Cloning and Expression in *Escherichia coli* of *Proteus vulgaris* Genes for 16S Ribosomal RNA

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In contrast to the established systems of plasmid-coded homologous ribosomal DNA (rDNA) cistrons in *Escherichia coli* little is known about the fate of heterologous rRNA. In order to study expression of foreign rDNA, rRNA cistrons from *Proteus vulgaris* were cloned in phage vector Charon 35, subcloned in pBR322 and transformed in *E. coli*. The inserts of two clones (pPM2 and pPM14) were characterized by restriction analysis and Southern hybridization. Each of them harboured a complete *rrn* cistron. The location of rRNA genes of clone pPM2 was also verified by R-loop analysis. The 5' flanking region of the 16S rRNA of pPM2 was sequenced and compared to the *E. coli* counterparts. High-level homologies exist in the functional parts of this region, e.g. promoters, box A and RNAase III recognition site. The copy number of pPM2 and pPM14 was estimated to be 8 and 10, respectively. Clones showed a markedly reduced growth rate (generation time about 57 to 70 min) as compared to the non-transformed cells (generation time 40 min). rDNA cistrons of *P. vulgaris* were properly expressed and the transcripts are processed as demonstrated by the presence of 16S rRNA from *P. vulgaris* in both ribosomes and 30S ribosomal subunits isolated from the transformed *E. coli* cells. The fraction of heterologous rRNA in ribosomes was about 25%.

INTRODUCTION

All previous studies investigating the function of ribosomal RNA during translation and the role these molecules play in the regulation of ribosomal proteins have been performed in *Escherichia coli* using plasmids carrying homologous rRNA cistrons with either unaltered (Ikemura & Nomura, 1977) or *in vitro*-altered primary structures (Gourse *et al.*, 1982; Stark *et al.*, 1982; Skinner *et al.*, 1985; Jemiolo *et al.*, 1985; Meier *et al.*, 1986). In most cases plasmid pKK3535 (Brosius *et al.*, 1981*a, b*), carrying the *E. coli rrnB* cistron, has been used, since with this system expression and incorporation of 16S and 23S rRNA in ribosomes has been demonstrated (Gourse *et al.*, 1982). Compared to the wild-type strain, the presence of additional copies of plasmid-coded unaltered or mutated rDNA genes resulted in a more or less dramatic reduction of doubling time.

Translation experiments, using *E. coli* r-proteins and 16S rRNA from different sources, have shown (Nomura *et al.*, 1968) the high degree of conservatism of ribosomal components, allowing their *in vitro* replacement even over large genealogical distances. However, a successful *in vivo* expression of a plasmid-coded *E. coli* 5S rRNA gene in *Pseudomonas putida* was only reported recently (Hartmann *et al.*, 1985).

In order to obtain a better insight into the fate of heterologous 16S rRNA we cloned *Proteus vulgaris* complete rDNA cistrons in *E. coli*. Recovery of *P. vulgaris* 16S rRNA from *E. coli* ribosomes is a prerequisite for the identification of rRNA expressed from a successfully transformed and functional heterologous *rrn* cistron in the *E. coli* genome.

