# Expression of plasmid encoded *Escherichia coli* 5 S ribosomal ribonucleic acid in *Pseudomonas putida*

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The recombinant plasmid pNRK 36, which represents the plasmids RSF 1010, a small multicopy plasmid of the incompatability group IncQ that confers resistance to streptomycin and sulfoamide to its host cells, and pKK 223-3, which contains the try-lac (tac) promoter followed by a polylinker and a DNA segment containing the 5 S rRNA (rrn B) with the ribosomal RNA transcription terminators, was employed to transform *Pseudomonas putida* 2440 cells. The plasmid encoded 5 S rRNA from *Escherichia coli* was transcribed and processed properly in *P. putida* cells thus demonstrating for the first time the expression of a plasmid encoded ribosomal RNA in a heterologous system. The fact that the *E. coli* 5 S rRNA was not incorporated into assembled ribosomes suggests that the in vivo incorporation of 5 S rRNA into the 50 S ribosomal subunit is closely linked to 23 S rRNA and/or ribosomal protein synthesis.

E. coli *5 S rRNA* *Plasmid encoding* *Transcription* Pseudomonas putida *Ribosomal assembly*

## 1. INTRODUCTION

RSF 1010 is a small (8.7 kb) multicopy plasmid of the incompatibility group IncQ that confers resistance to streptomycin and sulfonamide to its host cells [1]. It is very similar or identical with the 2 other representatives of the IncQ group, R 300 [2] and R 1162 [3]. One of the most striking properties of this plasmid is its extraordinary broad host-range among Gram-negative bacteria [4]. The vector, pKK 223-3 [5], contains the strong trp-lac (tac) promoter, which is followed by a polylinker derived from pUC-8 [6], which facilitates the positioning of genes behind the promoter and ribosomal binding site. The polylinker is followed by a DNA segment containing the 5 S rRNA (rrn B), followed by the strong rrn B ribosomal transcription terminators. The plasmid pKK 223-3, which is used as an expression vector for the insertion of genes to be expressed at high levels between the regulatable tac promoter [7–9] and the rrn B transcription terminators [10], was used for the introduction of a 4.5 S gene [11]. Strong promoters such as phage lambda P1, lac UV5, trp or the hybrid trp-lac (tac) promoter have been used to express and regulate cloned genes in *Escherichia coli*. However, for other Gram-negative bacteria, e.g. *Pseudomonas*, little information is available to date on the structure and function of promoters. The lac promoter of the transposon Tn 951 is active in *Pseudomonas putida* and *P. aeruginosa* [12], although in all cases, a much lower expression was observed compared to the expression in the original host. It was shown recently that overproduction of rRNA and free ribosomes produced a large repression of rRNA and tRNA synthesis from chromosomal genes, i.e. rRNA and tRNA operons are negatively regulated, either directly or through some intermediate, by free non-translating ribosomes [13].

To obtain a more detailed insight into the transcription, processing and possible incorporation of the plasmid encoded 5 S rRNA from *E. coli* into ribosomes from *P. putida* and other Gram-negative bacteria, we have constructed a plasmid which carries the constituents to be used as a broad host range vector.

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## 2. MATERIALS AND METHODS

Plasmid DNA was purified by Sepharose CL-4B column chromatography in 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and covalently closed circular DNA of plasmids by cesium chloride density gradient centrifugation in the presence of ethidium bromide [14]. *P. putida* 2440 is a derivative of the strain mt-2, defective in host-specific restriction [15]. *P. putida* competent cells were prepared and transformed as described [16] with some modifications: cells were grown to a density of $A_{600}$ = 0.2–0.25 in TY media and spun for 4 min at 5000 × *g* at 4°C. The cell sediment was resuspended in 1/5 of the original volume of sterile ice-cold 100 mM $CaCl_2$ solution, placed on ice for 25 min and centrifuged for 4 min at 5000 × *g* at 4°C. The component cells were resuspended in 1/20 of the original culture volume of cold sterile 100 mM $CaCl_2$ and approx. $1 \times 10^8$ cells in 0.1 ml were mixed with 0.2–2.0 μg DNA, and incubated on ice for 15 min, followed by incubations: 4 min at 42°C, 15 min on ice, 4 min at 42°C and finally 15 min on ice. To allow the bacteria to recover and to express antibiotic resistance, 1–2 ml TY media was added followed by an incubation at 37°C for 2 h. The entire transformation mixture was spread on a single plate. For antibiotic selection 2 mg/ml carbenicillin [1] and 100 μg/ml streptomycin were used for *P. putida*. Plates were scored after 24 h incubation at 37°C.

