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Restriction Enzyme Analysis of *Bacillus subtilis* Ribosomal Ribonucleic Acid Genes

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The organization of the ribosomal ribonucleic acid (rRNA) genes (rDNA) of *Bacillus subtilis* was examined by cleaving the genome with several restriction endonucleases. The rDNA sequences were assayed by hybridization with purified radioactive rRNA's. Our interpretation of the resulting electrophoretic patterns is strengthened by an analysis of a fragment of *B. subtilis* rDNA cloned in *Escherichia coli*. The results indicated that there are eight rRNA operons in *B. subtilis*. Each operon contains one copy of the sequences coding for 16S, 23S, and 5S rRNA. The sequences coding for 5S rRNA were shown to be more closely linked to the 23S rRNA genes than to the 16S rRNA genes.

Bacillus subtilis has proved to be an extremely useful system for the study of molecular mechanisms involved in temporal gene expression during phage development and bacterial differentiation (10). More recently it has been used by Pace and his colleagues to demonstrate that the study of *B. subtilis* 5S rRNA is also a highly suitable model system for the study of RNA processing (17). All of these applications have profited from the extensive genetic characterization *B. subtilis* has received (15).

We have begun a study of the structural organization of the rRNA genes to gain insight into the regulation of ribosome biosynthesis, to provide data pertinent to the RNA processing studies, and to provide defined substrates for in vitro transcription studies. Our strategy for this study has proceeded on two planes. First, we have examined the structural complexity and organization of the rRNA genes by hybridizing labeled rRNA to the electrophoretically separated fragments of total chromosomal DNA produced by various restriction endonucleases. We believe this analysis will allow us to calculate the specific number of rRNA genes and to predict the results of alternative cloning strategies. Second, we have analyzed *B. subtilis* DNA fragments cloned in *Escherichia coli*. This report suggests that data from these two types of analysis substantiate each other.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* 168T$^+$ or a derivative, MO-101-P (from W. Steinberg) were used for RNA and DNA preparation. No differences in organization of the genes (rDNA) coding for rRNA or in rRNA content were detected with these strains. *E. coli* WS100 and plasmid p12-E2 were obtained from W. Steinberg. p12-E2 is one of a collection of clones produced by inserting *Bam*HI-digested fragments of MO-101-P into the vector pBR313.

Chromosomal DNA preparation. DNA was prepared by a modified Marmur procedure (8).

Restriction enzyme digestion. Restriction endonucleases *Bam*HI and *Eco*RI were prepared by the procedure of T. A. Bickle et al. (2), except Sepharose 4B was used instead of agarose. *Hin*dIII and *Sma* were purchased from New England Biolabs. All digestions were incubated with a three- to fourfold excess of enzyme, using the buffer conditions described in the 1978 New England Biolabs catalog.

Electrophoresis of DNA. Electrophoresis of DNA in 0.8% or 1.0% agarose was performed as previously described (15), except that 3-mm horizontal slabs were used. The resulting patterns were stained with 1 μg of ethidium bromide per ml, visualized with UV light, and photographed as previously described (15). The patterns were quantitatively transferred to nitrocellulose sheets (Millipore Corp.) as described by Southern (19).

Hybridization reactions. [^{32}P]rRNA was incubated with the Southern transfers in RNA-driven reactions at 68°C for 18 h in 3× SSC and 0.1% sodium dodecyl sulfate (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate). Ten milliliters of hybridization buffer, containing 5 μg of ^{32}P-labeled RNA plus 15 to 18 μg of competing nonlabeled RNA, was sealed in a plastic bag with the filters. After hybridization, the Millipore sheets were washed, RNase treated, and autoradiographed as previously described (15).

Preparation of nonlabeled 16S and 23S rRNA. 16S and 23S rRNA were prepared by phenol extraction of ribosomal subunits as described by Edgell et al. (8). After extraction of 50S subunits, 23S rRNA was separated from 5S rRNA by centrifugation through a 5 to 20% sucrose gradient as described by Potter et al. (15). 16S and 23S rRNA were judged to be greater than 98% pure by electrophoresis in 2% agarose.

