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An indigenous posttranscriptional modification in the ribosomal peptidyl transferase center confers resistance to an array of protein synthesis inhibitors

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Abstract

A number of nucleotide residues in ribosomal RNA undergo specific posttranscriptional modification. The roles of most modifications are unclear, but their clustering in the functionally-important regions of rRNA suggest that they might either directly affect the activity or assembly of the ribosome or modulate its interactions with ligands. Of the 25 modified nucleotides in *E. coli* 23S rRNA, 14 are located in the peptidyl transferase center, the main antibiotic target in the large ribosomal subunit. Since nucleotide modifications have been closely associated with both antibiotic sensitivity and antibiotic resistance, the loss of some of these posttranscriptional modifications may affect the susceptibility of bacteria to antibiotics. We investigated the antibiotic sensitivity of *E. coli* cells in which the genes of eight rRNA modifying enzymes targeting the PTC were individually inactivated. The lack of pseudouridine at position 2504 of 23S rRNA was found to significantly increase the susceptibility of bacteria to peptidyl transferase inhibitors. Therefore, this indigenous posttranscriptional modification may have evolved as an intrinsic resistance mechanism protecting bacteria against natural antibiotics.

Posttranscriptional modifications in rRNA are ubiquitous. However, the functional significance of most of them remains obscure. Acquisition and maintenance through the course of evolution of a number of genes dedicated to rRNA modification in spite of the associated energetic and metabolic cost argue that the modified nucleotides in rRNA render competitive benefits for the species. Clustering of the modified nucleotides in the functional centers of the ribosome hint that some of them may help to fine-tune ribosome functions. Indeed, inactivation of certain modification enzymes was shown to either decrease the efficiency of protein synthesis or negatively affect ribosome assembly[1 - 5]. Yet, in most cases, the lack of individual posttranscriptional modifications only marginally affects cell growth [6; 7].

The ribosome is one of the evolutionarily-preferred antibiotic targets. A large variety of natural antibiotics of microbial origin bind to the ribosomes of sensitive organisms and inhibit protein synthesis thereby providing competitive advantage to the antibiotic producers. One of the common ways in which antibiotic producers avoid suicide is by expressing

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specific methyltransferase enzymes which modify rRNA residues in the drug target site, and thus prevent antibiotic binding to the ribosome [8; 9]. Acquisition of such genes by pathogens is one of the major causes of clinical resistance to a number of antibacterial drugs. However, not only acquisition of new rRNA modifications, but the lack of natural modifications may affect susceptibility of cells to antibiotics. In a few well-documented cases, the lack of natural modifications was shown to render cells resistant to specific antibiotics [10 - 12].

Most of the antibiotics that inhibit functions of the large ribosomal subunit act upon the peptidyl transferase center (PTC), the main catalytic site in the ribosome. The PTC is formed primarily by segments of domain V of 23S rRNA which are targeted by many rRNA modifying enzymes. Out of the 25 posttranscriptional modifications mapped in *E. coli* 23S rRNA, 14 modified nucleotides are located in domain V (Figure 1) [13; 14]. Genes responsible for eight of these modifications are known. In this study, we investigated, which of these PTC modifications affect susceptibility of the bacterial cell to PTC-targeting antibiotics.

Inactivation of rluC dramatically increases susceptibility of the cell to PTC-targeting antibiotics

E. coli strains with individually inactivated genes of eight rRNA modifying enzymes that target PTC (RluB, RluC, RluE, RluF, RlmB, RlmE, RlmL and RlmN) were acquired from the Keio collection [15]. Except for the *rlmE* gene, inactivation of the genes encoding the modifying enzymes did not notably affect growth of *E. coli* in LB medium; *rlmE* inactivation resulted in a moderate (two-fold) reduction in cell growth rate (data not shown). Comparison of susceptibility of the strains to seven PTC inhibitors showed that the lack of most ofposttranscriptional modifications had small (one dilution or less) effect on the minimal inhibitory concentrations (MIC) of some of the drugs (Table 1). The notable exception was the strain in which RluC was inactivated. Cells lacking this enzyme showed increased susceptibility to *all* the tested PTC inhibitors. Furthermore, in the *rluC*$^{-}$ strain, susceptibility to some antibiotics increased dramatically - up to 16-fold. This effect was clearly specific to the PTC-targeting antibiotics because *rluC* inactivation had no effect on the MIC of streptomycin, an antibiotic that targets the small ribosomal subunit (Table 1).