Agarose and polyacrylamide gel electrophoreses were performed by standard methods using Tris/EDTA/borate buffer. DNA bands were visualized and photographed under short-wave ultraviolet light. DNA fragments were recovered from gel slices by electroelution. Resistant bacteria were selected and grown in TY media or solid media at 37°C with 100 μg/ml streptomycin. The vectors RSF 1010 and pKK 223-3 were described in [5,17]. The restriction endonuclease *Pvu*II and the T4 DNA ligase were purchased from Bethesda Research Laboratories; all other enzymes were from Boehringer, Mannheim, and were used according to the instructions supplied by the manufacturer. Total RNA from *P. putida* 2440 was extracted and purified by Sephacryl S-200 gel filtration [18].

The 5 S rRNA containing fractions were labelled at the 3′- and 5′-ends as reported [19,20]. Labelled 5 S rRNA was purified by electrophoresis and then sequenced using the enzymatic digestion method of Donnis-Keller et al. [21]. The terminal nucleotides were determined by thin-layer chromatography of total $T_2$ and $P_1$ digests [22]. Ribosomes and ribosomal subunits were prepared as reported [23]. For densitometric analysis of autoradiographs a laser densitometer LKB 2202 Ultroscan was employed.

## 3. RESULTS AND DISCUSSION

To test the transcription, processing and possible incorporation of the plasmid encoded 5 S rRNA from *E. coli* (rrnB) in *P. putida* 2440 ribosomes, the plasmids RSF 1010 and pKK 223-3 were combined by blunt-end ligation utilizing the *Pvu*II site of the parental plasmids, resulting in the plasmid pNRK 36 (fig.1). The restriction-negative host strain *P. putida* 2440 ($res^-$, $mod^+$) was transformed by the calcium chloride method with the hybrid plasmid.

The growth rate of the strain containing the plasmid pNRK 36 is normal and is not affected compared to the strain without the plasmid, indicating that the production of unusual rRNA is not harmful in any obvious way to the *P. putida* cells. The ribosomes and the cytoplasm were analysed for *E. coli* like 5 S rRNA molecules as shown in figs 2–4.

The detection of *E. coli* plasmid encoded 5 S rRNA, compared to the chromosomal 5 S rRNA from *P. putida* was facilitated by the fact, that both rRNAs migrate in a 15% polyacrylamide gel to different positions (fig.2). The *E. coli* 5 S rRNA was eluted and end-labelled. The 5′- and 3′-ends of the molecule were analysed by employing enzymatic sequencing methods (fig.5) and the end-nucleotide determination by PEI-thin-layer chromatography (fig.6). Using these techniques we were able to determine that the molecule under investigation was indeed the 5 S rRNA from *E. coli*, i.e. the 5′- and 3′-ends of the molecule are identical with the respective ends of the mature 5 S rRNA (rrnB) from *E. coli*.

