Two-Dimensional Restriction Analysis of the Bacillus subtilis Genome: Gene Purification and Ribosomal Ribonucleic Acid Gene Organization

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Received for publication 7 July 1976

With two-dimensional restriction enzyme analysis we have been able to cleave the Bacillus subtilis genome and resolve the resulting deoxyribonucleic acid (DNA) segments into discrete bands on agarose gels. A general procedure for gene purification has been developed by coupling multidimensional restriction analysis with a biological assay for gene detection. The organization of ribosomal ribonucleic acid (rRNA) genes was studied by hybridizing 16S and 23S rRNA probes to the two-dimensional DNA banding patterns.

It is generally believed that there are certain principles common to differentiation in all organisms. These principles may be most easily examined in a simple prokaryotic organism that undergoes differentiation such as Bacillus subtilis. The eventual understanding of sporulation (and differentiation) is the goal of this work.

The in vitro study of isolated genes should prove to be a powerful approach to understanding differential gene expression. Various factors can be added to the in vitro system, and one can determine what control elements are involved in turning specific genes on and off. In this report we describe a general procedure for purifying genes. We have used this procedure to purify a single species of deoxyribonucleic acid (DNA) segment that contains the B. subtilis metB gene.

Translational controls (1, 8, 11, 12, 18) as well as transcriptional controls (13, 17) are probably involved in differentiation. Translational control could be the effect of altering transfer ribonucleic acid (tRNA) concentrations, messenger RNA (mRNA) masking, and/or the result of heterogeneities in translational machinery at either the protein or ribosomal RNA (rRNA) level. In this paper we present new information concerning the structure and organization of rRNA genes.

A simple procedure has recently been developed that allows the resolution of complex genomes into discrete bands of specific DNA segments (16). This procedure involves first cleaving the genome with one restriction enzyme and then fractionating the resulting DNA segments according to size on an electroelution device. The DNA in each fraction is then cleaved with another restriction enzyme, after which it can either be analyzed on a slab gel or again fractionated on an electroelution device. We have employed this procedure, termed multidimensional restriction analysis, in our study of the B. subtilis genome. By coupling multidimensional restriction analysis with a simple bioassay (the transformation assay), we have been able to purify unique DNA segments with known gene activity. By coupling multidimensional restriction analysis with the transfer procedure of Southern (20) and subsequent RNA-DNA hybridization, we have been able to study the organization of the genes coding for rRNA in B. subtilis.

MATERIALS AND METHODS

Bacterial strains and cell growth, labeling, and DNA extraction procedures. Bacillus subtilis strain 168T+ (a prototrophic transformant of the strain 168 originally obtained from J. Spizizen) was grown in a chemically defined modification of the growth medium proposed by Sterlini and Mandelstam (21). The final concentration of PO₄ was changed to 3 × 10⁻⁴ M, the medium was buffered with 0.1 M tris(hydroxymethyl)aminomethane (Tris, pH 7.2), 0.1% glucose was added, and 1× non-essential amino acids and minimal essential medium amino acids (Grand Island Biological Co.) were substituted for casein hydrolysate. The cells were grown with vigorous aeration at 37°C to a density of 10 cells/ml. Carrier-free ³²PO₄ (New England Nuclear Corp.) was then added to a final concentration of 250 μCi/ml, and the cells were grown an additional 1.5 h. Cell lysis and DNA extraction were performed as previously described (16).

No plasmid band of DNA was detected in this B. subtilis 168T+ strain (see also reference 14). The
soluble portion of cell extracts was subjected to electrophoresis in 0.5% agarose after precipitation of chromosomal DNA with 1.0 M NaCl. The gel was stained with ethidium bromide by incubating it for 2 to 3 h at room temperature in a solution of electrophoresis buffer containing ethidium bromide at a final concentration of 1 μg/ml. Visualization of the wet gels with ultraviolet light (366 nm) in a dark room was sufficient to visualize all three forms of plasmid pSC101 in *Escherichia coli* C600.5 as well as plasmids in other bacterial strains (Charles Moran, personal communication).