METHODS

Cloning of ribosomal DNA. Chromosomal DNA from *Proteus vulgaris* IFAM 1731 (IFAM = Institut für Allgemeine Mikrobiologie, Universität Kiel) was digested with *Bam*HI. Since fragments of size 9–20 kb are suitable for cloning in substitution phage vector Charon 35 (Loenen & Blattner, 1983) fragments larger than 8 kb were enriched by ultracentrifugation (10–30%, w/v, sucrose). Respective fractions were pooled and precipitated with ethanol. DNA from Charon 35 was digested with *Bam*HI and the *cos*-sites of the arms were ligated according to Maniatis *et al.* (1982). Arms were separated from stuffer fragments by 10–30% (w/v) sucrose gradient ultracentrifugation. Arms and restriction fragments from *P. vulgaris* were mixed under conditions which favour the generation of large concatamers. After ligation DNA was *in vitro* packaged (*in vitro* packaging extracts were a gift from K. Apel, Kiel) and plated on *E. coli* K802 or K803. Phage clones harbouring ribosomal cistrons were identified by plaque hybridization (Maniatis *et al.*, 1982) with 5'- γ -³²P-labelled 16S rRNA from *P. vulgaris* on Hybond N (Amersham/Buchler) (Reed & Mann, 1985). [γ -³²P]ATP was from NEN. DNA from single clones was extracted from phage minipreparations (Maniatis *et al.*, 1982) and digested with *Bam*HI. Fragments were separated on a 0.7% (w/v) agarose gel. Insert DNA was eluted with NA-45 DEAE paper (Schleicher and Schuell) and subcloned in plasmid pBR322. Competent cells of *E. coli* HB101 were transformed as described (Mandel & Higa, 1970; Dagert & Ehrlich, 1979). Bacterial clones containing 16S rDNA from *P. vulgaris* were identified either by colony hybridization (Grunstein & Hogness, 1975) or by Southern hybridization (Southern, 1975) of plasmid minipreparations (Birnboim & Doly, 1979). Detailed restriction and gene maps of two different recombinant plasmids (pPM2 and pPM14) were obtained by digesting the inserts with various restriction enzymes, followed by Southern hybridization with γ -³²P-labelled 16S and 23S rRNA from *P. mirabilis*. Maps were verified by *Bal*31 analysis of selected fragments (Perbal, 1984). Restriction enzymes were purchased from Boehringer Mannheim.

Expression of rDNA. Expression of heterologous rDNA in *E. coli* was determined by 16S rRNA fingerprint analysis. Cells were lysed and debris was pelleted (45000 g) in a Sorvall centrifuge. The supernatant was centrifuged (44000 r.p.m., 2 h, rotor type TFT 65.38) in a Contron ultracentrifuge to pellet ribosomes. These were phenol-extracted twice and the rRNAs precipitated with ethanol. Alternatively 16S rRNA was extracted from the small subunit of ribosomes (Traub *et al.*, 1971; Zablen & Woese, 1975). The nucleic acids were separated by preparative 2.8% (w/v) PAGE. 16S rRNA was electroeluted using the Biotrap apparatus (Schleicher and Schuell) as indicated by the manufacturer, extracted twice with phenol and precipitated with ethanol. RNAase T1 digestion, 5'-labelling of oligonucleotides with [γ -³²P]ATP and polynucleotide kinase, and the generation of a fingerprint were done as described previously (Stackebrandt *et al.*, 1985).

The fingerprint obtained was compared with those of pure 16S rRNA of *E. coli* HB101 and *P. vulgaris*. *P. vulgaris*-specific oligonucleotides were analysed further by partial alkaline hydrolysis and two-dimensional mobility shift analysis (Stackebrandt *et al.*, 1985). To estimate the ratio of homologous and heterologous oligonucleotides in the 'mixed' fingerprint, the corresponding material from selected spots was isolated from the thin-layer plate (Stackebrandt *et al.*, 1985) and the radioactivity determined in a scintillation counter (Philips).

Growth measurements were performed by transferring similar amounts of inocula of clones harbouring pPM2 and pPM14, *E. coli* HB101, *E. coli* (pKK3535) (Brosius *et al.*, 1981*b*) and *E. coli* (pBR322) in LB medium (Maniatis *et al.*, 1982) supplemented with 80 μ g ampicillin ml⁻¹ (except for HB101). Cells were shaken (250 r.p.m. at 37 °C) and cell density measured at 578 nm.

Sequencing of promoter region. The nucleotide sequence of the promoter region was determined by subcloning the 1.2 kb *Hind*III and the 1.8 kb *Bam*HI–*Bgl*II fragment from pPM2 in phage M13mp8 (Messing & Vieira, 1982). The sequencing reaction was carried out according to Sanger *et al.* (1977) using the universal primer (Boehringer Mannheim) and a synthetic primer approximately 250 bp upstream from the 5'-terminus of the 16S rRNA gene. Both [α -³²P]ATP and dATP α -³⁵S (NEN) were used as radionucleotides. Sequencing analysis was carried out on 6% (w/v) PAGE gels using an LKB sequencing apparatus.