Since some of the RNA processing signals that are present are missing, it is significant that the RNA produced from this minigene is processed properly to 5 S rRNA. This indicates that the rRNA processing enzymes in *P. putida* 2440 accept

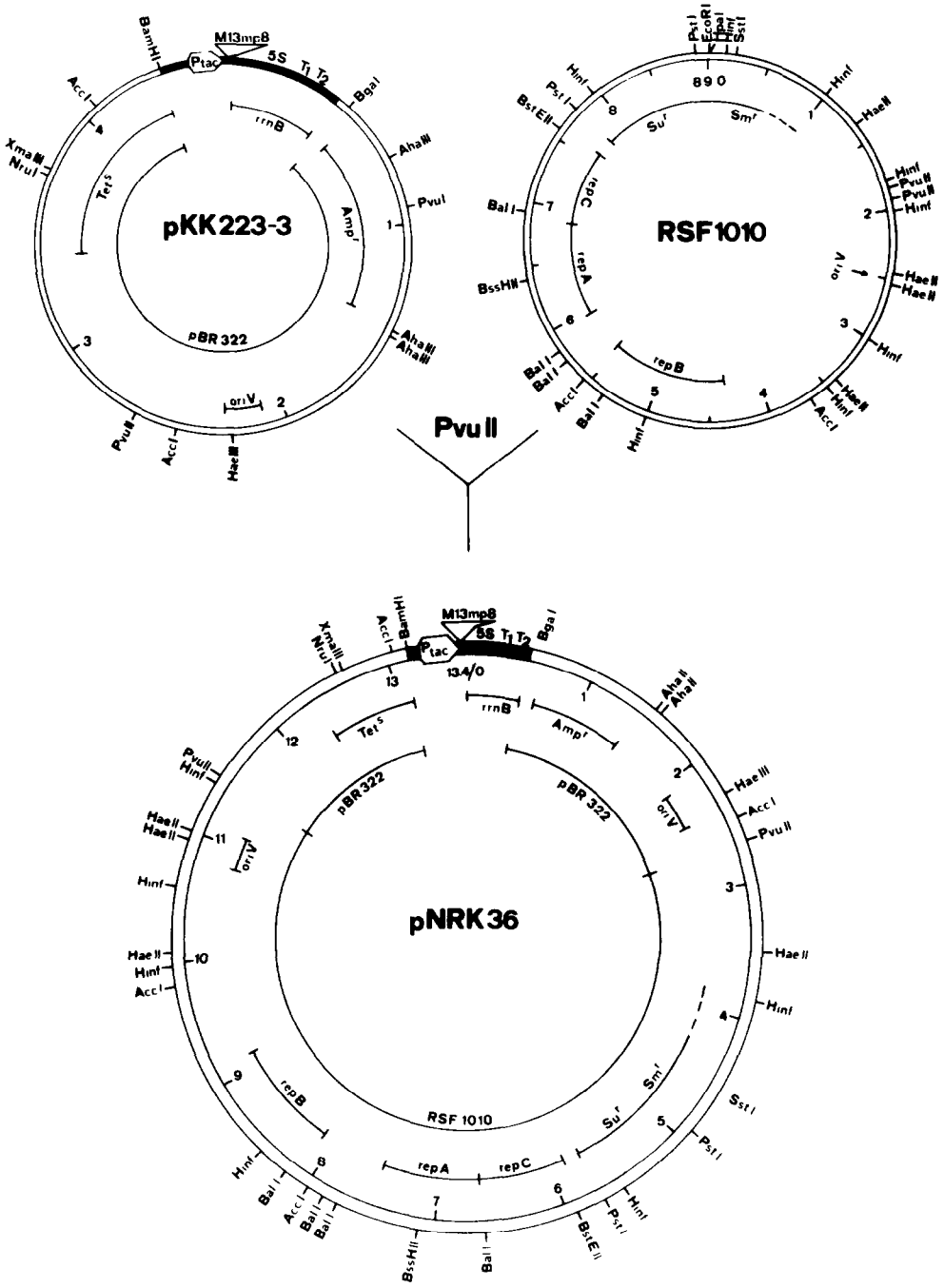


Fig.1. Diagrammatic representation of the construction of the broad-host plasmid pNRK 36. The restriction map of the RSF 1010 is derived from Scholz et al. [17] and that of pKK 223-3 from Brosius et al. [5]. The parental plasmids were digested with endonuclease *PvuII*, the DNA was treated with T4 DNA ligase and the ligated mixture was used to transform *P. putida* 2440 by the calcium chloride method.

