tRNA genes are found between the 16S and 23S rRNA genes in *Bacillus subtilis*

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ABSTRACT

There are at least nine, and probably ten, ribosomal RNA gene sets in the genome of *Bacillus subtilis*. Each gene set contains sequences complementary to 16S, 23S and 5S rRNAs. We have determined the nucleotide sequences of two DNA fragments which each contain 165 base pairs of the 16S rRNA gene, 191 base pairs of the 23S rRNA gene, and the spacer region between them. The smaller spacer region is 164 base pairs in length and the larger one includes an additional 180 base pairs. The extra nucleotides could be transcribed into tRNA^{Ile} and tRNA^{Ala} sequences. Evidence is also presented for the existence of a second spacer region which also contains tRNA^{Ile} and tRNA^{Ala} sequences. No other tRNAs appear to be encoded in the spacer regions between the 16S and 23S rRNA genes. Whereas the nucleotide sequences corresponding to the 16S rRNA, 23S rRNA and the spacer tRNAs are very similar to those of *E. coli*, the sequences between these structural genes are very different.

INTRODUCTION

Ribosomal ribonucleic acid (rRNA) operons in bacteria can contain tRNA genes which are co-transcribed with the rRNA. In *E. coli* these are found in the spacer regions between the 16S and 23S rRNA genes and distal to the 5S rRNA gene (1,2,3). To examine the generality of this pattern of organization in bacteria we chose to look for spacer tRNA genes in the rRNA genes (rDNA) of *B. subtilis*.

According to recent reports, the *B. subtilis* chromosome contains 8 or 9 rRNA gene sets (4,5). Each rRNA gene set contains a 16S, a 23S and a 5S rRNA gene; the gene order within a rRNA gene set is 16S-23S-5S (6,7). Some evidence for co-transcription of the entire gene set has been obtained (8), but no large precursor rRNA has been identified. It has been proposed that the DNA sequences between the 16S and 23S rRNA genes are identical to each other in *B. subtilis* (5,7,9) and that there are no tRNA genes in these spacer regions (spacer tRNA genes). However, this paper presents evidence that at least one, and most likely two, of the rRNA gene sets of *B. subtilis* contain

both tRNA^{Ile} and tRNA^{Ala} genes in the spacer region between the 16S and 23S rRNA genes.

MATERIALS AND METHODS

Strains

B. subtilis 168 was obtained from G. Chambliss (University of Wisconsin-Madison). *E. coli* C600 and HB101 were used for transformations, K802 was used for transfections.

DNA Preparation and Analysis

DNA was extracted from *B. subtilis* cells using a procedure based on that of Marmur (10), and was further purified by CsCl density gradient centrifugation. Plasmid DNA and lambda DNA were prepared as described (11,12,13). Lambda Charon 15 DNA was a gift from D. Moore.

DNA ligase, polynucleotide kinase, and restriction enzymes were purchased from P.L. Biochemicals, New England Biolabs and Bethesda Research Laboratories and used as directed.

DNA fragments were separated by electrophoresis in 0.7-1.2% agarose gels (14), localized by staining with ethidium bromide, and transferred to nitro-cellulose filters using the method of Southern (15). Lambda and plasmid DNAs cut with a variety of restriction enzymes were used as size markers on the gels.

γ -³²P-ATP was prepared according to the method of Johnson and Walseth (16), using ³²PO₄⁻³ obtained from New England Nuclear Corp. DNA fragments were 5' end labeled using γ -³²P-ATP and polynucleotide kinase, and prepared for sequencing according to the method of Maxam and Gilbert (17).

Homology between various DNA sequences was examined using a computer program based on that described by Fitch (18,19).

RNA Preparation

After overnight growth in L broth, *B. subtilis* cells were transferred to low phosphate MOPS (20) minimal medium (0.2% glucose and 0.25 mM phosphate), and grown until the phosphate supply was nearly depleted. This culture was diluted about 20 fold into fresh low phosphate MOPS minimal medium, and grown to an OD₄₅₀ of 0.1. Then 200 μ C/ml of ³²PO₄⁻³ was added and the cells were harvested after 3-4 generations of growth. The cells were pelleted, resuspended in one half volume TSE (0.01 M NaCl, 1 mM EDTA, 0.02 M Tris pH 7.6) and incubated on ice with 0.8 mg/ml lysozyme for 10 minutes. SDS was added to 1% and the cells were warmed to room temperature until lysis occurred (usually about 3 minutes). The lysed cells were phenol extracted twice and the nucleic

acids were ethanol precipitated with 100 μ g carrier RNA (yeast tRNA). 16S and 23S rRNA were separated on a 5-20% sucrose gradient (1), 5S rRNA and tRNAs were further purified by two-dimensional polyacrylamide gel electrophoresis as described by Ikemura and Dahlberg (21), except that the first dimension was 14% polyacrylamide (19:1 acrylamide:bisacrylamide), run for 5 1/2 hours at 400 volts and the second dimension was 20% polyacrylamide (19:1), 5 M urea, run for 20-22 hours at 400 volts.

