THE NUMBER OF 5 S rRNA GENES IN *BACILLUS SUBTILIS*

Hideki KOBAYASHI and Syozo OSAWA+

Department of Biology, Faculty of Science, Kyushu University, Fukuoka, 812 and +*Laboratory of Molecular Genetics, Department of Biology, Faculty of Science, Nagoya University, Nagoya, 464, Japan*

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1. Introduction

In *Escherichia coli*, the 16 S, 23 S and 5 S rRNA genes together with some tRNA genes are organized as one transcriptional unit (operon) and the number of such operon is 7 [1,2]. They are distributed at different places on the left half on the *E. coli* chromosome map [3]. In each operon, the number of each rRNA gene is 1, with an exception of *rrnD* where 2 tandemly repeated 5 S rRNA genes exist at the end of the operon [4]. Therefore, the number of the gene is 8 for 5 S rRNA, while it is 7 for 16 S and 23 S rRNAs.

In *Bacillus subtilis*, the majority of rRNA genes is located near the replication origin of the chromosome [5–7], and the 16 S, 23 S and 5 S rRNAs are transcribed in this order as 1 transcriptional unit [8]. Heteroduplex analyses [7] revealed that several rRNA operons are clustered, each being separated by heterologous spacers of various length. This paper presents evidence indicating that the copy number of the 5 S rRNA genes of *B. subtilis* is at least 10. Since each 5 S rRNA gene is closely linked to the 23 S rRNA genes, the number of the rRNA operon is in all probability 10 at least, and this number for the 5 S rRNA gene is not due to the presence of tandemly repeated 5 S rRNA genes in any rRNA operon.

2. Experimental

2.1. *Isolation of 5 S rRNA and its 3'-end labelling*

The 5 S rRNA was prepared by phenol method from ribosomes. The rRNAs extracted from 70 S ribosomes were applied on sucrose gradient (2.5–12.5%) containing 50 mM sodium acetate (pH 4.8) and 100 mM NaCl, and centrifuged at 24 000 rev./min for 16 h. The 5 S rRNA fractions were precipitated with ethanol and electrophoresed by 10% polyacrylamide gel containing 7 M urea, 50 mM Tris–borate (pH 8.3) and 1 mM EDTA. The intact 5 S rRNA was eluted from gel-slices in 500 mM ammonium acetate, 0.1% SDS and 0.1 mM EDTA at 37°C overnight. The elute was freed from polyacrylamide by Millipore filtration, ethanol precipitated, dissolved in distilled water, and then used for the 3'-end labelling.

A probe preparation is as follows: The 5 μg 5 S rRNA (135 pmol) was labeled with [5'-^{32}P]pCp in a 10 μl reaction mixture containing 50 mM Hepes (pH 7.5), 3.3 mM dithiothreitol, 15 mM $MgCl_2$, 10% dimethyl sulfoxide, 10 μg/ml bovine serum albumin, 525 pmol ATP, 4 U of T4 RNA ligase and 35 pmol [5'-^{32}P]pCp at 0°C for 20 h [9]. After the reaction, the labeled RNAs were subjected to electrophoresis on 7 M urea–10% polyacrylamide gel. The intact 5 S r[3'-^{32}P]RNA extracted from the gel was recovered by ethanol precipitation with a carrier (100 μg yeast tRNAs) and dissolved in 5 × SSC (SSC; 150 mM NaCl, 15 mM sodium citrate)–50% formamide (spec. act. 2×10^6 cpm/μg).

2.2. *Hybridization*

Bacillus subtilis DNA was prepared as in [10] with a slight modification. The total chromosomal DNA (15 μg) was completely digested with *Eco*RI, *Sma*I, and *Bam*HI in 50 μl reaction mixture containing 33 mM Tris–acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 μg/ml bovine serum albumin. The double digestion was carried out in the same buffer simultaneously. The digests were applied to 0.8% agarose gel. The electrophoretically separated DNA was subjected to alkali denaturation and transferred to the filter (Millipore HAWP) according to [11]. Hybridization

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was carried out at 40°C for 18 h. After the incubation, the filter was washed with 5 × SSC–50% formamide, treated with RNaseA, and autoradiographed [12].