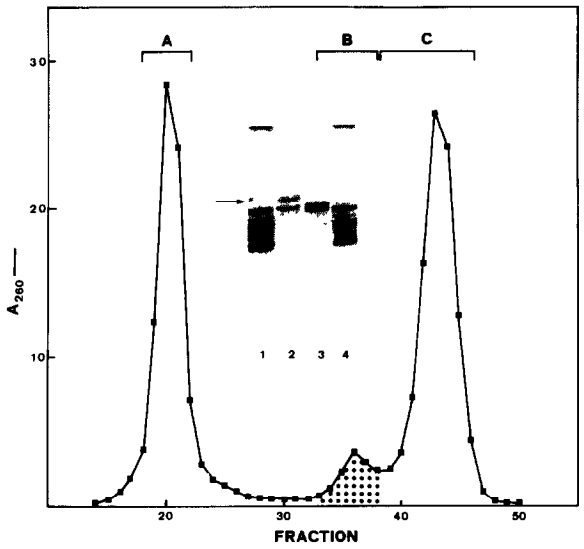


Fig.2. Filtration of RNA from *P. putida* 2440 carrying pNRK 36. To isolate 5 S rRNA, a sample was dissolved in buffer A (100 mM Tris-HCl, pH 8.0, 500 mM NaCl and 10 mM $MgCl_2$), applied to a column (2.6 × 80 cm) of Sephacryl S-200 and eluted with buffer A. The brackets designate the fraction collected for further analysis. The polyacrylamide gel inserted demonstrates: (1) the sample loaded onto the column, (2) the fraction collected under B, (3) a 5 S rRNA from *Pseudomonas* as reference and (4) total rRNA prepared from *P. putida* 2440 not carrying the plasmid pNRK 36.

the plasmid transcript and that in vivo the rRNA processing enzymes can work independently and outside of the assembled ribosome. It is interesting to note that the plasmid encoded *E. coli* 5 S rRNA (17%), compared to the chromosomal encoded *P. putida* 5 S rRNA (83%), appears in significantly lower quantities as judged by isolation from phenol extracted *Pseudomonas* cells carrying the plasmid, contrary to the data presented with a hybrid plasmid encoded 5 S rRNA in a homologous *E. coli* system [24,25], than would be expected from a multicopy plasmid. This suggests either that the chromosomal rRNA promoter is more efficient than the tac plasmid promoter or that the chromosomal rRNA is more stable than the plasmid RNA, or a combination of both. Further it cannot be excluded that the data presented here reflect a higher affinity for the protein-RNA interaction of *Pseudomonas* constituents, which is not reflected by in vitro 50 S reconstitution experiments [27].