Purified tRNAs were fingerprinted with RNase T1 according to the method of Sanger (22,23). Digestion with RNase T2 used the methods of Nishimura (24).

Hybridizations

Hybridizations using rRNAs as probes were performed in 6xSSC (20xSSC is 3M NaCl, 0.3M NaCitrate pH 6.8) at 68°C. An excess of unlabeled competitor rRNA (16S rRNA in the case of 32 P-23S rRNA and vice versa) was usually added to the hybridization mixture. After overnight incubation the filters were washed at 68°C in 6xSSC several times, treated with 10 μ g/ml RNase in 2xSSC at 37°C for 30 minutes, washed in 2xSSC, then in 0.1xSSC at room temperature, and dried prior to autoradiography.

Hybridizations using 5' end labeled DNA probes were performed in 6xSCP (20xSCP is 2M NaCl, 0.6M Na_2HPO_4 , 0.02M EDTA, pH 6.2), 1% sarkosyl at 68°C. After overnight incubation the filters were washed in 1% sarkosyl, 5mM Tris pH 8 several times.

The hybridizations with 4-8S RNA used filters prepared by a method described by Weiner (25).

Cloning

DNA fragments were cloned into the lambda phage Charon 15 (26) or the plasmid pBR322 (27). λ phage libraries were screened as described (28). Plasmid libraries were screened by a modification of the method described by Grunstein and Hogness (29). Colonies of plasmid containing cells were transferred to Whatman 540 paper and placed in 0.5N NaOH for only 3 minutes to reduce the background from heterologous hybridization to *E. coli* rRNA genes. The filters were then washed in 2xSCP several times. The colonies were screened by hybridization with 32 P-rRNA in 5xSSPE (20xSSPE is 20mM Na_2EDTA , 0.1M Na_2HPO_4 , 0.1M NaH_2PO_4 , 3M NaCl), 0.3% SDS, 1xDenhardt's solution (30) at 68°C overnight and washed as described above except that 0.1% SDS was included in the first wash in 6xSSC at 68°C. All work with recombinant DNA was carried out in accordance with the appropriate NIH guidelines.

RESULTS

Characterization of rRNA Gene Sets

The rRNA genes of *B. subtilis* were first characterized by hybridization of ^{32}P labeled *B. subtilis* rRNA to Southern blots of genomic *B. subtilis* DNA as shown in Figure 1.

The number of rRNA gene sets was determined by counting the number of fragments to which both 23S and 5S rRNA hybridized; such fragments represent the 23S-5S (or 3') ends of rRNA gene sets. Figures 1a and 1b show the results obtained when EcoRI and PstI, respectively, were used to cleave the

Figure 1. Hybridization Analysis of *B. subtilis* rRNA Gene Sets. *B. subtilis* DNA was cut with the restriction enzymes EcoRI (a), PstI (b), SmaI (c) or HindIII (d) and transferred to nitrocellulose filters after agarose gel electrophoresis. The hybridization probes were uniformly ^{32}P -labeled 16S rRNA, 23S rRNA, and 5S rRNA as indicated. rDNA fragments indicated with a star were cloned and used for restriction enzyme mapping. rDNA fragments marked with arrows are the spacer fragments. Size markers indicated on the left apply to both (a) and (b). Size markers to the right of (c) apply only to (c). The dashes to the right of the 5S rRNA hybridization pattern in (a) and (b) indicate the bands which were counted to determine the minimum number of rRNA gene sets. Bands which were judged to contain two co-migrating rDNA fragments are marked with two dashes. The bands marked in (d) hybridized to both 23S and 5S rRNA. Figure 6 (b) shows more clearly the pattern of hybridization using HindIII digested DNA and 16S and 23S rRNAs as probes.