3. Results and discussion

Bacillus subtilis DNA was digested with several restriction endonucleases. The digests were separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to 5 S r[3′-^{32}P]RNA by the Southern method [11]. The 3′-end of the purified 5 S rRNA was labeled with ^{32}P with [5′-^{32}P]pCp and T4 RNA ligase as in [9] and used as a probe for the detection of fragments carrying rRNA genes.

The hybridization of 5 S r[3′-^{32}P]RNA to *Eco*RI-, *Sma*I- and *Bam*HI-digested DNA fragments gave 10, 8 and 9 radioactive bands, respectively (fig.1). Table 1 summarizes the size of DNA fragments that were hybridized to 5 S rRNA. The *Sma*I-5.5 kilobases and *Bam*HI-12 kilobases fragments were more intense as compared with others, suggesting that these bands contain different fragments of the same size, or one kind of fragment containing 2 or more 5 S rRNA genes. Then, the DNA was further digested with 2 endonucleases with combinations of *Eco*RI–*Sma*I, *Sma*I–*Bam*HI and *Bam*HI–*Eco*RI. In all the cases, the band-number was 10. Since the 3′-end-labeled intact 5 S rRNA was used as a probe, the number of bands directly represents a minimum copy number of 5 S rRNA genes.

To see whether each of these 5 S rRNa genes is linked to a 23 S rRNA gene, or some 5 S rRNA genes exist as a tandem repeat in certain rRNA operon(s), a similar Southern hybridization experiment was carried

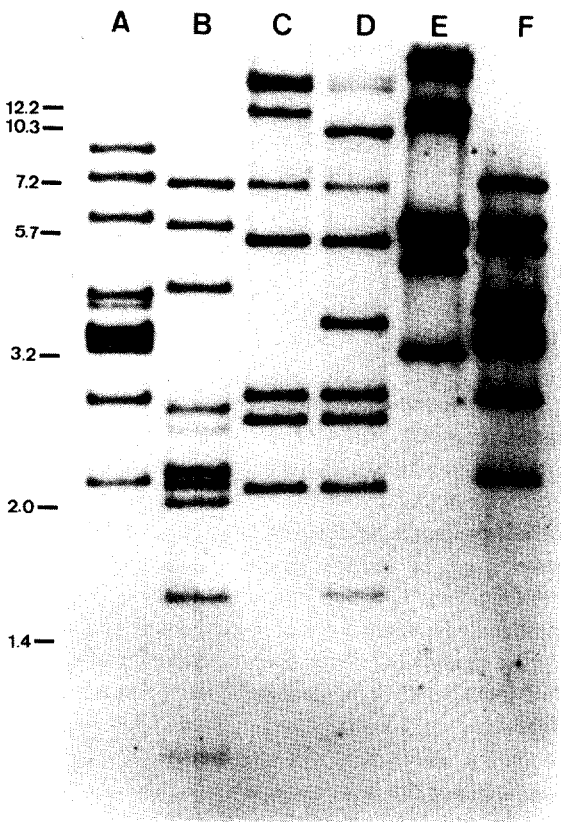

Fig.1. Autoradiograms of *B. subtilis* DNA fragments containing 5 S rRNA genes. The endonuclease-digested DNAs were hybridized to 3′-labeled 5 S rRNA by the Southern method [11]. The experimental conditions are given in section 2 in detail. The molecular size of the reference *Eco*RI–λ*met*DNA is expressed in kilobases on the left of the column: single digestion, (A) *Eco*RI, (C) *Sma*I, (E) *Bam*HI; double digestion, (B) *Eco*RI–*Sma*I, (D) *Sma*I–*Bam*HI, (F) *Bam*HI–*Eco*RI.