^{32}P-labeled 16S and 23S rRNA. ^{32}P-labeled rRNA was prepared by labeling exponentially growing cells with 0.5 mCi of $^{32}PO_4$ per ml as described by Potter et al. (15). At an optical density at 500 nm of 1.0, 20 ml of labeled cells was harvested by centrifugation and redissolved in 5 ml of 50 mM Tris (pH 8.0), 50 mM EDTA, 15% sucrose, and 1 mg of lysozyme per ml. After incubation of 30 min at 37°C, 40 μl of diethylpyrocarbonate (Eastman) and 40 μg of proteinase K were added. Next, 0.25 ml of 10% sodium dodecyl sulfate was added, and the mixture was allowed to stand for 5 min at room temperature. The solution was then adjusted to 0.5 M potassium acetate and cooled on ice for 30 min to precipitate sodium dodecyl sulfate, protein, and chromosomal DNA. After centrifugation at 12,000 × *g* for 15 min, the RNA was precipitated from the supernatant solution with 2 volumes of 95% ethanol. The precipated RNA was redissolved in 0.14 ml of 1/10E buffer and 0.1% sodium dodecyl sulfate. This mixture was separated by electrophoresis through 2% agarose in E buffer at 10 V/cm until the bromophenol blue marker had migrated 20 cm. E buffer contains 0.04 M Tris, 0.02 M sodium acetate, and 0.001 disodium EDTA with the final pH equal to 7.2.

The RNA bands were stained with 1 μg of ethidium bromide per ml and visualized with UV light. The bands were sliced from the gel with a razor blade, and the gel slice was rinsed with deionized water. Each gel slice was then crushed by extrusion through a 20-ml disposable syringe without a needle. The crushed gel slice was placed into a 250-ml flask with 20 ml of 4× SSC and 0.2% sodium dodecyl sulfate, and the RNA was eluted by diffusion while shaking for 6 to 10 h at room temperature. The crushed gel was next filtered from the RNA solution by passage through a 0.45-μm-pore Nalge disposable filter.

^{32}P-labeled 5S rRNA. 5S rRNA was prepared by an electrophoresis method analogous to 16S and 23S rRNA except that the 5S preparation was phenol extracted three times and separated by electrophoresis through a 10% acrylamide gel in E buffer. 5S rRNA purified in this manner was shown to be identical to 5S rRNA isolated from 50S ribosomal subunits by comparing the RNase T_1 digestion products of each on two-dimensional acrylamide gels by the procedure of DeWachter and Fiers (7).

Electroelution. A 20-mg sample of *Bam*HI-digested *B. subtilis* DNA was electroeluted through a discontinuous electroelution device as described by Polsky et al. (14). The total digest was collected into 700 15-ml fractions.

Plasmid preparation. Plasmid DNA was prepared by cesium chloride-ethidium bromide centrifugation after amplification with chloramphenicol as described by Clewell and Helinski (4).

R-loop analysis. R-loops were formed in 70% formamide as described by Berget et al. (1). The reaction mixture was diluted 10-fold into a 70% formamide hyperphase. A 40% hypophase was used for spreading, followed by staining and shadowing with platinum/palladium and carbon according to Davis et al. (6).

DNA strand separation. A 1-μg sample of plasmid p12-E2 was completely digested with endonuclease *Bam*HI in a total reaction volume of 40 μl. The mixture was heated at 100°C for 10 min, then quickly cooled to 4°C and immediately applied to a horizontal agarose gel, where it was electrophoresed as described for double-stranded DNA. After the bromophenol blue marker had migrated 8 cm, the bands were visualized, photographed, and transferred to nitrocellulose as described in the preceding section.