The modification status of U2504 in the PTC affects sensitivity to antibiotics

In contrast to most bacterial rRNA modifying enzymes which target one specific nucleotide, RluC converts three uridine residues in 23S rRNA (at positions 955, 2504 and 2580) to pseudouridines [16; 17]. In order to identify the specific residue which was responsible for the observed hypersusceptibility effects, two of the U's targeted by RluC, U955 and U2580, were mutated to Cs. These residues are involved in formation of Ψ-G base pairs and their mutation to C does not disrupt the general base pairing scheme in the corresponding rRNA regions. The mutations were introduced in the pLK35 plasmid [18] and the resulting plasmid, pUCUC (Amp^{r}), was introduced in the strain SQ171. In this strain, all the chromosomal *rrn* alleles are inactivated and wild type rRNA is expressed from the *rrn* genes present on the pHK-sacB plasmid (Kan^{r}) [19; 20] (Quan and Squires, personal communication). After plasmid

exchange, the selected Amp^r/Kan^s strain carrying the pUCUC plasmid expressed only mutant ribosomes with the U-to-C transversions at positions 955 and 2580 in 23S rRNA, as was verified by primer extension (not shown). SQ171 cells expressing mutant 23S rRNA from the pUCUC plasmid grew with a doubling time similar to that of cells carrying the wild type pLK35 plasmid. Furthermore, the mutations U955C and U2580C on their own had only a small effect on antibiotic sensitivity (Table 1).

The *rluC* gene in the SQ171 cells expressing mutant ribosomes was then inactivated by transducing the *rluC::kan* allele from the corresponding strain in the Keio collection [21]. In this strain, inactivation of *rluC* affected the conversion of only a single uridine residue (U2504) to pseudouridine as was verified by primer extension (Figure 2). The lack of this single-nucleotide modification significantly increased susceptibility of the cells to PTC-targeting inhibitors. The most pronounced effects in comparison with the *rluC*+ control were observed with clindamycin (16-fold), linezolid (8-fold) and tiamulin (4-fold) (Table 2).

Antibiotic resistance could drive acquisition and maintenance of some rRNA modification enzymes

Our results reveal that one specific posttranscriptional modification in the PTC of the *E. coli* ribosome, conversion of U2504 to Ψ, notably increases cell resistance to several antibiotics. This 23S rRNA residue is exposed in the PTC active site and it is a component of the binding site of several PTC-targeting inhibitors [22 - 26].

The importance of the U2504 modification for ribosome function is uncertain. Inactivation of *rluC* produces no discernible change in the bacterial growth at several tested laboratory conditions [16; 17] (and our data). Furthermore, even though *rluC* is found in genomes of various α–proteobacteria, it is not universally conserved and many bacterial species (as well as most archaea and eukaryotes) lack the gene arguing that the ribosome can efficiently carry protein synthesis without pseudouridilation of U2504. Thus no data currently exist that would link RluC, and more specifically, Ψ2504, to any particular ribosomal function.

Many natural antibiotics that bacteria might encounter in the environment target the PTC. Resistance to these antibiotics, especially if it comes without loss of fitness, should notably benefit the cell. Therefore, it is conceivable that certain rRNA modification mechanisms could be gained and retained in the course of evolution as a way to protect the ribosome from the action of these inhibitors. From our data, it appears that among other indigenous rRNA modifying enzymes acting upon the nucleotides in the PTC, RluC is the best candidate for this role.

RluC could have originally evolved in a microbial producer of one of the protein synthesis inhibitors and then was ‘snatched’ by other organisms. Alternatively, it could have evolved or is still evolving from one of the cellular pseudouridine synthases which generate specific Ψ’s for the sake of improving ribosome function. In contrast to most bacterial rRNA modification enzymes which show high specificity and target one specific rRNA position, RluC modifies three uridine residues in the *E. coli* ribosome, all located at a notable distance from each other in the ribosome spatial structure and within seemingly divergent rRNA

structural motifs. It is unclear whether the main function of the RluC enzyme is to modify only one of the three U's (U2504?) and the other two modifications are simply a result of RluC promiscuity or if RluC has been evolutionary optimized to target all three of the residues it acts upon.

In conclusion, we believe that our data illuminate a possibility that some of the indigenous posttranscriptional modifications of rRNA can be viewed as an intrinsic antibiotic resistance mechanism. It is generally possible that even those rRNA modifications which play important functional role in the modern ribosome could have been originally acquired as antibiotic resistance mechanisms.

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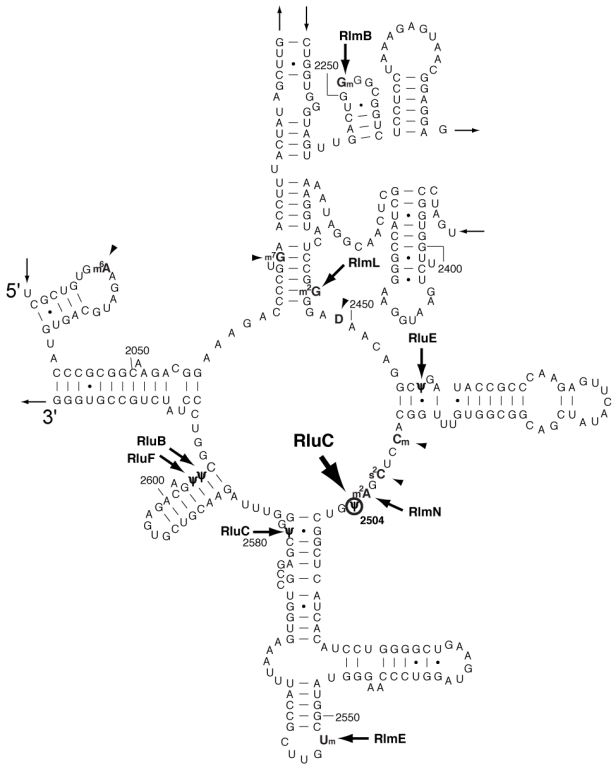

Figure 1.