Multidimensional restriction analysis procedure. The procedure used was essentially identical to that previously described (16). The 32P-labeled DNA was first digested with one restriction enzyme and then fractionated on an electrophoretion device similar to that described by Lee and Sinheimer (10). This device is essentially a 12-mm-diameter disc gel apparatus containing 0.8% agarose with a mechanism at the bottom for collecting the DNA segments as they are electrophoretically separated and driven out of the gel. About 2.5 μg of unlabeled carrier DNA from the bacteriophage λDNA was added to each fraction and the salt concentration was increased to 0.15 M NaCl. The DNA in each fraction was then precipitated with 2 volumes of isopropanol and kept at −20°C for at least 12 h. The DNA was then pelleted by spinning in a Sorvall HB-4 rotor at 8,000 rpm for 25 min. The DNA pellets were air dried and dissolved in digestion buffer for the second enzyme. After the second digestion the DNA was analyzed on agarose slab gels as previously described (16). The slab gels were then stained with ethidium bromide (1 μg/ml), and the banding pattern of the lambda DNA in each channel was examined as a check for complete digestion with the second enzyme. The gels were then dried and used for autoradiography (6). Complete digestion with the first enzyme was tested by removing a portion of the initial digestion mixture and adding a small amount of lambda DNA labeled to a high specific activity with 32P. This portion was then analyzed by agarose gel electrophoresis following by autoradiography. If the lambda DNA in this sample was completely digested, then this was assumed to indicate that the *B. subtilis* DNA in the initial digestion mixture was also completely digested.

Transformation assay. A simple spot test transformation assay was employed to test for the presence of the *metB* gene. *B. subtilis* strain BR151, auxotrophic for lysine, tryptophan, and methionine, was grown to competence (2) and used in the assay. The competent cells were spread on an agar plate containing the minimal ingredients for growth except for the amino acid methionine. A 5-μl amount of DNA solution that had been concentrated by isopropanol precipitation as described before and resuspended in a small volume of sterile saline citrate (0.15 M NaCl plus 0.015 M sodium citrate (SSC)) was then spotted on the competent cells, and the plates were incubated for 2 days. The appearance of colonies indicated the presence of the *metB* gene in the DNA solution. Colonies were then streaked onto plates lacking lysine and onto plates lacking trypto-
Photographic reproduction. To enhance the photographic reproducibility of autoradiographs, the contrast was increased and the X-ray film background was simultaneously decreased by contact-printing the autoradiograph onto Techifax double-coated, black, direct positive Diazochrome film no. KBKD (Scott Graphics Inc., Holyoke, Mass.). The autoradiograph was placed in a glass photographic contact printer, exposed to long-wave ultraviolet light from two 15-watt GEF15T8BL light bulbs at a distance of 12 inches (ca. 30.48 cm) for 40 min, and developed in a sealed jar containing concentrated NH₄OH vapor. The resulting "diazo-direct positive" was photographed on a Lucite light box (or X-ray viewer), using Kodak high-contrast copy film to produce the negative for the figures shown here.

RESULTS

Two-dimensional banding patterns. Figure 1 shows a two-dimensional restriction analysis pattern of B. subtilis DNA. In this case the ³²P-labeled DNA was first cleaved with the EcoRI enzyme and then fractionated on an electroelution device containing a 1% agarose gel. The DNA in each resulting fraction was then digested with the SmaI enzyme and analyzed on a 1% agarose slab gel. Figure 1 is an autoradiograph of these dried slab gels.

Very small DNA segments migrate rapidly through the gel of the electroelution device and are collected in the first fractions. Because of their small size, most of these DNA segments do not contain cleavage sites for the second enzyme and, therefore, migrate as a dense unresolved band on the slab gel. In later fractions the DNA segments are longer and have more cleavage sites for the second enzyme. This results in a reduction in the intensity of the dense unresolved band in later fractions.

Reversing the order in which the restriction enzymes are used does not alter the specific DNA segments that are produced in the end, but it does change the resulting two-dimensional banding pattern. Generally it is preferable to perform the first cleavage with the enzyme that will introduce relatively few breaks into the DNA. This reduces the number of small DNA segments initially produced and reduces the number of DNA segments that will escape cleavage by the second enzyme. The net result is an improved separation of DNA segments in the second dimension. Figure 2, for example, shows a two-dimensional banding pattern in which the B. subtilis DNA was first digested with SmaI (an enzyme that puts in fewer breaks than EcoRI) and then with EcoRI.

Fig. 1. Two-dimensional banding pattern of B. subtilis DNA. In this case the EcoRI enzyme was used first and the SmaI enzyme was used after electroelution. The first fractions are at the left. Size markers are EcoRI cleavage products of lambda c1857 S7 DNA. The molecular weights of the size markers shown (10⁶) were determined by Thomas and Davis (22). These two-dimensional banding patterns are composite pictures of autoradiography of several slab gels.
It is clear that the dense unresolved bands shown in Fig. 2 are much less intense than those in Fig. 1.

**Gene purification.** By simply adding a biological assay to the multidimensional restriction analysis procedure, it becomes possible to purify genes. We have employed a transformation assay to test for the presence of the *metB* gene.

