JOURNAL OF BACTERIOLOGY, Nov. 1979, p. 742-744
0021-9193/79/11-0742/03$02.00/0

Vol. 140, No. 2

Organization of Transfer and Ribosomal Ribonucleic Acid Genes in *Bacillus subtilis*

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Received for publication 10 August 1979

The structural relationship between the transfer ribonucleic acid (tRNA) and the ribosomal RNA (rRNA) genes of *Bacillus subtilis* has been studied by restriction endonuclease analysis of total chromosomal deoxyribonucleic acid (DNA) and characterization of DNA fragments cloned in *Escherichia coli*. The DNA sequences encoding rRNA and tRNA were assayed by hybridization to radioactive RNA. The results support the conclusion that the tRNA genes are interspersed between and closely linked to the rRNA genes of *B. subtilis*. They probably do not appear between the 16S and 23S rRNA genes as in *E. coli*.

Density transfer experiments were used by I. Smith and his colleagues to demonstrate that the DNA sequences coding for rRNA and tRNA are located near the origin of chromosome replication in *Bacillus subtilis* (6). These experiments could not resolve whether the rRNA genes are grouped next to a cluster of tRNA genes or whether the two gene sets are interspersed. We have recently used restriction enzyme analysis of *B. subtilis* chromosomal DNA and *B. subtilis* DNA fragments cloned in *Escherichia coli* to show that there are eight rRNA operons each consisting of the sequences coding for 16S rRNA, 23S rRNA, and 5S rRNA (4). In this report, we show that portions of each heterologous spacer between rRNA operons code for tRNA's.

Figure 1 examines the purity of the RNAs used as probes, showing the results of two-dimensional electrophoresis of the RNase T_1 digestion products of the 5S RNA and the 4S RNA prepared by electrophoresis on 10% acrylamide gels. The 5S RNA yields a simple pattern (Fig. 1A), indicating a relatively pure preparation of 5S RNA. This pattern is identical to the pattern resulting from analysis of a RNase T_1 digestion of 5S rRNA isolated from 50S ribosomal subunits. The more complex pattern seen in the digestion of 4S RNA (Fig. 1B) indicates that the 4S preparation contains a mixture of RNA species, as would be expected for the tRNA's. Most of the oligonucleotide sequences found in the 4S RNA preparation were not found in the 5S RNA preparation.

B subtilis 168 DNA was digested with sufficient restriction endonuclease *Sma* for the reaction to go to completion. The resulting fragments were separated by electrophoresis through 0.8% agarose, and the pattern was transferred to a nitrocellulose filter by the procedure of Southern (7). These filters were then used in RNA-driven hybridization reactions to assay for DNA fragments complementary to 5S rRNA or 4S RNA. In each hybridization reaction an excess of nonlabeled 16S and 23S rRNA was included, as described previously (4). After hybridization the filters were treated with RNase and washed, and autoradiographs were produced as seen in Fig. 2.

Each restriction enzyme fragment of DNA that hybridized 5S rRNA appeared to hybridize tRNA. In fact, most of tRNA was hybridized to fragments that also encoded 5S rRNA. The remaining tRNA hybridized to fragments that appeared to represent the 16S end of two rRNA operons. It therefore appears that there is no *Sma* recognition site between the DNA sequence coding 5S rRNA and the DNA sequences coding for many of the tRNA's. This linkage has also been demonstrated with restriction enzymes *Eco*RI and *Bam*HI (data not shown). It should be noted that no tRNA hybridizes to the 2.2-Kilobase *Sma* fragment that we have shown to contain the 16S-23S junction of the rRNA operons (4). Therefore, there are probably no tRNA genes in the spacer region between the genes for the 16S and 23S rRNA's, as found in *E. coli* (5).

It is possible that the apparent linkage of 5S and tRNA genes is the result of comigration of restriction fragments separately encoding 5S or tRNA. This is unlikely in all cases, and in fact, analysis of a cloned fragment of *B. subtilis* DNA in *E. coli* also shows that tRNA genes are linked to 5S rRNA genes. We recently described the structure of plasmid p12-E2, which contains a