In vitro labeling of DNA. After cleavage of p12-E2 with *Bam*HI, the 3′ ends were labeled using T4 DNA polymerase and [^{32}P]dATP in the presence of nonlabeled dCTP according to the conditions of I. R. Lehman (10). Cleavage of this labeled DNA was followed by conventional restriction enzyme analysis. DNA was uniformly labeled using *E. coli* DNA polymerase I and the nick translation conditions of Maniatis et al. (12).

Physical containment. All experiments involving recombinant DNA molecules were conducted in accordance with the *Guidelines for Research Involving Recombinant DNA Molecules* issued by the National Institutes of Health 23 June 1976.

RESULTS

B. subtilis DNA was digested with *Bam*HI or *Sma* restriction endonuclease, and the resulting fragments were separated according to size by agarose gel electrophoresis. The resulting patterns were transferred to nitrocellulose sheets, and the DNA fragments complementary to rRNA were assayed by hybridization with ^{32}P-labeled RNA (Fig. 1 and 2).

The intense 6-kilobase (kb) band in the *Bam* digest (Fig. 1) was resolved as two bands hybridizing to 23S rRNA or 5S rRNA and three bands hybridizing to 16S rRNA by using selected electroeluted fractions from this part of the digestion pattern (Fig. 3). The more rapidly migrating fraction collected from the electroelution (no. 170) contains less of the slower-migrating bands; therefore, the rapid band is not obscured from view. We therefore resolved the *Bam* digest into eight bands hybridizing 23S rRNA and eight bands hybridizing 16S rRNA. The banding pattern resulting from hybridizing 5S rRNA to *Bam* fragments is similar to the 23S *Bam* pattern (Fig. 1).

Hybridization of rRNA to the electrophoretically separated fragments from *Sma* cleavage of *B. subtilis* DNA is shown in Fig. 2. The most obvious feature of the patterns is that the 2.2-kb band is very intense in the 16S and 23S patterns but is completely absent in the 5S pattern. We also observed that, in the 16S *Sma* pattern, bands at 18 and 3 kb appear more intense than do bands at 30, 14, 3.3, and 2.8 kb. This immediately implied that bands at 18, 3, and 2.2 kb are probably composed of multiple fragments having identical mobility. The implication of this pattern will be discussed later.

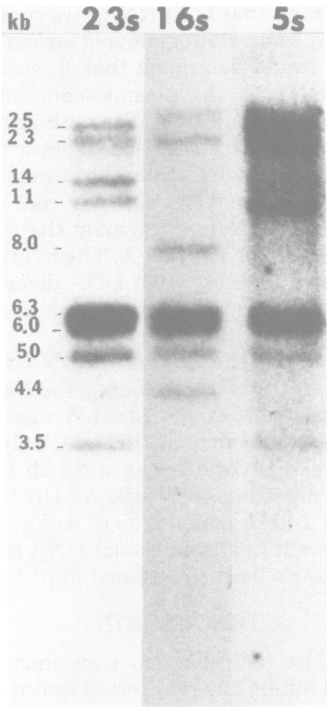

FIG. 1. *Autoradiographic localization of the 23S, 16S, and 5S rRNA coding sequences in the BamHI digestion of B. subtilis DNA. The DNA was digested with BamHI and then electrophoresed in 0.8% agarose. The DNA fragments were transferred to a nitrocellulose filter and hybridized with ^{32}P-labeled 23S, 16S, or 5S rRNA. Phage λ DNA digested with HindIII was used as a molecular weight marker. Some bands are purposely overexposed to show less intense bands.*

Analysis of cloned DNA. To complement our restriction enzyme digest of total *B. subtilis* DNA, we analyzed fragments of the *B. subtilis* chromosome cloned in *E. coli*. Strain 12-E2 was graciously provided from the clone bank of W. Steinberg. This clone was constructed by ligating *Bam*-digested *B. subtilis* DNA into the *Bam* site of the *E. coli* plasmid pBR313 (W. Steinberg, personal communication). Plasmid p12-E2 was identified by W. Steinberg as a clone carrying *B. subtilis* rDNA sequences by in situ hybridization with ^{32}P-labeled *B. subtilis* rRNA.

