel: 423-439-2022; Fax: 423-439-2030; Champney@etsu.edu.

to inhibit 30S and 50S subunit assembly in various bacteria, including *E. coli* and *Staphylococcus aureus* (Mehta and Champney 2002; Mehta and Champney 2003).

During subunit biogenesis, 16S, 23S, and 5S rRNA transcripts and ribosomal proteins combine to form intermediate precursors. The rRNA in the precursor particles is cleaved and processed by endo- and exoribonucleases to produce the mature subunits (Kaczanowska and Rydén-Aulin 2007; Deutscher 2009). The RNase enzymes involved in the processing of rRNA are indicated in Figure 1. In addition to maturation, ribonucleases are involved in the degradation of rRNA. When an inhibitor, such as an antibiotic, is present in a bacterial cell, specific RNases are utilized to degrade the rRNA to eliminate the stalled precursor (Champney 2006).

It has been previously shown that strains of *E. coli* deficient for RNase E, RNase II, or polynucleotide phosphorylase (PNPase) displayed an increased sensitivity to erythromycin and azithromycin (Usary and Champney 2001; Silvers and Champney 2005). These mutant *E. coli* strains were shown to accumulate 23S rRNA and demonstrated a reduced rate of subunit resynthesis after antibiotic removal.

Bacterial RNases have been recently described as potential new antimicrobial target (Eidem et al. 2012). The RNase inhibitor VRC has been shown to impair ribosomal subunit formation in both *S. aureus* and *E. coli* (Frazier and Champney 2012a; Frazier and Champney 2012b). The present work was conducted to see if elimination of rRNA processing RNases enhanced the sensitivity of cells to aminoglycoside antibiotics. This work shows that *E. coli* strains deficient for RNase III, RNase E, RNase G, or RNase PH have an increased sensitivity to neomycin and paromomycin, reflected in reduced subunit synthesis and enhanced rRNA turnover.

Materials and methods

Analysis of cellular growth and viability

Escherichia coli strains used are listed in Table 1. Cultures were grown at 37°C (or 32°C for *ts* mutants) in tryptic soy broth (TSB). Strains SK5665, SK5729, SK6639 and SK7622 were supplemented with 4 μg/mL thymidine during growth. Growth rates were measured as an increase in cellular density over time using a Klett-Summerson colorimeter. Neomycin or paromomycin were added to cultures at 10μg/mL based on their IC_{50} values (Mehta and Champney 2002). All cultures were grown for two cellular doublings to approximately 4×10^8 colony forming units (CFU)/mL. Cellular viability was determined by TSB agar colony counting after serial dilution (Jett et al. 1997).

Ribosomal subunit assembly assay

Cell cultures were grown in TSB and at a Klett reading of 20, neomycin or paromomycin were added to the cells. After 15 minutes of growth with the antibiotics, ^{3}H uridine (30 Ci/mmol, American Radiochemicals) at a concentration of 1μCi/mL and uridine at 2μg/mL were added. The cells were allowed to grow for two cellular doublings. At that time, uridine was added to 50μg/mL and the cells were incubated for an additional 15 minutes. Cells were collected by centrifugation and stored frozen at −70°C.

Cellular lysates were prepared with lysozyme and DNaseI as previously described (Silvers and Champney 2005). The samples were centrifuged through 5–20% sucrose gradients in S buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM Mg acetate, 50 mM NH_4Cl) in an SW41 rotor at 187813 × *g* for 3.5 hours. Following centrifugation, fractions were collected by pumping the gradient through an ISCO Model UA-5 absorbance monitor set at 254nm. The fractions

were collected into vials and mixed with 3mL Scintisafe gel before measuring the ^{3}H uridine radioactivity by liquid scintillation counting.

Agilent Bioanalyzer analysis of total RNA

Bacterial cells were grown as described above with neomycin or paromomycin at 10μg/mL. At a density of 4×10^{8} cells/mL, the cells were collected by centrifugation and RNA was prepared from the cell pellet. Total RNA from cell samples was isolated by phenol/chloroform extraction and ethanol precipitation (Rio et al. 2011). Typically 0.5 to 1μg of RNA was examined using an Agilent Bioanalyzer 2100 and the RNA 6000 nano chip. Integrated gel peak areas were determined with Agilent software.