As usual, the $^{32}$PO$_4$-labeled DNA was first digested with a restriction enzyme (in this case, *SmaI*) and then fractionated on an electroelution device (with a 0.8% agarose gel). The DNA in each fraction was subsequently tested for the presence of the *metB* gene. The DNA in the fraction of interest was then cleaved with the second enzyme (*EcoRI*) and again fractionated on an electroelution device (0.9% agarose). Figure 3 shows the resulting radioactivity and biological activity distributions for two separate preparations of this gene. The peak of biological activity was found to be associated with a peak of radioactivity.

These peaks can be matched with specific bands in the two-dimensional banding pattern. The purified gene (from the fraction with the peak of biological activity after the second electroelution) was analyzed by electrophoresis with *EcoRI*-digested DNA from the fraction containing the *metB* gene after the first electroelution. The resulting banding patterns are shown at the top of Fig. 3. The purified gene was found to have the same apparent mobility as the top band of a doublet. Apparently this doublet was not well resolved on the electroelution device, and this explains why the peak of biological activity was found in the top portion of the corresponding peak of radioactivity.

The size of the DNA segment containing the *metB* gene was estimated to be 2,500 base pairs. The position of the corresponding band in the complete two-dimensional pattern is shown in Fig. 2.

It is worth noting that the radioactivity distributions are not identical in the two purifications depicted in Fig. 3. In fact, some large peaks present in one profile are absent in the other. This is because during the first electroelution two DNA segments of a nearly identical size can be collected into the same fraction or into adjacent fractions, depending on the precise moment at which the fraction collector shifts. Thus, on one occasion two bands might be in the same channel, whereas on a separate occasion they will be found in adjacent channels. This presents a complication in multidimensional restriction analysis that we have not been able to eliminate.

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**Fig. 2. Two-dimensional banding pattern of *B. subtilis* DNA.** In this case the DNA was digested first with *SmaI* and then with *EcoRI*. The size markers are the same as those for Fig. 1. Triangles point to bands that hybridize to either 16S or 23S rRNA. The locations of bands too faint to be seen in this autoradiograph are not marked. Bands 5, 15, 20, and 21 (see Fig. 4 for numbering) only become visible after hybridization to rRNA labeled to a higher specific activity with $^{32}$PO$_4$. The thick arrow in the center points to the bands containing the *metB* gene. The two thin arrows at the right edge point to two long horizontal lines formed by bands of similar mobilities in adjacent channels (see text). The electroelution device contained 0.8% agarose, and the slab gels contained 1.4% agarose.
We should add that there are frequently "overlaps" during electroelution. A single species of DNA segment can be collected into two adjacent fractions. The faint bands sometimes seen at high-molecular-weight positions are the result of this overlap.

rRNA genes. Figure 2 shows the two-dimensional pattern that results when B. subtilis DNA is first cleaved with SmaI and then EcoRI. Southern (20) has developed a procedure for transferring DNA segments from gels to nitrocellulose sheets, and Fig. 2 is, in fact, an autoradiograph prepared from four such sheets. The DNA used was labeled to a low specific activity with $^{32}$PO$_4$ and the autoradiograph shown in Fig. 2 was developed after a 5-day exposure. The DNA fragments present in nitrocellulose sheets were then hybridized to rRNA (16S or 23S), which was labeled to a high specific activity with $^{32}$PO$_4$. After hybridization the sheets were again used for autoradiography, but this time the exposure period was reduced to about 12 h. Figure 4 shows a resulting pattern. We thus identified those bands containing sequences homologous to 16S and 23S rRNA. The results are summarized in Fig. 2 and 4 and Table 1.

DISCUSSION

Gene purification. Through multidimensional restriction analysis we have been able to cleave the B. subtilis genome and resolve the resulting DNA segments into discrete bands on agarose gels. Restriction enzymes have proven to be extremely useful tools in the study of viral genomes, and it is hoped that this usefulness might now be extended to the study of more complex genomes.

Gene purification through multidimensional restriction enzyme analysis is straightforward
and applicable to any system for which there is an appropriate biological assay or hybridization probe. The multidimensional approach is an extension of important earlier work (9), in which a 60-fold enrichment of some genes, including metB, was achieved after digestion of B. subtilis DNA with a single restriction enzyme and electrophoresis on a gel.