Figure 4 shows a map of restriction enzyme sites on the *B. subtilis Bam* fragment from plasmid p12-E2. This map was constructed by determining the size of the products resulting from single, double, and triple digestions. After complete *Bam* cleavage, partial *Hin*dIII digestion products were analyzed. The end pieces were identified by in vitro labeling of the 3′ ends of the *Bam* fragment, followed by digestion with *Hin*dIII. The fragments complementary to 23S or 5S rRNA were identifed by hybridization to Southern sheets as described for the chromosomal DNA digest (Fig. 5). Figure 4 shows that 23S rRNA hybridizes to the 2.7-kb *Bam/Hin*dIII fragment on both sides of the RI site. The 23S RNA did not hybridize to the 2.1-kb *Bam/Hin*dIII fragment. 5S RNA hybridized only to the 2.2-kb *Bam/Hin*dIII/RI fragment. These results are consistent with hybridization to single enzyme digest of p12-E2.

To determine how much of the 2.7-kb *Bam/Hin*dIII fragment codes for 23S rRNA, direct electron microscopic visualization of R-loops was used. In the 70% formamide buffer used, RNA-DNA hybrids are more stable than DNA-DNA strands; therefore, the rRNA displaces the homologous DNA strand, resulting in a loop of single-stranded DNA (1). Complete and partial *Bam* digests of plasmid p12-E2 were used in R-loop reactions with 23S rRNA. With complete *Bam* digest, the R-loops are found on the end of

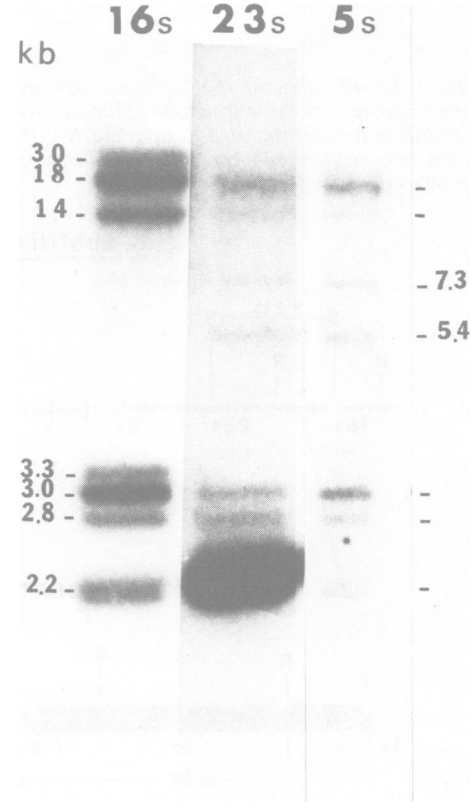

FIG. 2. *Autoradiographic localization of the 23S, 16S, and 5S rRNA coding sequences in the Sma digestion of B. subtilis DNA. The procedure outlined in Fig. 1 was followed, except the DNA was digested with the restriction endonuclease Sma.*

the 6-kb fragment, resulting in a forked structure rather than an actual loop. In partial digestions both loops and forks were found. Measurements of 10 R-loops show that 23S rRNA is complementary to 2.5 ± 0.1 kb of p12-E2. Figure 6 shows a partially digested plasmid dimer containing both a loop and a fork structure.

If the 5S and the 23S genes found on p12-E2 are part of the same transcriptional unit, one would expect that they should be coded for by the same DNA strand. After the insert of p12-E2 was cut out with *Bam*, it was denatured, and the strands were separated by electrophoresis on a neutral gel. Hybridization with 23S and 5S rRNA showed that both RNAs are complementary to the same slower-moving strand (Fig. 7).