Analysis of 16S rRNA and 23S rRNA fragmentation by Northern blot hybridization

Biotinylated 16S and 23S specific probes were constructed as previously described (Silvers and Champney 2005). The 16S (241 bp) and 23S (146 bp) DNA probes were amplified from plasmid pKK3535 DNA (Brosius et al. 1980) using the polymerase chain reaction. The 16S primers used were 16S F: GGA GGA AGG TGG GGA TGA CG and 16S R: ATG GTG ACG GGC GGT GTG (nt. nos. 1173–1414). The 23S primers used were 23S F: TAG GGG AGC GTT CTG TAA G and 23S R: CCC ATT AAC GTT GGA C (nt. nos. 1188–1334). The primers were from Life Technologies. PCR products were purified by extraction with equal amounts of phenol and chloroform before precipitating with 2 volumes of ethanol. The pellets were resuspended in 30μL of sterile water. The purified DNA probes were labeled with biotin using the Label-IT biotin labeling kit (Mirus) per the manufacturer's instructions (Silvers and Champney 2005).

Six micrograms of total RNA was denatured by heating at 55°C for 10 minutes and separated on a 5% TAE PAGE gel as previously described (Rio et al 2011). A biotinylated RNA standard was run in each gel to provide an estimate of RNA fragment sizes. The intact 16S and 23S rRNAs were evident by ethidium bromide staining of the gel. This region of the gel was removed prior to Northern hybridization to permit a more sensitive detection of the rRNA fragments. After destaining overnight in sterile water, the RNA was transferred onto Nytran nylon membranes using a Turbo blot apparatus (Schleicher & Schuell). The membranes were pre-hybridized in 15mL of 1X pre-hybridization solution (MRC, Inc.) at 42°C for 30 minutes. The membranes were then hybridized overnight at 42°C with 10mL hybridization buffer (50% formamide, 5X SSC, 0.1% sarkosyl, 0.02% SDS and 200μg/mL BSA), 1X background quencher (MRC, Inc.), and 4pmol of the denatured 16S or 23S specific probe (Silvers and Champney 2005). After hybridization, the membranes were washed and the probe detected with horseradish peroxidase using the North2South chemiluminescent hybridization kit (Pierce Chemical Co.). Analysis of the rRNA fragmentation was conducted by imaging membranes in the G Box Imager (SynGene).

Results

RNases play a key role in the processing, maturation and turnover of rRNA (Deutscher 2009; Davies et al. 2010). Figure 1 shows the important rRNA processing enzymes in *E. coli.* This figure also indicates that the aminoglycoside antibiotics neomycin and paromomycin bind to both 16S and 23S rRNAs (Sutcliffe 2005; Foster and Champney 2008; Scheunemann et al. 2010). It was hypothesized that *E. coli* cells lacking essential processing RNases would show an increased sensitivity to these compounds. Cell viability assays were performed to determine whether *E. coli* mutants deficient for any of eight RNases showed an enhanced sensitivity to these antibiotics. As Table 2 shows, *E. coli* mutants deficient for the rRNA processing RNases all showed enhanced growth sensitivity to neomycin compared with wild type cells. Most of the mutants were also reduced in their relative sensitivity to

paromomycin as well. Strains deficient for the degradative RNases I, II and PNPase showed results similar to that of the wild type *E. coli* cells and were not examined further. Strains with mutations affecting the rRNA processing enzymes RNase III, E, G and PH were particularly sensitive to both aminoglycosides.

Aminoglycoside antibiotics can bind to both 30S and 50S ribosomal subunits (Sutcliffe 2005; Borovinskaya et al. 2007; Foster and Champney 2008; Długosz and Trylska 2009; Scheunemann et al. 2010). We therefore tested to see if growth with the aminoglycosides impaired the assembly of either subunit compared with the untreated cells. A reduction in the net synthesis of both the 30S and 50S ribosomal subunits was observed for the wild type and each mutant strain examined. Figure 2 shows the sucrose gradient profiles for six of the strains. For untreated control cells the ratio of ^{3}H-rRNA in the 50S and 30S subunits was 2:1 as expected. Both aminoglycosides promoted a reduction in 30S and 50S subunit amounts in all of the RNase mutant strains. 50S subunit amounts were reduced to between 10 and 60% of the untreated control cells while 30S subunit amounts were reduced to between 35 and 75% of the control levels (Fig. 2). The aminoglycosides were most effective against an *E. coli* strain lacking RNase III and one lacking the four RNases I, II, PNPase and PH (Fig. 2b and 2f). Growth with the aminoglycosides also stimulated an increase in radiolabeled RNA in the top gradient fractions. After treatment with the antibiotics, there was a 30 to 200% increase in the RNA in the top gradient fractions (Fig. 2). This increase is indicative of rRNA degradation (Silvers and Champney 2005). In each case, the labeled RNA lost from the subunit regions was accounted for by an equivalent amount present in the top gradient fractions.