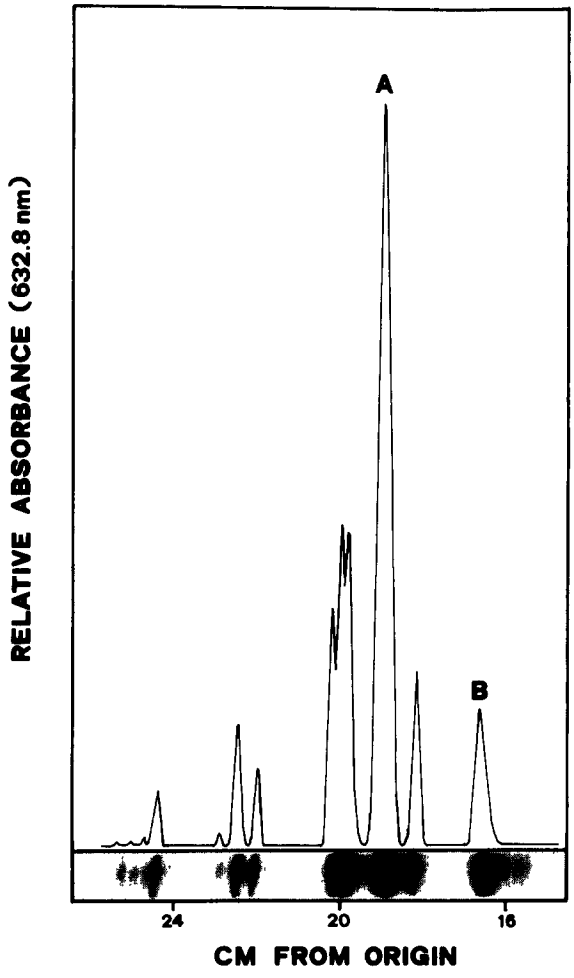


Fig.3. Scanning profile of 5'-end labelled rRNA (see fig.2 Sephacryl S-200 filtration, pool B). A, 5 S rRNA from *P. putida* 2440; B, plasmid encoded 5 S rRNA from *E. coli* (rrn B).

The *E. coli* 5 S rRNA might be subjected to substantial degradation in the cytoplasm, due to the lack of protection against ribonuclease attack by 5 S rRNA binding proteins. Indeed it was observed that newly synthesized rRNA by plasmid encoded rrnB operon fused to the lambda P1 promoter/operator is not stable in the homologous *E. coli* system, suggesting that the availability of ribosomal proteins is not sufficient, resulting in unprotected rRNA which is accessible to degradation. Furthermore it was suggested that the synthesis of ribosomal mRNA is inhibited after induction of the P1-rrnB [13].

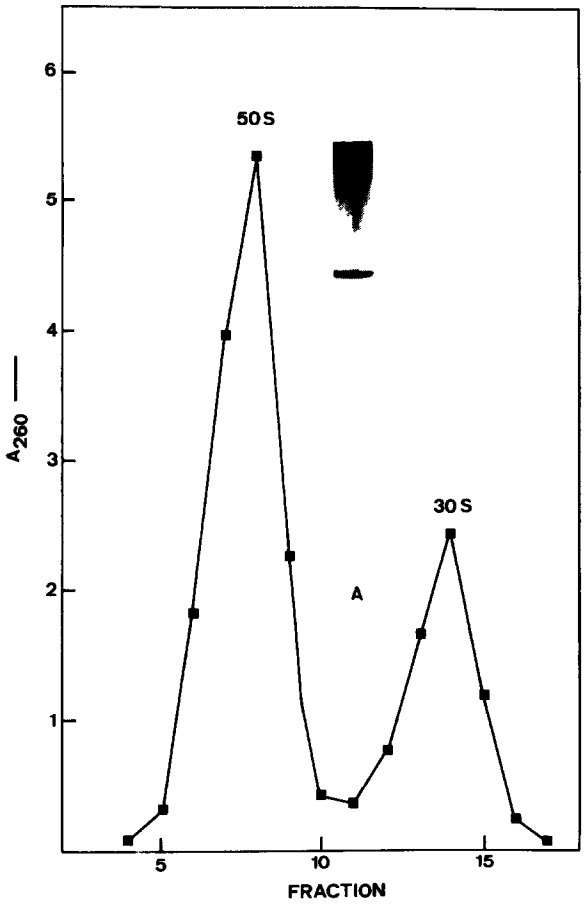


Fig.4. Sucrose gradient centrifugation of ribosomes isolated from *P. putida* 2440 carrying pNRK 36. To analyse the 50 S ribosomal subunit for incorporation of the plasmid encoded 5 S rRNA from *E. coli*, the 50 S ribosomal subunit was extracted and analysed by polyacrylamide gel electrophoresis (A). As shown in the inset, beside the chromosomal 5 S rRNA from the host no plasmid encoded *E. coli* 5 S rRNA can be observed.