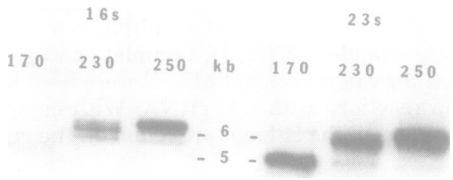

FIG. 3. *Localization of the 23S and 16S rRNA coding sequences in three fractions of an electroelution profile of BamHI-digested B. subtilis DNA. Each fraction was re-electrophoresed in 1% agarose and assayed as described in Fig. 1.*

Since it could be argued that *B. subtilis* and *E. coli* rDNA in the plasmid-containing host (WS100) might both hybridize with rRNA from *B. subtilis*, we are compelled to show that this cloned fragment comes from the chromosome of *B. subtilis* and not *E. coli*. *E. coli* (WS100) contains no 6.3-kb *Bam* fragment that codes for 23S rRNA but not 16S rRNA. The hybridization pattern for *E. coli* (WS100) DNA digested with *Bam* is the same as the pattern published for *E. coli* by Kiss et al. (9).

To show that the *B. subtilis* DNA fragment was not rearranged upon cloning, the 1-kb *Bam*/*Hin*dIII fragment of the plasmid was radioactively labeled in vitro with DNA polymerase. This fragment hybridized to a 6.3-kb fragment in a *Bam* digestion of *B. subtilis* DNA. Therefore this 1.1-kb fragment exists within a 6.3-kb *Bam* fragment in chromosomal DNA as well as within a 6.3-kb fragment cloned in p12-E2.

DISCUSSION

DNA-RNA hybridization experiments by I. Smith and others have suggested that the rRNA genes of *B. subtilis* are present in 7 to 10 copies (17). The density transfer experiments by Oishi and Sueoka (13) and Smith et al. (17) show that approximately 80% of the DNA that hybridizes

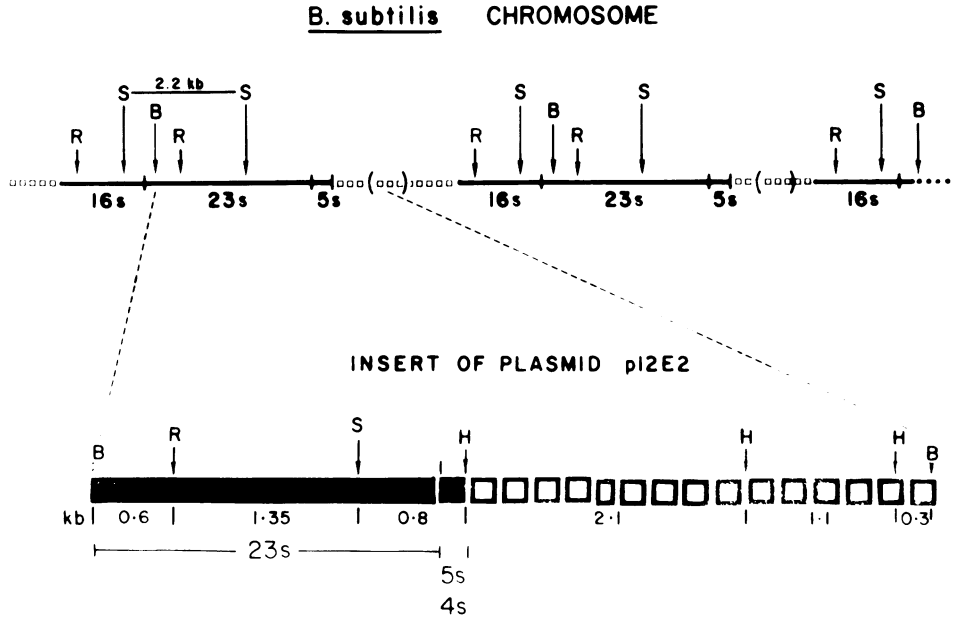

FIG. 4. *Restriction endonuclease map of cloned B. subtilis fragment (bottom) and proposed restriction enzyme map for rRNA operons of the B. subtilis chromosome (top line). Dark lines represent coding sequences; open squares represent heterologous spacer sequences. The top line shows only two and a half of the eight rRNA operons. R, EcoRI cleavage site; S, Sma; B, BamHI; H, HindIII.*