The loss of rRNA processing RNases is predicted to lead to the accumulation of 16S and 23S rRNA precursor species. Total RNA was isolated from each strain after growth without and with the aminoglycosides and the status of 16S precursor rRNA in the cells was examined by Agilent chip analysis (Fig. 3). The precursor 16S rRNA (17S) is 148 nucleotides larger than the mature 16S (1542 nucleotides) and is easily distinguished in the Agilent gel. The precursor to 23S rRNA is only 10 to 16 bases larger than the mature molecule and cannot be distinguished by this procedure (Kaczanowska and Rydén-Aulin 2007). Most of the mutant strains showed an enhanced accumulation of 16S precursor after growth with neomycin or paromomycin (Table 3). The largest increase in 16S precursor rRNA was seen in *E. coli* mutants deficient for RNase E, PH or R where more than 20% of the total 16S RNA was present in the precursor form (Table 3). The Agilent gels also indicated enhanced rRNA degradation in some mutants after aminoglycoside treatment (Fig. 3, small RNA).

Ribosomal RNA degradation and turnover in the mutant strains was examined further. Fragmentation of 16S and 23S rRNA stimulated by antibiotic treatment was examined by Northern hybridization analysis. Figure 4 shows the result of Northern blots hybridized with a 16S rRNA probe, and Figure 5 shows the same *E. coli* RNA samples examined for rRNA fragmentation with a 23S rRNA probe. Enhanced 16S rRNA fragmentation was observed in each of the antibiotic treated samples in compared to the untreated control. *E. coli* deficient for RNase III, R+PH, G and I/II/PNPase/PH displayed the greatest increase in 16S rRNA fragmentation when compared with the mutant strain controls grown without neomycin or paromomycin (Fig. 4). Fragmentation of 23S rRNA was also detected by Northern hybridization analysis for the wild type and RNase mutant strains (Fig. 5). The largest increases in 23S rRNA were observed in strains deficient for RNase III, I/PH/R, and I/II/PNPase/PH after antibiotic treatment These results enhance the observations made by the sucrose gradient analysis indicating rRNA degradation after antibiotic treatment.

Discussion

Ribonucleases function in both rRNA processing and degradation (Deutscher 2009). RNases III, E, G, and PH are involved in the processing of precursor rRNA during subunit synthesis (Davies et al. 2010); Fig. 1). Elimination of specific RNases by mutation was predicted to enhance the effectiveness of antibiotics which stall ribosomal subunit formation. *E. coli* cells with a mutation in the gene for RNase E showed an enhanced sensitivity to erythromycin and the accumulation of a precursor to the 50S subunit (Usary and Champney 2001). *E. coli* cells deficient for RNase II or PNPase were increased in their sensitivity to azithromycin and showed an impairment of 23S rRNA function (Silvers and Champney 2005). Turnover of the rRNA was stimulated and 50S ribosomal subunit formation was impaired by azithromycin treatment. In addition, recovery of subunit formation after antibiotic removal was delayed. Based on these findings, it was hypothesized that *E. coli* cells deficient for specific processing RNases would display an increased sensitivity to aminoglycosides.

The wild type strain with a full complement of processing RNases showed a significant decrease in cell viability and a decrease in the formation of both subunits after antibiotic treatment (Table 2 and Fig. 2). A small amount of 16S precursor rRNA was found and enhanced rRNA fragmentation was apparent in the hybridization analysis.