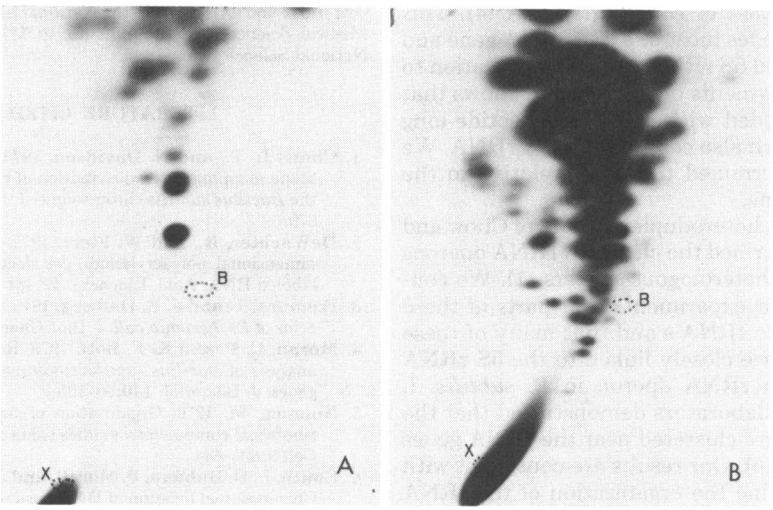

FIG. 1. *Two-dimensional electrophoresis of RNase T_1-digested RNA preparations. (A) 5S RNA; (B) mixture of 4s RNAs (tRNA's). X, Xylene cyanol marker; B, bromophenol blue marker. B. subtilis 168 was grown for three generations in a low-phosphate medium with 0.25 mCi of $^{32}PO_4$ per ml as previously described (4). After harvest and lysozyme treatment the cells were broken by the addition of sodium dodecyl sulfate to 2.5% in the presence of 10 μg of proteinase K per ml and 0.3% diethylpyrocarbonate. After phenol extraction and ethanol precipitation, the 4S and 5S RNAs were purified by electrophoresis on 10% acrylamide gels as previously described (4). In this report 4S RNAs are designated as tRNA's (3). ^{32}P-labeled 5S or 4S RNA was digested with a 10-fold excess of RNase T_1 (Sigma), and the products were analyzed by two-dimensional electrophoresis in acrylamide as described by DeWachter and Fiers (2). The only alteration made in this procedure was to use Tris-borate (pH 8.3) rather than Tris-acetate as the running buffer in the second dimension.*

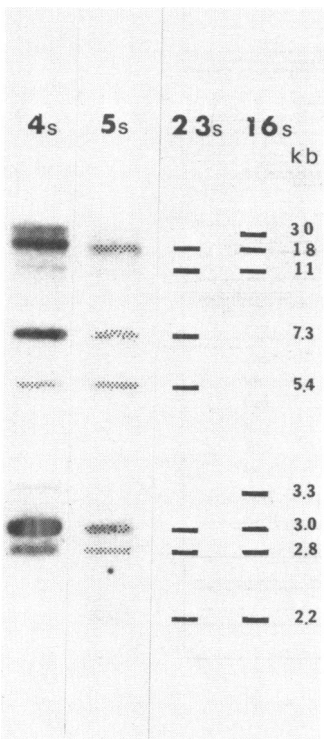

FIG. 2. *Autoradiographic localization of the 5S and tRNA (4S) coding sequences in a Sma digestion of B. subtilis DNA. The DNA was digested with Sma and then electrophoresed in 0.8% agarose. Purification of B. subtilis 168 DNA, restriction endonuclease digestion of DNA, and electrophoresis of the resulting fragments have been described previously (4). After electrophoretic separation, DNA fragments were denatured and transferred to nitrocellulose strips by the procedure of Southern (7). Hybridization of ^{32}P-labeled 5S or 4S RNA to the Southern filters was carried out in 3× SSC (SSC:0.15 M NaCl–0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate. Typically a filter containing 15 μg of the B. subtilis DNA fragments produced by endonuclease Sma digestion was sealed in a plastic bag containing 10 ml of buffer, 2 to 5 μg of ^{32}P-labeled RNA (~1×10^6 cpm/μg), and 20 μg each of nonlabeled 23S rRNA and 16S rRNA. After 18 h at 68°C, the filters were extensively washed, treated with RNase, and prepared for autoradiography as previously described (4). Phage λ DNA digested with HindIII was used as a molecular weight marker. Also depicted is a schematic representation of the 16S and 23S rRNA hybridization profile of Sma fragments as previously published (4). The addition of a mixture of nonlabled 23S rRNA and 5S rRNA does not affect the hybridization profile of ^{32}P-labeled 4S RNA.*

*Bam*HI fragment of *B. subtilis* DNA (4). This fragment encodes most of a 23S rRNA gene and a closely linked 5S rRNA gene. Hybridization to restriction fragments of this plasmid shows that tRNA is encoded within a 400-nucleotide-long fragment which also codes for the 5S rRNA. We have not determined the order relative to the 23S rRNA gene.

The elegant heteroduplex studies of Chow and Davidson described the clustered rRNA operons separated by heterologous spacers (1). We conclude from our experiments that parts of these spacers encode tRNA's and that many of these tRNA genes are closely linked to the 5S rRNA genes of each rRNA operon in *B. subtilis*. I. Smith and collaborators demonstrated that the tRNA genes are clustered near the rRNA genes of *B. subtilis* (6). Our results are consistent with those and define the organization of the tRNA genes and rRNA genes one step further.

Special thanks go to the late W. Steinberg for sharing with us several clones from his collection. We thank S. Bachenheimer for his help with the two-dimensional electrophoresis.

This work was supported by Public Health Service grants GM-07092 and CG-144 from the National Institute of General Medical Sciences and by grant no. PCM78-09814 from the National Science Foundation.

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Monthly	12	mem.$22;nonmem.$115

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