Table 1
The number and size of DNA fragments carrying 5 S rRNA

Enzymes	Bands (no.)	Size of fragments (kilobases)									
*Eco*RI	10	9.0	7.6	6.1	4.4	4.1	3.7	3.5	3.3	2.9	2.2
*Sma*I	8	18	16	11.5	7.3	5.5[a]	2.9	2.7	2.1		
*Bam*HI	9	28	20	12[a]	11	6.0	5.8	5.6	4.9	3.3	
*Eco*RI–*Sma*I	10	7.4	5.9	4.5	2.8	2.6	2.3	2.1	2.0	1.6	1.0
*Sma*I–*Bam*HI	10	18	16	10.5	7.3	5.5	3.8	2.9	2.7	2.1	1.6
*Bam*HI–*Eco*RI	10	7.6	6.1	5.5	4.4	4.1	3.7	3.5	3.3	2.9	2.2

[a] Intense bands DNA sizes (kilobases) were determined by co-electrophoresis of the *Eco*RI-digested λ*met*DNa of known size

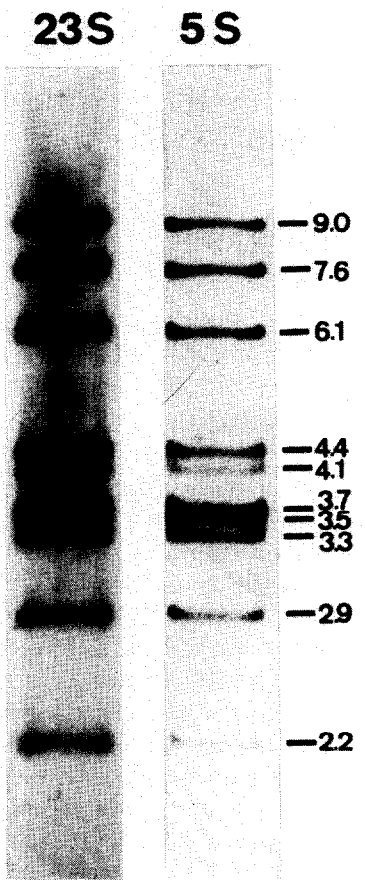

Fig.2. Autoradiograms of *B. subtilis Eco*RI-DNA fragments carrying 23 S and 5 S rRNAs. The 23 S rRNA was isolated by phenol extraction from the 50 S ribosomal subunits separated by sucrose gradient centrifugation. The procedures followed were as in section 2, except that 3% polyacrylamide gel was used to recover the 3′-labeled intact 23 S rRNA. The hybridization of 23 S r[3′-^{32}P]RNA was carried out in the presence of 100-fold of unlabeled 16 S rRNA as a competitor. Specific activity is as follows: 23 S rRNA, 2×10^5 cpm/μg; 5 S rRNA, 2×10^6 cpm/μg. The numbers represent the length of the DNA fragments (in kilobases).

out using 23 S r[3′-^{32}P]RNA. To avoid a cross contamination between 23 S and 16 S rRNA, the 23 S rRNA was prepared from the 50 S ribosomal subunits. When the 23 S r[3′-^{32}P]RNA was hybridized to the *Eco*RI-digested DNA fragments, 10 radioactive bands were observed. Furthermore, the banding pattern was identical with those of 5 S rRNA (fig.2). The result indicates that both the 23 S and 5 S rRNA genes are included in the same *Eco*RI-fragments, suggesting a complete linkage of all the 5 S rRNA genes to the 23 S rRNA genes. The *Sma*I- and *Bam*Hi-digested DNA gave 8 and 9 radioactive bands, respectively and the patterns were again the same with those of 5 S rRNA (not shown).

In [13] 8 rRNA genes were detected in *B. subtilis* by the Southern method using rRNAs totally labeled with ^{32}P as a probe. Here, the good separation of DNA fragments on agarose gels and the use of 3′-labeled probes made it possible to detect 10 5 S and 23 S rRNA genes which are closely linked to each other on chromosome. The determination of the copy number for 16 S rRNA gene and its linkage to the 23 S or 5 S rRNA is extremely difficult because the *Eco*RI-fragment containing the 3′-end of 16 S rRNA gene seems to be so small in size that it cannot bind to the nitrocellulose filters. However, this does not seriously affect the conclusion drawn here.

The gene copy number for rRNA is 7 (8 for 5 S rRNA) in *E. coli* [1,4] and 2 in *Mycoplasma capricolum* [14]. Thus the number of rRNA genes is variable in different bacteria.

Acknowledgements

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