The first attempt to analyse the expression of a plasmid encoded 5 S rRNA gene in a homologous system was described in [24,25]. It was reported that transformation of an RNA processing mutant (rne, RNase $E^-$) of *E. coli* with the plasmid pJK3$\Delta$ resulted in full-length transcripts from both promoters. The recombinant plasmid pJR3$\Delta$, contains a tandem promoter from the ribosomal transcription unit rrnA from *E. coli*, a hybrid 5 S rRNA gene, consisting of the 5'-end region of the first and of the 3'-end part of the second 5 S rRNA gene from the rrnD cluster and portions from the 16 S and 23 S rRNA gene [24,25]. These transcripts were processed to precursors of 5 S rRNA in vivo at the permissive temperature, but only about 20% of these transcripts are processed to mature 5 S rRNA. This arrangement resulted in a hybrid 5 S rRNA which contains a C-A pair in helix I and served as a marker for detecting the plasmid encoded 5 S rRNA. Transformation of wild type ($rne^+$, N3433) *E. coli* cells with the plasmid resulted in 5 S rRNA which was processed properly. Attempts were made to demonstrate that the plasmid encoded 5 S rRNA was indeed incorporated into the ribosome.

It is important to notice that in our opinion it is not sufficient to analyse only the total cellular extract or the ribosomal fraction but to analyse the 50 S ribosomal subunit. Plasmid encoded fully processed mature *E. coli* 5 S rRNA may associate with the ribosomal fraction at least during the preparation, indicating an unspecific affinity to the ribosomal subunit proteins (not shown). In analysing the isolated 50 S ribosomal subunit we were unable to detect any incorporation of the processed *E. coli* 5 S rRNA into *Pseudomonas* ribosomes and we would like to suggest that the ribosome assembly mechanism in vivo might not allow the entrance of a foreign 5 S rRNA and/or 5 S rRNA-protein complex and that the ribosomal assembly might be, as a prerequisite, linked to the transcription of a complete rRNA operon.

*P. putida* 2440 does not contain a lacI gene. To check the function of the tac promoter in *Pseudomonas*, the lacI gene was introduced to a RSF derivative carrying the tac promoter, it was shown that upon induction with ITPG a considerable increase in the level of resistance was observed [26].

Investigations are in process to introduce a complete plasmid encoded operon from *E. coli* in *P. putida* 2440 and to make the tac promoter inducible by ITPG.