RNase III is responsible for the initial cleavage of the precursor rRNA (Deutscher 2009). This study found that the addition of neomycin or paromomycin to *E. coli* cells deficient for RNase III led to an approximate 90% reduction in cell viability (Table 2). Examination of ribosomal subunit assembly showed that when either antibiotic was added, the amounts of 30S subunits were reduced by more than 60% and the 50S subunit level was reduced by approximately 85% (Fig. 2). RNA analysis by Agilent chip showed that there was a large increase in the 16S precursor, an indication of subunit precursor accumulation (Table 3; (Silvers and Champney 2005). Finally, analysis of 16S and 23S rRNA by Northern blotting hybridization revealed an increase in the 16S rRNA fragmentation and a change in the fragmentation pattern of 23S rRNA (Fig. 4 and Fig. 5). These results are consistent with the important role of RNase III in generating the rRNA precursors from the primary transcript.

RNases G and E function in processing of the 5′ end of 16S rRNA (Li et al. 1999). RNase G is also involved in processing of 23S rRNA (Song et al. 2011; Gutgsell and Jain 2012). We found that the absence of either RNase led to a decrease in cell viability when neomycin or paromomycin were present. The loss of RNase E appeared to have a greater on cell viability reduction than the absence of RNase G (Table 2). In both *E. coli* strains, there was a decrease in the 30S and 50S ribosomal subunit amounts with neomycin appearing to have a stronger effect in assembly reduction than paromomycin (Fig. 2). Agilent chip analysis revealed a larger increase in 16S precursor for RNase E deficient cells but no apparent 16S precursor for RNase G deficient cells (Table 3). Further analysis of 16S and 23S rRNA by Northern blotting hybridization demonstrated that both RNase E and G deficient cells showed enhanced fragmentation of 16S and 23S rRNAs (Fig. 4 and Fig. 5).

RNase PH is involved in 23S rRNA processing through cleavage of the 3′ end. Without this cleavage additional RNases, such as RNase T, cannot complete the maturation process (Gutgsell and Jain 2012). A double mutant strain lacking the processing RNase PH and the degradative enzyme RNase R showed impairment in the formation of both subunits by both sucrose gradient analysis and by the accumulation of precursor 16S rRNA. Also very large increases in the fragmentation of both RNA species were seen. The loss of RNase PH in a quadruple deficient mutant lacking the degradative enzymes RNase II and PNPase led to a very severe reduction in cell viability when neomycin or paromomycin were present. This strain also showed an approximate 80% reduction in the assembly of both the 30S and 50S

ribosomal subunit when the aminoglycosides were used. Lastly, analysis of bacterial RNA by Agilent chip revealed the presence of the 16S precursor with and without antibiotic treatment (Table 3). Significant amounts of 16S and 23S rRNA oligonucleotides were also found under both circumstances (Fig. 4 and Fig. 5). In both of these mutant strains, impairment of rRNA processing and degradation significantly enhanced the antibiotic effectiveness. Ribonuclease I, also missing in both strains, is a periplasmic enzyme and is not normally involved in ribosomal RNA processing (Gesteland 1966).

The clearest results in this study were observed with strains containing single mutations in RNases III, E, and G. The results indicating major roles for the processing enzymes RNase III, E and G in antibiotic sensitivity are apparent. The interpretation of the results with the strain defective for RNases PH, and R and the strain missing four enzymes is less certain. Both strains showed major effects resulting from antibiotic treatment but the combination of nuclease mutations precludes a clear understanding of the role of the missing individual proteins. It is well established that in ribonuclease defective mutants, other ribonucleases can complement the defect in numerous ways. Studies have shown that due to the homology between RNases E and G, the loss of RNase E in *E. coli* cells can be complemented by an overexpression of RNase G (Lee et al. 2002). Additionally, when RNase E is absent in *E. coli* cells, there is an increase in the degradative enzyme, RNase R, indicating that the loss of one RNase involved in RNA degradation can lead to the up-regulation of another RNase involved in rRNA turnover in the bacterial cell (Cairrão and Arraiano 2006).

The enzymes identified in this study, which increase sensitivity to aminoglycosides, are different from the RNases shown to affect erythromycin and azithromycin sensitivity. The difference in enzymes in the two cases may result, in part, from the differences in the rRNA transcription and subunit assembly sequence. Aminoglycosides affect both 30S and 50S ribosomal subunit synthesis while macrolides impair only affect 50S ribosomal subunit assembly (Champney and Tober 2000; Usary and Champney 2001; Mehta and Champney 2002). During transcription, 16S rRNA is transcribed first and initiates 30S precursor assembly prior to 23S and 5S rRNA synthesis for 50S precursor formation. Aminoglycoside antibiotic stalling of 30S subunit formation leads to the accumulation of a 21S precursor, whose rRNA will not be further processed in specific RNase deficient mutants. Macrolide antibiotic stalling of 50S assembly generates a 32S precursor to the 50S particle which can be removed from the cell by the rRNA degradative enzymes like RNase II and PNPase as observed (Silvers and Champney 2005).