Under certain circumstances multidimensional restriction analysis might allow gene purification without the use of a biological assay or hybridization probe. For example, B. subtilis mutants containing a variety of large deletions in the tryptophan operon are available (3). The mobilities of the resulting DNA segments containing these deletions would be altered, and therefore a simple comparison of normal and mutant banding patterns should allow the identification of the band(s) containing the tryptophan operon. The band(s) could then be cut from the gel and the DNA removed by the "freeze squeeze" method (23).

Thus, the banding pattern itself is a detailed fingerprint of the genome and conveys useful information. It is interesting to note that there is an unexpected peculiarity in the banding patterns. Arrows in Fig. 2 point to two long lines formed by bands of apparently identical mobilities in adjacent channels. These lines extend across the entire width of the pattern at the mobilities of the arrows. Similar lines have been observed in E. coli two-dimensional banding patterns (16). The significance of this observation, if any, is not yet known.

rRNA genes. It has been known for some time, as a result of saturation hybridization studies (19), that there are several (5 to 10) copies of 16S and 23S rRNA genes per B. subtilis genome. Colli et al. (5) later showed that the 16S genes are closely linked to 23S genes. Chow and Davidson (4), using an electron microscope, conducted an elegant study of B. subtilis DNA which was first denatured and then allowed to partially renature. Chow and Davidson concluded that the gene sets (each gene set contains one 16S gene and one 23S gene) are separated by spacer DNAs that vary in both size and sequence. These spacers were assigned sites and the gene sets were arranged into several clusters or linkage groups.

On the basis of the data presented above we have concluded that the SmaI and EcoRI restriction enzyme cleavage sites within rRNA gene sets are positioned as shown in Fig. 5. The arguments supporting this map are discussed below.

Figure 5 shows that the initial cleavage with SmaI will divide each gene set into a 16S end piece, a 23S end piece, and a center section. EcoRI cleavage of the center section then divides it into two DNA segments. One of these DNA segments will hybridize to only 23S rRNA and is represented by band 2 in Fig. 4. The other center section DNA segment will hybridize to both 16S and 23S rRNA's and is represented by band 1 in Fig. 4. Estimates of the multiplicity (number of copies per genome) of these DNA segments were made by scanning autoradiographs of the DNA banding patterns with a Joyce Loebl microdensitometer. These estimates are only approximate, but it was found that there are about 10 copies of these center-section DNA segments generated per genome (see Table 1). This number agrees well

![FIG. 5. Map of the relative positions of EcoRI and SmaI cleavage sites within the rRNA gene sets. A and B represent the heterogeneous DNA sequences external to the gene sets. The distances between cleavage sites are known (100 daltons), but the positions relative to the ends of the gene sets are only estimated.](http://jb.asm.org/)

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a Bands are numbered as in Fig. 4.
b Sizes of smaller DNA segments were determined with HaeIII cleavage products of simian virus 40 DNA as size markers.
c Estimates of multiplicity were made by scanning the autoradiographs of the DNA banding patterns shown in Fig. 4 with a Joyce Loebl microdensitometer. These values are only approximate. Very faint bands were simply assigned multiplicities of 1.
with previous estimates of the multiplicity of rRNA gene sets (4, 19).

It is important to note that bands 1 and 2 are always found in the same channel(s) in the two-dimensional restriction analysis, and the sizes of the DNA segments in bands 1 and 2 sum to the size of the DNA segments in this fraction before EcoRI cleavage. This is compatible with the map of restriction enzyme cleavage sites shown in Fig. 5.

The end-piece DNA segments generated by two-dimensional restriction analysis will vary in size because of heterogeneities in the external DNA environments of the gene sets. These end pieces, therefore, are found at different locations in the two-dimensional banding pattern. We, in fact, find five distinct bands that will hybridize to only 23S rRNA (not including the highly reiterated band 2).

Chow and Davidson (4), as mentioned before, have shown that the spacer DNAs connecting gene sets vary in both size and sequence. We find that two of these spacers contain no SmaI or EcoRI cleavage sites. The result is that there are two cases in which the 16S and 23S end-piece DNA segments are still connected by spacer DNA after two-dimensional restriction analysis. Bands 4 and 6, in particular, are found to hybridize to both 16S and 23S rRNA. One can estimate the differences in the sizes of these spacers by assuming that in each of these DNA segments there is an equal amount of 16S and 23S end-piece DNA. One then simply subtracts the size of the smaller DNA segment from that of the larger. The spacer in band 4 is thereby found to be 230 base pairs smaller than the spacer in band 6. This agrees fairly well with the figures obtained by Chow and Davidson (4). They estimated that the smallest spacer is 140 base pairs in length and the next smallest is 280 base pairs in length. The size difference would therefore be 140 base pairs.