DNA. We note that in every digest which was probed with both 5S and 23S rRNAs, the 5S rRNA hybridized only to fragments to which 23S rRNA also hybridized. Thus we feel that each 5S rRNA gene is closely associated with a 23S rRNA gene and that the number of 5S rRNA genes is a measure of the number of rRNA gene sets. Both 5S and 23S rRNAs hybridized to nine distinguishable fragments in EcoRI (Figure 1a) and PstI (Figure 1b) digests of genomic DNA. This is easiest to see when 5S rRNA is the probe; the bands of hybridization are indicated with a dashed line to the right of the lane with the 5S rRNA hybridization pattern. In both digests, one band appeared much darker than the others, indicating the presence of two co-migrating rDNA fragments; these bands are marked with two dashed lines. Thus there were a total of at least nine and possibly ten rRNA gene sets.

The fragments marked with stars in Figure 1 were cloned (see below) and subjected to restriction enzyme mapping and hybridization analysis. The results of these experiments and genomic Southern blot analysis (Figure 1) allowed construction of the maps shown in Figure 2.

HindIII sites at the 3' ends of some of the rRNA gene sets were predicted from the sequences of the 5S rRNA precursors (31). In order to determine how many rRNA gene sets contained this HindIII site, 23S rRNA was

Figure 2. Restriction Map of a *B. subtilis* rRNA Gene Set. The portions cloned as pSS-P and pLS-P are indicated. Within these clones, the regions between the SmaI sites were sequenced. The locations of the 5' ends of the 16S and 5S rRNA genes and the 3' end of the 23S rRNA genes are approximate. The HindIII site in parentheses at the 3' end of the rRNA gene is absent from five of the rRNA gene sets.

hybridized to B. subtilis DNA which had been cleaved with HindIII. As shown in Figure 1d, 23S rRNA hybridized to six size classes of fragments; the pattern of hybridization of 5S rRNA was identical. The intensity of hybridization to the smallest size class of fragment (3.2 kb) indicated that it contained multiple rDNA fragments. These fragments extended from near the 5' end of the 23S rDNA through the 5S rDNA (Figure 2). 23S rRNA also hybridized to five fragments which were larger than 3.2 kb, presumably due to the absence of one or both of the HindIII sites at the ends of the 3.2 kb fragment. Since sequence analysis of 5S rRNA precursors (31) indicated that at least some rRNA gene sets lacked the HindIII site near the 5S rRNA gene, we concluded that five of the rRNA gene sets in B. subtilis did not contain a HindIII site at the 3' end.

Characterization of the 16S-23S rDNA Spacer Regions

The spacer fragments, which overlap the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene, can be identified using the results shown in Figure 1. When EcoRI is used to cut the DNA, both 16S and 23S rRNA hybridize to the 1.2 and 1.4 kb rDNA fragments (Figure 1a), whereas 5S rRNA does not hybridize to these fragments. Two rDNA fragments with similar hybridization characteristics are obtained when the restriction enzymes PstI (Figure 1b, 3.1 and 3.3 kb fragments) or SmaI (Figure 1c, 0.5 and 0.7 kb fragments) are used. These fragments are henceforth referred to as spacer fragments and are marked with arrows in Figure 1.

The possibility of additional spacer fragments was eliminated by a hybridization analysis of EcoRI cut genomic DNA using as a probe a cloned 1.2 kb EcoRI small spacer fragment (data not shown). Only the 1.2 kb and 1.4 kb rDNA fragments, which had already been identified as spacer fragments (see above and Figure 1a), showed homology to this probe. Therefore these two size classes of fragments contained all the 3' ends of the 16S rRNA genes and all of the 5' ends of the 23S rRNA genes which were found in the genome.

Because each of the three restriction enzymes produced two sets of spacer fragments differing in size by 0.2 kb, the heterogeneity in fragment size appeared to result from the presence of extra DNA sequences in some gene sets rather than from a difference in the location of restriction enzyme cleavage sites within the structural genes. To test this possibility the 3.1 and 3.3 kb rDNA fragments resulting from PstI digestion of the DNA were cloned after ligation into pBR322. These clones are subsequently referred to as pSS-P (small spacer- PstI) and pLS-P (large spacer- PstI). Restriction enzyme mapping of the cloned fragments revealed that each contained two SmaI

sites. Upon cleavage with *Sma*I, pSS-P generated a 0.5 kb *Sma*I fragment, whereas pLS-P generated a 0.7 kb *Sma*I fragment. Each plasmid also generated another fragment after digestion with *Sma*I; these were both the same size and contained both the pBR322 sequences and the sequences flanking the excised 0.5 kb or 0.7 kb *Sma*I fragments. The 0.5 and 0.7 kb fragments corresponded in size to the two spacer rDNA fragments seen in Figure 1c. Further restriction enzyme analysis showed that the differences between pSS-P and pLS-P were confined to the region between the 16S and 23S rRNA genes (data not shown).