Secondary structure of central loop segment of domain V of the *E. coli* 23S rRNA [27]. Posttranscriptionally modified residues shown in bold and indicated by arrows (when the enzyme responsible for the modification is known) or arrowheads (when the corresponding enzyme is yet unknown). Enzymes responsible for individual modifications were described in the following references: [7; 16; 17; 28 - 32]. Two out of three RluC targets are located in domain V (positions 2504 and 2580). Ψ 2504, whose presence renders cells resistant to several peptidyl transferase targeting antibiotics, is circled.

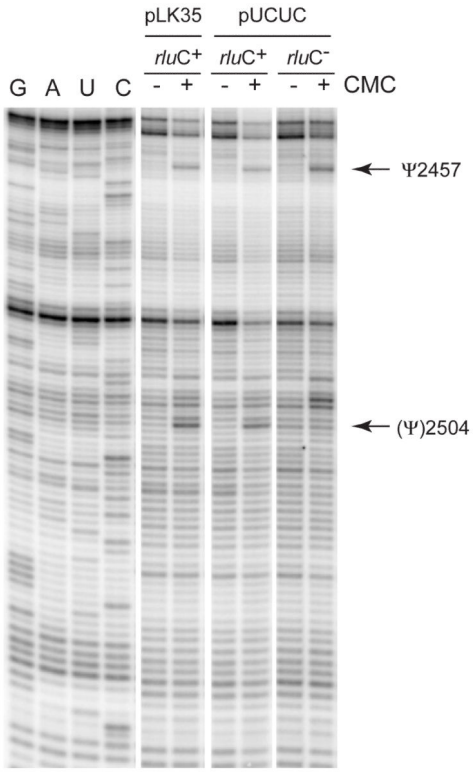

Figure 2.

Status of the U2504 modification in the strains expressing 23S rRNA with the U955C and U2580C mutations. The strain SQ171 lacking chromosomal *rrn* alleles [20] was transformed with either pLK35 plasmid expressing wild type rRNA or plasmid pUCUC carrying *rrnB* operon with the mutations U955C and U2580C in 23S rRNA gene. After plasmid exchange and verification that the strain expresses exclusively mutant ribosome, the *rluC::kan* allele was transduced from the strain JW1072 from the Keio collection [15]. The status of U2504 modification was verified using the procedure of Ofengand *et al.* [33] based on selective modification of pseudouridine residues by N^3-1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (CMC). Samples that are modified (+) or not modified (−) with CMC are indicated. G,A,U,C – sequencing lanes. Note the lack of the CMC-modified band at position 2504 in the RNA sample prepared from the *rluC*$^-$ derivative of SQ171 cells expressing U955C/U2580C double-mutant 23S rRNA. Ψ residue at position 2457, which is introduced by RluE, remains unaffected.

Table 1

Minimal inhibitory concentrations (μg/ml) of antibiotics for the *E. coli* strains lacking individual rRNA-modifying enzymes.

Antibiotic [a] Strain	Tiamulin	Chloramphenicol	Clindamycin	Linezolid	Hygromycin A	Sparsomycin	Dalfopristin/ quinupristin	Streptomycin
Wild type	1024 [b]	8	512	1024	512	32	256	32
RluB⁻	1024	4	256	512	256	16	256	32
RluC⁻	256 [c]	4	64	64	256	16	128	32
RluE⁻	1024	8	512	1024	512	16	256	32
RluF⁻	1024	8	512	1024	256	16	256	32
RlmB⁻	1024	8	512	1024	512	32	256	32
RlmE⁻	1024	8	256	1024	256	8	256	32
RlmL⁻	1024	8	512	1024	256	32	256	32
RlmN⁻	512	8	512	1024	256	16	256	32
SQ171/pLK35	64	8	512	512	256	16	512	nd
SQ171/pUCUC	32	4	1024	256	256	8	256	nd
SQ171/pUCUC RluC⁻	8	2	64	32	128	8	128	nd

[a] All the tested antibiotics, except for streptomycin which was used as a control, target the peptidyl transferase center in the large ribosomal subunit.

[b] MIC's were tested using the microbroth dilution procedure in 96-well plates {Standards, 2003 #5065}.

[c] A two-fold change in MIC is highlighted by a bold font; change in MIC that exceeds two-fold compared to the appropriate control is highlighted with a gray color of the corresponding table slots.