Determination of plasmid copy number. This was done by separating 5 μ g *Bam*HI-digested total DNA of *E. coli* harbouring plasmid pPM2 or pPM14 on a 0.7% (w/v) agarose gel, adjacent to samples containing copies of *Bam*HI-digested plasmid DNA ranging from an amount equivalent to the total amount of chromosomal DNA to 256 times this amount. Plasmids were purified from RNA by treatment with RNAase T1 (Calbiochem-Behring) and RNAase A (Boehringer Mannheim), followed by centrifugation through 1 M-NaCl (Maniatis *et al.*, 1982). Each sample was adjusted to 5 μ g by adding the appropriate amount of *Bam*HI-digested salmon sperm DNA. After separation, fragments were blotted to Hybond N. The membrane was hybridized with 5'-³²P-labelled 16S rRNA from *P. vulgaris* and exposed to an X-ray film. Hybridization conditions were stringent to minimize cross-hybridization with *E. coli* chromosomal rDNA cistrons. The signal strength of the plasmid bands of the clones was compared to those of the purified plasmids (K. Apel, personal communication).

R-loop analysis. DNA of isolated *Bam*HI insert of pPM2 was crosslinked by treatment with Trioxsalen (Sigma), followed by UV-irradiation. After hybridization with 16S and 23S rRNAs, the DNA was modified by glyoxal

(a) pPM2

(b) pPM14

Fig. 1. Restriction maps of the cloned *Proteus vulgaris* *rrn* cistrons. The location of the 5S rRNA gene was deduced from the position of the corresponding gene in the *E. coli* cistrons. (a) Plasmid pPM2, 15 kb. The order of the 3' terminal restriction sites *ClaI*, *HindIII* and *HindII* could not be resolved. (b) Plasmid pPM14, 12 kb.

(Sigma). The resulting R-loops were spread with cytochrome *c* and Pt/C rotary-shadowed (Cech & Pardue, 1976; Casey & Davidson, 1977; Kaback *et al.*, 1979; Woolford & Rosebush, 1979). Electron microscopy was done with a Philips EM 300; length measurements were done with a Morphomat (Zeiss).

RESULTS AND DISCUSSION

Proteus vulgaris was chosen as a source for testing the expression of heterologous rDNA in *E. coli*. On the basis of 16S rRNA cataloguing, both organisms are members of the gamma subdivision of purple phototrophic bacteria and their non-phototrophic relatives, sharing a moderate degree of relationship (S_{AB} value 0.68).

Cloning and sequence comparison

Three different rDNA cistrons from *P. vulgaris* were cloned in phage vector Charon 35. The sizes of the inserts were about 7.5, 10.5 and 13.5 kb. The two smaller inserts were subcloned in plasmid vector pBR322, but attempts to subclone the 13.5 kb insert failed, possibly due to the fragment size (Perbal, 1984). Fig. 1 presents gene and restriction maps of the two recombinant plasmids pPM2 and pPM14.

The locations of the rRNA genes within the insert of pPM2 were verified by R-loop analysis (Fig. 2). The total length of the insert (10.5 kb) as determined by restriction analysis is in accordance with the dimensions determined after electron microscopy.

Both recombinant plasmids possess a complete rDNA cistron including promoters and terminators, a prerequisite for proper transcription and processing in the host. In the case of pPM14 this was not obvious from the restriction map but could later be demonstrated by recovery of pPM14-coded 16S rRNA in *E. coli* ribosomes.