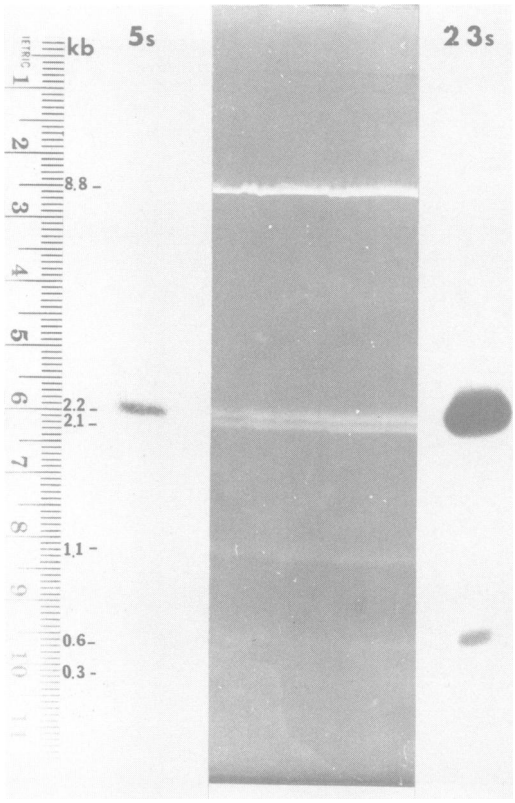

FIG. 5. *Localization of the 23S and the 5S rRNA coding sequences on p12-E2. Plasmid p12-E2 was digested with BamHI, HindIII, and EcoRI restriction endonucleases, and the digest was analyzed on 1% agarose gels. The DNA fragments were transferred to nitrocellulose and hybridized to 23S rRNA or 5S rRNA. This figure shows the ethidium bromide-stained gel (middle) and the autoradiographs of the filters used in the hybridizations. The molecular-weight standards used were λ DNA cleaved with HindIII or EcoRI and φX174 DNA (RF) cleaved with HaeIII.*

to rRNA replicates very early, suggesting that these genes are clustered near the origin of chromosome replication. The remaining 20% of hybridizable material replicates very late, suggesting the existence of a second chromosomal locus. Transcriptional studies by Zingales and Colli (20) show that the rRNA genes are organized in transcriptional units transcribed in the order 16S rRNA, 23S rRNA, and finally 5S rRNA. An elegant heteroduplex experiment by Chow and Davidson (3) provides evidence that the rRNA operons are clustered but separated by heterologous spacers of various lengths.

We began our interpretation of the restriction enzyme analysis of *B. subtilis* chromosomal DNA with the assumption that restriction enzyme recognition sites are more likely to be conserved within the rRNA operons than in the heterologous spacers surrounding each operon. The validity of this assumption will be finally proved only by analyses of each purified rRNA operon.

The *Bam*HI digestion pattern shows eight fragments hybridizing 23S rRNA and eight fragments hybridizing 16S rRNA. Since some fragments hybridize only 16S or only 23S rRNA, we suggest that a *Bam* recognition site exists near the 16S–23S junction within each operon (see Fig. 4). At least two events can lead to the appearance of bands that seem to hybridize both 16S and 23S rRNA. One is that there may be fortuitous comigration of two DNA fragments, one coding for 16S rRNA and the other for 23S rRNA. Another is that a 16S gene and a 23S gene may occur on the same fragment when there is not a *Bam*HI cleavage site within the heterologous spacer region between two operons with similar polarity. We believe that both the first and second types of event occur in *Bam*-digested *B. subtilis* DNA. An example of the first type can be found in the 6-kb region, and an example of the second may be seen in the 5-kb band.

If one accepts this model of one recognition site for the *Bam*HI endonuclease per operon, then there must be eight rRNA operons in each bacterial chromosome. The banding patterns for 23S and 5S rDNA's are identical in *Bam*HI digest; therefore, 5S is more closely linked to 23S than to 16S, as is predicted by transcriptional studies that show the operon is transcribed in the order 16S, 23S, and finally 5S rRNA (20). Colli and his colleagues have also demonstrated the map order of 16S, 23S, and 5S rRNA genes by hybridization of rRNA's to randomly sheared fragments of DNA fractionated by Cs_2SO_4-$HgCl_2$ isopycnic centrifugation (5).