The present research has identified rRNA processing RNases as important in the effectiveness of aminoglycoside antibiotic inhibitory activity. Inhibition of processing RNases by small molecule inhibitors (Frazier and Champney 2012a; Frazier and Champney 2012b) or RNA interference methods could represent an attractive new antibiotic target which can enhance the effectiveness of other antimicrobial agents.

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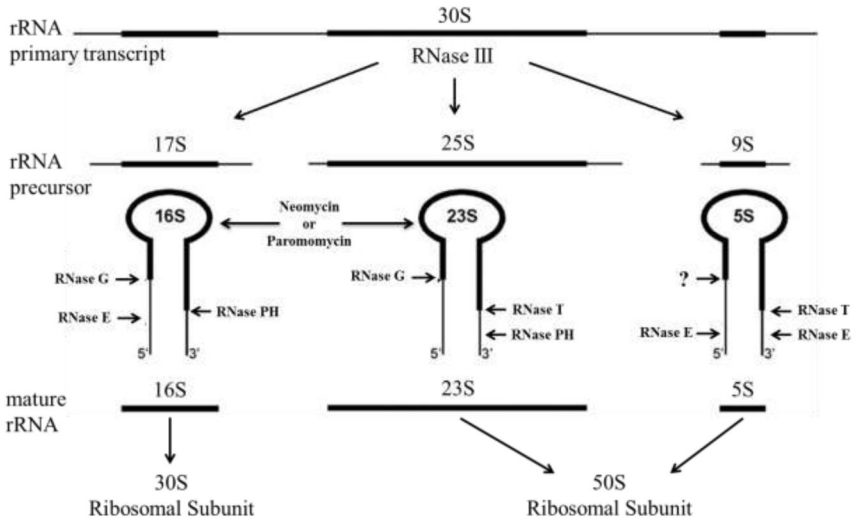

Fig. 1.

Bacterial rRNA processing pathways. The major rRNA processing enzymes are indicated. The binding of both aminoglycosides to 16S and 23S rRNAs is also shown (modified from (Davies et al. 2010). John Wiley and Sons used with permission.

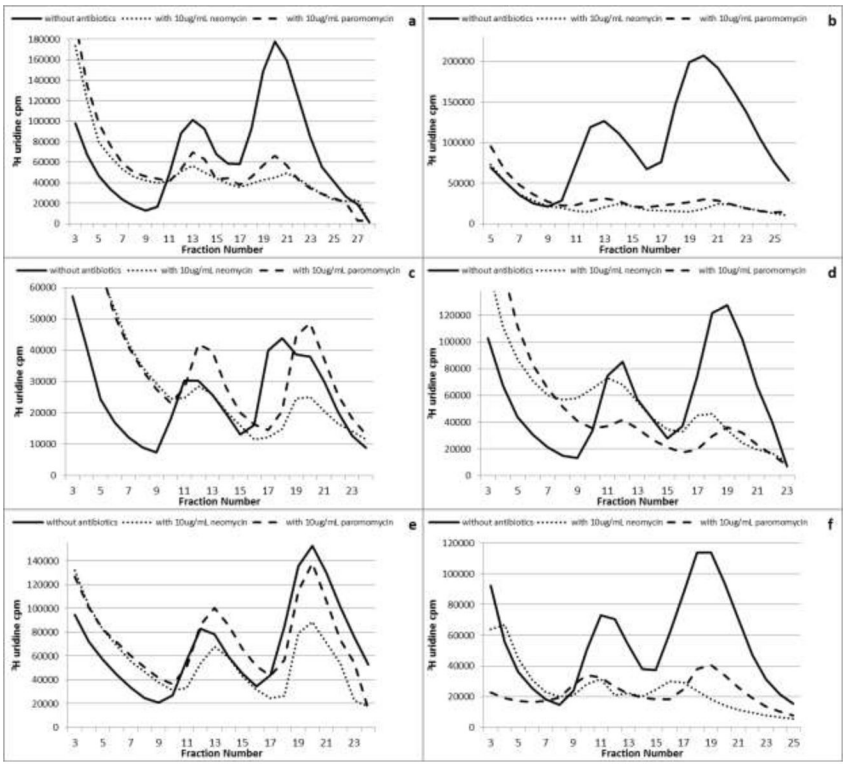

Fig. 2.