Band 19, which also hybridizes to both 16S and 23S rRNA, is the result of near comigration of two end-piece DNA segments. Careful examination of Fig. 4 shows that band 19 overlaps across three channels. The band in the left of these three channels has a slightly greater mobility than the band at the right. Hybridization studies (not shown) demonstrate that the left band hybridizes to only 16S rRNA, whereas the band at the right hybridizes to only 23S rRNA. Band 19 is therefore the result of near comigration of a 16S end-piece DNA segment and a 23S end-piece DNA segment. Band 19 does not contain an uncleaved spacer DNA.

It is now possible to count a total of eight 23S end pieces. There are the five in bands 12, 13, 16, 17, and 18 plus the three in bands 4, 6, and 19. This agrees well with our previous estimate of 10 rRNA gene sets per genome derived from the multiplicity of DNA segments in bands 1 and 2.

The interpretation of the bands that hybridized to only 16S rRNA is complicated somewhat by the existence of the EcoRI cleavage site within this end piece (Fig. 5). The result is that the second digestion, with EcoRI, will cleave each 16S end piece into two DNA segments that will hybridize to 16S rRNA. One of these DNA segments is small and constant in size, whereas the other is larger and variable in size because of the contiguous heterogeneous external DNA.

Band 3 in Fig. 4 is thought to be one of these small DNA segments resulting from EcoRI cleavage of a 16S end piece of rDNA. Other hybridization studies (insert of Fig. 4) have demonstrated that in this case the larger resulting 16S DNA segment is obscured by the very dense band 1 in Fig. 4. The sizes of the large DNA segment and the DNA segment in band 3 sum to the size of the DNA segments in this fraction before cleavage with EcoRI.

A similar result is found for the other 16S end pieces. The EcoRI treatment in the second dimension of analysis cleaves a small piece of DNA, which migrates at the same mobility as band 3. Band 5, for example, is thought to be a smaller DNA segment cleaved from the 16S end piece in band 4. Band 7 is thought to contain DNA cleaved from the 16S end piece in band 6. It is important to note that in both of these cases the sizes of the small and large 16S DNA segments correctly sum to the size of the DNA segments in that respective fraction before EcoRI cleavage.

DNA segments in bands 9, 15, and 21 are also thought to be the result of EcoRI cleavage of 16S end-piece DNA segments found in bands 8, 14, 19, and 20.

There is, however, one apparent exception to the map presented in Fig. 5. Bands 10 and 11 (Fig. 4) hybridize to only 16S rRNA, but in the channel in which they are located there is no band at the mobility of bands 3, 5, 7, 9, 15, and 21. There are several possible explanations of this anomalous observation. Perhaps bands 10 and 11 are the result of a microheterogeneity in the sequence of DNA coding for 16S rRNA. In principle, only two base changes (one to eliminate one cleavage site and one to create another) are necessary to cause the observed pattern change. Or possibly the DNA in bands 10 and 11 has evolved from a 16S rRNA gene to fulfill a different function and yet retains some homology to the 16S rRNA probe. Bands 10 and 11 are in fact much less intense than the other bands (bands 6, 12, and 18) of large DNA seg-
ments that hybridize to 16S rRNA. Another possible explanation is that bands 10 and 11 are the result of a stable mRNA that copurifies with 16S rRNA.

Careful examination of Fig. 4 reveals that there are other faint bands that were not assigned numbers. These bands were generally thought to be either the result of overlap from adjacent fractions or the result of the small amount of radioactivity present in the DNA itself.

If one ignores bands 10 and 11, then it is possible to count a total of eight 16S end pieces. Bands 4, 6, 14, 19, and 20 each contain one 16S end piece. There is one 16S end piece obscured by band 1, and band 8 is estimated to contain two end pieces, making a total of eight. This number agrees well with the number of 23S end pieces and with the multiplicity estimate of bands 1 and 2. We cannot, however, conclude that there are exactly eight rRNA gene sets per genome because of uncertainties in the multiplicity estimates of the end-piece DNA segments. There is a minimum of 8 gene sets, but there could possibly be as many as 10 or 11.

The identification of the bands containing rRNA genes might aid future efforts to purify these DNA segments. Fine structure analysis of the rRNA genes and their DNA environments would of course be greatly simplified if pure DNA segments were available.

ACKNOWLEDGMENTS

This work was supported by grant NP 131A from the American Cancer Society and Public Health Service grants GM 21313, GM 01138, GM 00685, and GM 07092 from the National Institute of General Medical Sciences. S.P. was the recipient of a Public Health Service predoctoral traineeship and a Stan and Leora fellowship.

LITERATURE CITED