We believe our results (Fig. 4) are consistent with the model that endonuclease *Sma* cleaves each rRNA operon twice, once in the 16S gene and once in the 23S gene. This pattern results from the generation of a homogeneous middle fragment of 2.2-kb size encoding 16S and 23S rRNA but not 5S rRNA, eight end pieces hybridizing 23S rRNA and 5S rRNA, and eight pieces from the 16S end of each operon. The intensity of bands at 18 and 3.0 kb in the 16 S *Sma* hybridization indicates that they may be doublets. Since an RNase treatment completes each hybridization reaction, the intensity of a band on the autoradiograph will be approximately proportional to the amount of coding sequence present in that band. According to our model (Fig. 4), the 2.2-kb fragment in the *Sma*

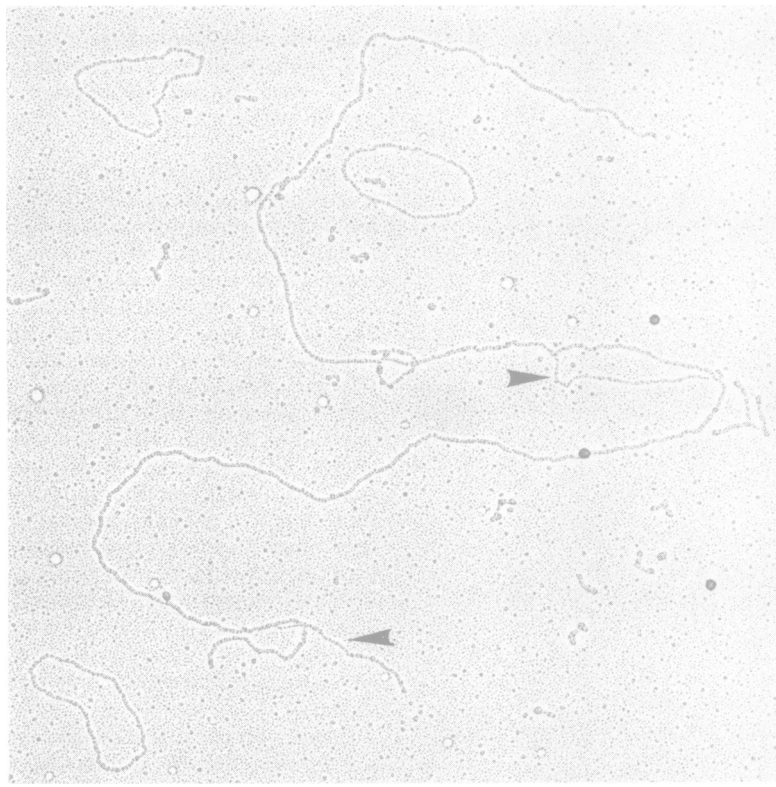

FIG. 6. *R-loop resulting from hybridization of 23S rRNA to p12-E2 which had been incompletely digested with BamHI. Photo shows a dimer of p12-E2 that has been cut once with BamHI. This plasmid dimer has one R-loop in the middle of the molecule and one on the end of the molecule, resulting in a forked structure. φX174 (+ strand) circular DNA is used as a size reference for single-stranded DNA.*

digest contains eight copies of the middle region of the rRNA operons. This middle-region fragment probably contains more 23S rRNA sequences than 16S rRNA sequences, since the relative intensity of the 2.2-kb fragment is so much greater in hybridization with 23S rRNA than with 16S rRNA.

Cloning and analysis of all of the rRNA operons of *B. subtilis* is our ultimate goal, but, based on the heteroduplex experiments of Chow and Davidson (3) and our own preliminary data, we expect that any one rRNA operon will be structurally analogous to the others.