Sucrose gradient profiles of ^{3}H uridine labeled ribosomal subunits isolated from *E. coli* cells grown with or without 10μg/mL antibiotics. Gradient profiles for wild type (a), RNase III deficient (b), RNase E deficient (c), RNase I/PH/R deficient (d), RNase G deficient (e), and RNase I/II/PNPase/PH deficient (f). Results are the means of 2 gradient profiles.

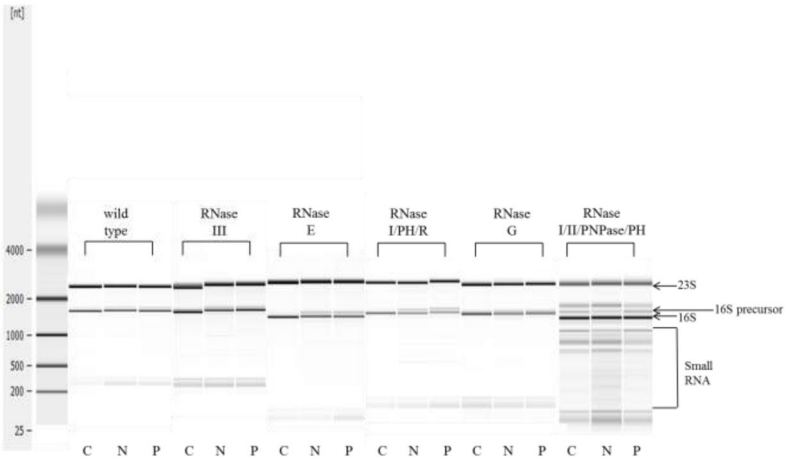

Fig. 3.

Agilent gel analysis of total RNA from wild type and mutant cells grown with and without antibiotics. Control (C), neomycin (N), paromomycin (P)

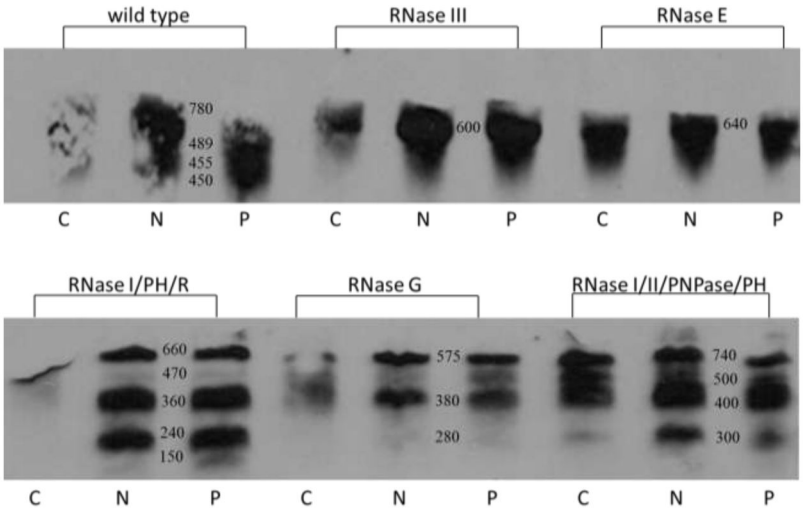

Fig. 4.

Northern blot analysis of 16S rRNA fragmentation for wild type and RNase mutant *E. coli* cells. RNA was isolated from cells grown without and with antibiotics. RNA sequences were identified by hybridization with a 16S DNA probe. Control (C), neomycin (N), paromomycin (P)