Sequencing the Spacer Region

The regions between the *Sma*I sites in each cloned spacer rDNA fragment were sequenced in order to determine the basis for the difference in their sizes. The strategy used in sequencing these two *Sma*I fragments is outlined in Figure 3 and the DNA sequences obtained are shown in Figure 4. The sequenced regions extend from 165 bases upstream of the 3' end of the 16S rRNA genes through the spacer regions and 191 bases into the 23S rRNA genes. Regardless of which strand was being studied, determination of the DNA sequence in a 6 nucleotide region of the 16S rDNA was difficult, due to compression of the DNA fragments on the sequencing gels. This region is indicated by brackets in Figure 4.

The large spacer fragment contains a block of 175 base pairs of DNA which are not found in the small spacer fragment. If this sequence is transcribed into RNA as part of a rRNA precursor, two regions of this transcript could be folded into a cloverleaf structure of a tRNA^{Ile} and a tRNA^{Ala},

Figure 3. Summary of the Strategy Used for DNA Sequencing. The arrows indicate the origin and direction of each sequence analysis.

Figure 4. The DNA Sequence of pLS-P and pSS-P. The entire DNA sequence of the internal SmaI fragment of pSS-P is shown. Differences found in the sequence of pLS-P are shown in the line below: insertions are boxed and deletions are indicated by triangles. The general locations of the 3' end of the 16S rRNA gene and 5' end of the 23S rRNA gene are indicated by dashes above the DNA sequence. The six base pairs enclosed in brackets (base pairs 90-95) were not determined unambiguously. Restriction enzyme cleavage sites mentioned in the text are shown above the DNA sequence. The tRNA genes within the 175 base pair insert of pLS-P are indicated.

respectively, as shown in Figure 5a.

A portion of one of the possible secondary structures for transcripts originating from each of the two different spacer regions is shown in Figure 5b. In these structures the tRNA sequences are located within a loop at the end of a long double-stranded stem that is common to transcripts from both the

Figure 5. Possible Secondary Structures of Spacer Region Transcripts. (a) shows cloverleaf representations of the tRNA^{Ile} and tRNA^{Ala} which could be transcribed from the DNA in the spacer region of pLS-P. (b) shows one of the possible secondary structures for part of the spacer region of pSS-P and the spacer region surrounding the tRNAs in pLS-P. The tRNAs of pLS-P are indicated by line drawings labeled tRNA^{Ile} and tRNA^{Ala}. The arrows show the ends of the inserted sequence of pLS-P and the corresponding site of the insertion in pSS-P. The structures shown correspond to the regions between nucleotides 214 and 281 of Figure 4 and are based on visual inspection of the DNA sequences.

large and small spacers. The ends of the pLS-P specific sequences are indicated with arrows in the pLS-P transcript, as is the site in the pSS-P transcript which corresponds to the location of the large insertion.

The Number of rRNA Gene Sets with Spacer tRNAs

The DNA sequences of the two spacer regions showed that some but not all of the spacer regions contained tRNA genes. The intensity of hybridization to the spacer fragments in Figure 1a indicated that about one quarter of the rRNA gene sets contained large spacer regions. In order to establish how many of these large spacer fragments existed we hybridized ^{32}P -labeled 16S and 23S rRNAs, and DNA probes containing parts of the tRNA^{Ile} or tRNA^{Ala} genes to genomic DNA which had been cut with either BamHI or HindIII. Both of these enzymes cut within the 23S rDNA but not within the spacer region or the 16S rDNA (see Figure 2). The tRNA^{Ile} specific probe was completely internal to the tRNA^{Ile} gene, extending from the TaqI site to the HaeIII site indicated above the DNA sequence of pLS-P in Figure 4. A hybridization probe corresponding to the tRNA^{Ala} gene produced a more complex pattern of hybridization because of the presence of many tRNA^{Ala} genes in the genome; however, the results obtained with this probe (data not shown) were entirely consistent with those obtained using the tRNA^{Ile} specific probe (described below).