Analysis of p12-E12 has shown that the restriction sites in this operon are present in the order we predicted from digests of total DNA. Based on *Bam*HI cleavage of total DNA we predicted a *Bam* site near the junction of the 16S and 23S rRNA coding region within an operon. The R-loop analysis shows that p12-E2 codes for approximately 2.5 kb of 23S rRNA, which is almost all of the 3 kb needed for the 23S rRNA; therefore, the *Bam* site is near the 16S–23S junction of the operon.

The 23S gene of p12-E2 is closely linked to a 5S gene encoded by the same strand of DNA, as would be expected if the 23S and 5S genes were part of the same operon. Chow and Davidson (3) showed that there was no heterologous spacer greater than 50 bases long between the 16S and 23S genes of a single operon, and we have shown that the non-rRNA coding region of p12-E2 is a unique sequence of DNA too long to be an intraoperon spacer between 23S and 16S rRNA genes. The 23S and 5S rRNA genes of p12-E2 are therefore part of one rRNA operon, and the *Bam*HI recognition site in the 23S coding region is the site between the 16S and 23S genes as predicted from the *Bam* digestion of total DNA.

We predicted that there should be a *Sma* cleavage site within each 23S gene, and there is one in the 23S gene of p12-E2. Based on the intensity of the bands in the *Sma* digest of total DNA, we suggested that the 2.2-kb middle fragment contained mostly 23S gene and a little of the 16S gene. The *Sma* cleavage site in p12-E2 is approximately 2 kb from the 16S–23S junction; therefore, only a very small fragment of 16S gene may be present in the *Sma* 2.2-kb fragment of total *B. subtilis* DNA. If we assume

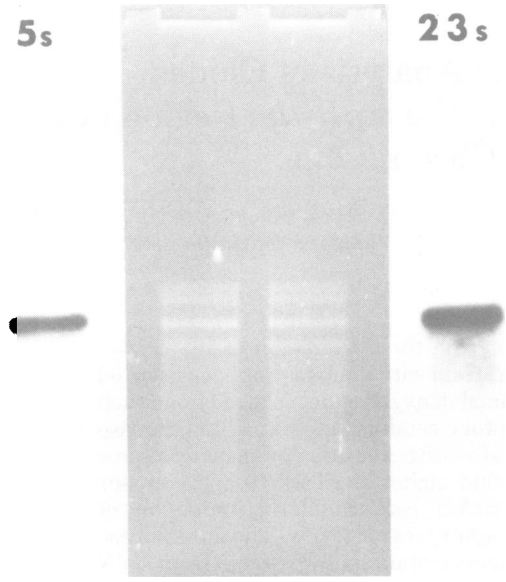

FIG. 7. *Localization of 5S and 23S rRNA coding sequences on the separated DNA strands of p12-E2. After digestion of p12-E2 with BamHI, the DNA was heat denatured, and strands were separated by electrophoresis on a neutral 1% agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized to 5S or 23S rRNA. Controls show that the top two bands are the separated DNA strands of pBR313 (the parental plasmid).*

that 1.5 kb is required to code for the 16S rRNA and that 0.2 kb of this gene is present in the 2.2-kb fragment, then each single 16S band should represent 1.3 kb. If there are eight middle fragments at the 2.2-kb band, each containing 0.2 kb of coding sequences, we would expect the 2.2-kb band to be more intense than each of the other bands representing single 16S genes. The width of the 2.2-kb band in the *Sma* digest of total DNA may reflect some small heterogeneity in the middle regions of the rRNA operons. The 2.2-kb *Sma* fragment contains two-thirds of each 23S gene, multiplied by eight genes; therefore, it is at least 16 times as intense as the other 23S bands seen in the *Sma* digest of total *B. subtilis* DNA. Preliminary analysis of five other clones and the previous reports of Potter et al. (15) are all consistent with our conclusions, but a complete analysis of all the cloned rRNA operons will be necessary for confirmation of this description of the organization of the genes.

ACKNOWLEDGMENTS

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