The 5' flanking region of *P. vulgaris* rRNA clone pPM2 (Fig. 3) contains putative promoters, a discriminator region, a box A region responsible for antitermination function (Morgan, 1986) and the 5' region of the stem containing the recognition site of RNAase III. The dimensions of these functional parts were adopted from published *E. coli* sequences. The promoter-leader

Fig. 2. R-loop analysis of 16S and 23S rRNA. Electron micrograph (a) and schematic representation (b) of the location of 16S and 23S rRNA genes in the *Bam*HI insert of pPM2. DNA and RNA are indicated as solid and dashed lines, respectively.

region of the *P. vulgaris* *rrn* operon consists of two promoters (P1 and P2), separated by 107 bp (5' of -10 regions). Both promoters exhibit high sequence homology with those of *E. coli*. While the -10 region of P1 is identical to the homologous regions of all *E. coli* cistrons, the -10 region of P2 is identical to those of *rrnD* and *rrnE* (Young & Steitz, 1979; de Boer *et al.*, 1979). The -35 regions (12 bp long according to Brosius *et al.*, 1981a) of both promoters are in no case identical to any of the *E. coli* counter-parts. Limiting the -35 region to the central sequences (Lamond & Travers, 1985) the homology is obvious.

Expression of heterologous 16S rRNA

Plasmid copy number was determined (Fig. 4) and under exponential growth conditions it was about 8 and 10 for pPM14 and pPM2, respectively, per haploid *E. coli* genome. These numbers are of the same order of magnitude as the number of rRNA genes in the host cell, previously determined to be 7 (Kenerley *et al.*, 1977; Kiss *et al.*, 1977).

The transcription of functional heterologous 16S rRNA can be verified by demonstrating its presence in isolated *E. coli* ribosomes. Fig. 5 shows that part of the RNAase T1 16S rRNA fingerprint of *E. coli* (pPM2) displaying oligonucleotides of pentamer size and larger. Selected spots representing large oligonucleotides of the 16S RNA of *E. coli* and *P. vulgaris*, respectively, are marked by different symbols. The extent of labelling of the oligonucleotides depends primarily upon the ratio of homologous and heterologous rRNAs in the ribosomes of transformed *E. coli* cells, but differences in the label of 5' nucleotides might also be influenced by the composition of the nucleotide sequence itself (Stackebrandt *et al.*, 1985). A quantitative measurement of isolated oligonucleotides should therefore allow a crude estimation of the amount of *P. vulgaris* 16S rRNA. Clone pPM2 was analysed for undissociated ribosomes, whereas 30S subunits of clone pPM14 were isolated prior to the analyses. The portions of the heterologous 16S rRNA from both clones were in the range of 20–30% of total 16S rRNA.

The finding that only about a quarter of the ribosomes contain heterologous 16S rRNA contrasts with the approximately 1:1 ratio of 16S rRNA genes. Possible explanations are as follows. (1) A sub-optimal recognition of the heterologous promoter region by DNA-dependent

Fig. 3. Comparison of the primary structure of the promoter, leader region and flanking sequences of the *rnnB* cistron of *E. coli* (Brosius *et al.*, 1981*a*) (line *a*) and a rDNA cistron from *P. vulgaris* (pPM2) (line *b*). Regions of known function are marked as follows: thin line, -35 region of promoters and box A; heavy line, -10 region of promoters; boxed, RNAase III recognition site; shadowed, 5' region of 16S rDNA gene.

RNA polymerase and/or of 16S rRNA processing signals by RNAase III. These possibilities seem unlikely since, as stated above, these sites show a high degree of homology to the corresponding *E. coli* sequences (Brosius *et al.*, 1981a). However, no information is available about an upstream enhancer sequence in *P. vulgaris* known to have an influence on the promoter strength of the *E. coli* *rrn* cistrons (Lamond, 1985). (2) A delayed heterologous r-

Fig. 4. Determination of plasmid copy number by agarose gel electrophoresis (a) and Southern hybridization (b). (a) Lane A, *Hind*III-digested λ phage; lane B, 5 μ g *Bam*HI-digested chromosomal DNA from *E. coli* HB101(pPM14); lane C, *Bam*HI-digested pPM14 equivalent to the total amount of chromosomal DNA from strain HB101(pPM14) applied in lane B; lanes D–K, increasing amounts of pPM14 DNA, each lane doubling the amount of the previous lane. *Bam*HI-digested salmon sperm DNA was added to each assay in lanes C to K to a total of 5 μ g. Bands corresponding to 7.5 kb inserts and to 4.4 kb pBR322 are designated 1 and 2, respectively. (b) Autoradiogram of DNA–RNA hybrids between 5'- γ - 32 P-labelled 16S rRNA from *P. vulgaris* and the *Bam*HI digests of chromosomal and pPM14 DNA in (a). The concentration of pPM14 in lane F (DNA equivalent to eight times the amount in lane B), which in signal strength is equivalent to that of lane B, gives an approximation of the plasmid copy number per haploid *E. coli* chromosome.