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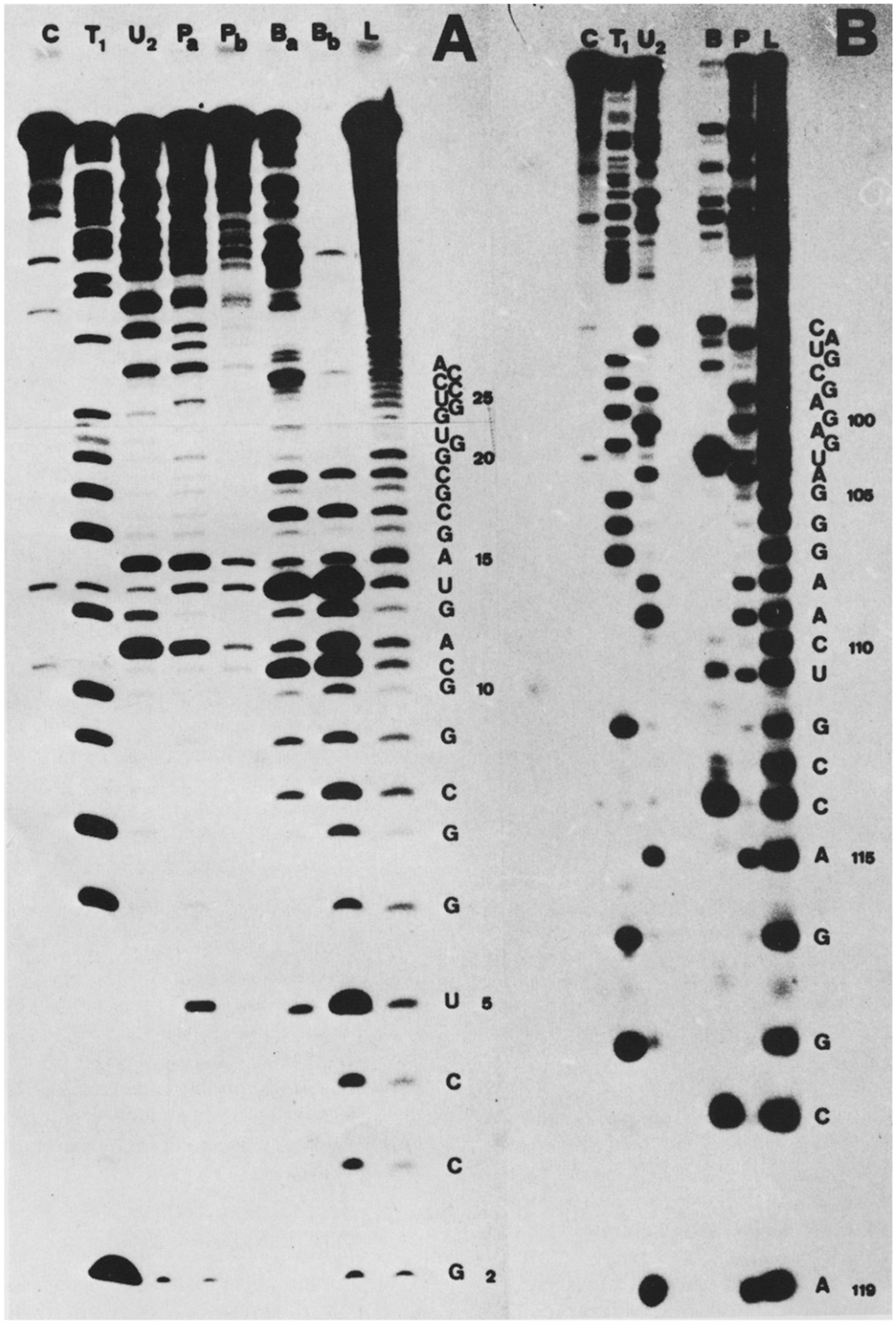


Fig.5. Sequencing data for the plasmid encoded 5 S rRNA from *E. coli* (rrnB) in *P. putida*, determined by partial digestion of 5′- (A) or 3′- (B)end labelled probes, according to Donnis-Keller et al. [21]. The reaction products were fractioned by electrophoresis through 25% polyacrylamide/7 M urea/50 mM Tris-borate, pH 8.3/1 mM EDTA.

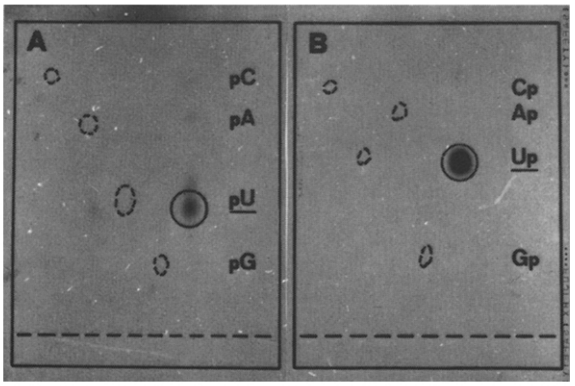


Fig.6. Thin-layer chromatography of a $T_2$ RNAse digest ($3'$-$^{32}$P)-labelled 5 S rRNA (A) and $P_1$ digest of ($5'$-$^{32}$P)-labelled 5 S rRNA (B). Plate dimensions were 10 × 6 cm and the chromatography media were (i) 0.5% formic acid, and (ii) 0.15 M lithium formate, pH 3.0. The migration positions of the four $5'$-nucleotide phosphates, i.e. $3'$-nucleotide phosphates are indicated.

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