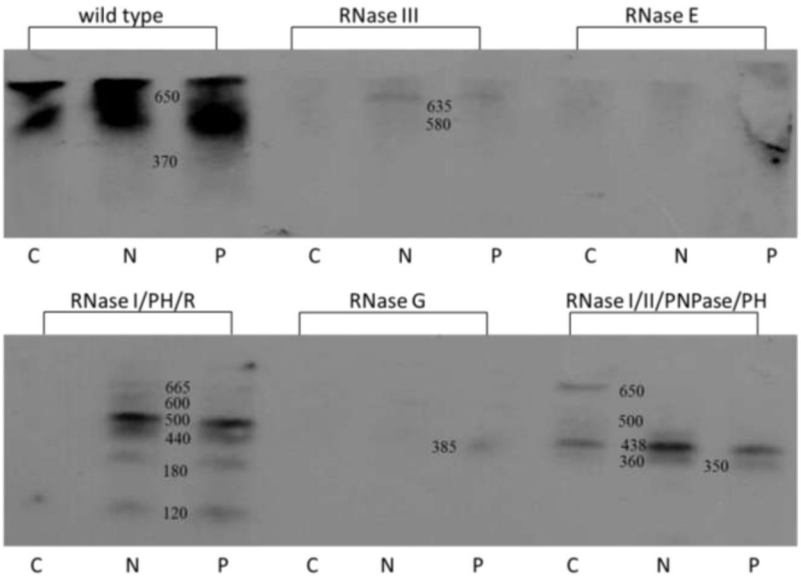

Fig. 5.

Northern blot analysis of 23S rRNA fragmentation for wild type and RNase mutant *E. coli* cells. RNA was isolated from cells grown without and with antibiotics. RNA sequences were identified by hybridization with a 23S DNA probe. Control (C), neomycin (N), paromomycin (P)

Table 1

E. coli strains used in this study

Strain	Phenotype	Genotype	Reference or source
SK901	None	F- malA thi-	(Kushner et al. 1977)
D10-1	I	HfrH met- rna-1 relA	(Gesteland 1966)
SK7622	III	F-thyA715 rncD38::kanR	(Babitzke et al. 1993)
SK5665	E	F-thyA715 rne1	(Arraiano et al. 1988)
SK4803	II	F-gal thi ton sup hasdR4 endAsbcB15 rnb296	(Donovan and Kushner 1986)
N7060	I/II/PNPase	F- metB1 tryA451 rpsl478 rna919rnb464 pnp13	(Weatherford et al. 1972)
MG1655 I^- R^-	I/PH/R	Δ rna Δrnr::cam rph-1	(Chen and Deutscher 2010)
GW11	G	F- zce-726::Tn10, TetR, rng::cat CmR	(Li et al. 1999)
SK6639	PH/PNPase	F-thyA715, CmR, pnp-200, rph-1, λ-	(Cheng and Deutscher 2003)
SK5729	I/II/PNPase/PH	F- thyA715, rna-19, rnb-500, pnp-7, λ-, rph-1	Sidney Kushner

Table 2

Effect of aminoglycosides on viability of wild type and mutant *E. coli* cells

		CFU ($\times 10^7$/mL)		
Strain	**RNase mutation**	**Control**	**Neomycin**	**Paromomycin**
SK901	None	133±28.8	35±12 (26.3)	4±0.7 (3.0)
SK7622	III	157±41.5	9±6.7 (5.7)	4±1.9 (2.5)
SK5665	E	112±8.5	4±0.5 (3.6)	1±0.01 (0.9)
MG1655 I^-R^-	I/PH/R	762±19.7	88±56.0 (11.5)	32±3.3 (4.2)
GW11	G	505±77.7	117±44.2 (23.2)	12±4.4 (2.4)
SK5729	I/II/PNPase/PH	100±28.4	3±1.9 (3.0)	2±0.6 (2.0)

Results are the means ± standard error (n=2). The percentage of the control cell number for each strain is listed in parenthesis.

Table 3

Distribution of precursor 16S rRNA by Agilent gel analysis

		% 16S rRNA Precursor		
Strain	**RNase mutation**	**Control**	**Neomycin**	**Paromomycin**
SK901	None	0.0	0.3	1.0
SK7622	III	0.3	8.9	3.6
SK5665	E	0.0	7.4	12.0
MG1655 I^-R^-	I/PH/R	0.8	21.8	33.1
GW11	G	0.0	0.0	0.0
SK5729	I/II/PNPase/PH	32.7	33.9	28.4

The percent of 16S precursor RNA relative to total 16S RNA was computed by analysis of the gel lanes in Fig. 3 using Agilent software. Results are the means of 2 gel analyses.