protein/rRNA binding, due to possible differences in the protein-binding nucleotides of the *P. vulgaris* 16S rRNA primary structure; in this case the heterologous rRNA might be more susceptible to RNAases than the homologous RNA. However, this influence seems to be less dominant since in an *in vitro* study on heterologous r-protein/rRNA interaction in bacterial ribosomes a strong heterologous binding has been reported for the *Proteus vulgaris* and *E. coli* system (Daya-Grosjean *et al.*, 1973).

Growth rate of strains carrying heterologous rrn cistrons

While *E. coli* HB101, containing no plasmid, has a doubling time of 40 min, the doubling time increased to 57 and 70 min for clones harbouring pPM2 and pPM14, respectively. No increase of doubling time was observed in pBR322-containing cells (39 min), while *E. coli* (pKK3535) had a doubling time of 45 min. The latter two values agree with those found by Jemiolo *et al.*, (1985). The most likely explanation for the increased doubling time is a significant influence of the additional 10 or so cistrons on the regulation of the ribosomal components, including tRNAs (Gourse *et al.*, 1983; Gourse & Nomura, 1984; Jinks-Robertson *et al.*, 1983; Siehnell & Morgan, 1985). As discussed by Gourse *et al.* (1982), reduced functional efficiency of ribosomal subunits or a lowered fidelity of translation may also be responsible for the prolonged generation times.

Concluding remarks

The system introduced here is appropriate as a starting point for future attempts to establish a single copy of heterologous rDNA cistron in the *E. coli* genome. This, in the long run, would enable us to answer questions about the stability of foreign rRNA cistrons; about recombination events (Brosius *et al.*, 1981a) and accompanying problems concerning the maintenance of almost perfect uniformity of rDNA cistron primary structures within a single strain; and about the effects of the heterologous system on the mutation rate of the 16S rDNA primary structure of

Fig. 5. RNAase T1 oligonucleotide fingerprint of 16S ribosomal RNA isolated from 30S ribosomal subunits of *E. coli* (pPM14). Only oligonucleotides of pentamer size and larger are shown. Circled spots were isolated and the sequence of the oligonucleotides determined by the mobility shift method (Stackebrandt *et al.*, 1985). Spots designated by small and capital letters contain *E. coli*- and *P. vulgaris*-specific oligonucleotide, respectively. Spots containing a mixture of oligonucleotides are designated by numbers. Oligonucleotide sequences were as follows: a, ACCUCAUAAAG; b, AACCUUACCUUG; c, AUAAACUACUG; d, AUAAACUUG; A, AACCUUACCUUACCUUG; B, AACUCAUAAAG; C, AACCCAUAUG; D, AUAAACCG; E, ACAUCCAG; F, ACAAG; G, UCAAUUUAAAG; H, AAUCCUUUAG; 1, UUAUAAACUCAAUUG; 2, CAACCUUUAUCCUUG; 3, AACUCAAAAG; 4, CCUAACACAUG; 5, UUAUUAUCCUUUG; 6, CUUACCAUUUG; 7, AUACAAAG; 8, UCACACCAUG; 9, CAAAAG; 10, CUUAAACCUUG; 11, UUAUUAUUG; 12, AAUAUUG. The spot marked with an asterisk contains the fragment UUAUUAUCCUUUAUCCUUAUUG, which is an extended version of a sequence found in catalogues of both organisms, i.e. UUAUUAUCCUUUG, spot 5 (position 463 in the *E. coli* sequence).

the transformed *P. vulgaris* cistron compared with those of the donor organism. The successful integration of a heterologous rRNA cistron in the *E. coli* genome (H. Niebel & E. Stackebrandt, unpublished) using lysogenic phage λ gt11 (Young & Davis, 1985) has been achieved and this represents a promising starting point.

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