As shown in Figures 6a and 6b, the tRNA^{Ile} specific probe hybridized to three size classes of rDNA fragments. One class, indicated with a D (distal), probably corresponded to the 3' end of a rRNA gene set since both 23S and 5S rRNA also hybridized to a fragment of the same size. This result was also obtained when several other restriction enzymes were used (data not shown). The other two size classes of rDNA fragments which hybridized to the spacer tRNA^{Ile} specific probe corresponded in size to fragments which hybridized to both 16S and 23S rRNA, and thus appeared to be spacer fragments. These are labeled with an S (spacer) in Figures 6a and 6b.

The results shown in Figures 6a and 6b indicated that there were at least two rDNA spacer fragments which contained tRNA^{Ile} genes. However, these experiments did not establish the exact number of large spacer fragments since both BamHI and HindIII generated two co-migrating rDNA fragments (indicated by the stars in Figures 6a and 6b). In each digest, one or both of these unresolved fragments could contain a tRNA^{Ile} gene. (Quantitation based on intensities of hybridization in various bands was unreliable due to a large difference in the sizes of the tRNA^{Ile} hybridizing fragments.)

The number of spacer tRNA containing fragments was determined by purifying the 2kb-3kb sized fragments of HindIII digested *B. subtilis* DNA and

Figure 6. Determination of the Number of Spacer Regions Containing tRNA Sequences. *B. subtilis* genomic DNA cut with BamHI or HindIII was size fractionated and probed by hybridization with 32 P-labeled 16S rRNA, 23S rRNA or a tRNA^{Ile} specific DNA probe as indicated. Fragments hybridizing to the tRNA^{Ile} specific probe are labeled S (spacer) or D (distal) in (a) and (b). Bands which could contain either one or two rDNA fragments with spacer tRNAs are indicated with stars. The brackets in the left lanes of (b) and (c) indicate the 2.0-2.5 kb fragments resulting from cleavage of the DNA with HindIII. In (c), these purified fragments are shown before (left lane) or after (right lane) cleavage with PstI. The 1.1 and 0.9 kb fragments indicated in this lane are the sizes predicted for large and small spacer rDNA fragments, respectively. Hybridization of 23S rRNA to the spacer fragments in (b) is low because the HindIII cleavage site is only about 90 nucleotides from the sequence corresponding to the 5' end of 23S rRNA (see Figure 4). Also in (b), the faint hybridization of 16S rRNA to a 3.2 kb fragment is due to lack of sufficient 23S rRNA competitor in this particular experiment (see figure 1d).

redigesting them with PstI. Southern blot analysis of the resulting fragments, using 16S rRNA as a probe, showed that redigestion of the three HindIII fragments (2.3, 2.3, and 2.1 kb in size, respectively) (Figure 6c, left lane)

with PstI generated two 0.9 kb spacer rDNA fragments and one 1.1 kb spacer rDNA fragment (Figure 6c, right lane). In this case, quantitation on the basis of hybridization intensity was possible since the three fragments were nearly the same size. These HindIII-PstI rDNA fragments corresponded in size to those predicted for spacer regions which lacked (0.9 kb) or contained (1.1 kb) tRNA genes (see the map of Figure 2). Thus only one of the three 2kb-3kb sized rDNA fragments generated by cleavage with Hind III (one of the 2.3 kb rDNA fragments) contained a tRNA^{Ile} gene. This fragment and the 7.4 kb Hind III fragment were the only two rDNA fragments having the hybridization characteristics expected for rDNA spacer fragments which also contain tRNA^{Ile} genes (labeled S in Figure 6b). Therefore, we concluded that two of the rRNA genes in *B. subtilis* contained a spacer tRNA^{Ile} gene.

Expression of Spacer tRNA Genes

If the spacer DNA sequences are actually transcribed into tRNAs, it should be possible to isolate the corresponding tRNAs directly from cells. Therefore, labeled 4-8S RNAs of *B. subtilis* were hybridized to nitrocellulose filters carrying pLS-P and pSS-P DNA. The RNAs which hybridized were eluted from the filters, and analyzed by two-dimensional polyacrylamide gel electrophoresis and RNase T₁ fingerprinting. No small RNAs hybridized specifically to pSS-P but three tRNA sized RNA species hybridized specifically to pLS-P. The RNase T₁ fingerprints of two of these were identical to fingerprints predicted from the sequences of the spacer tRNA^{Ile} and tRNA^{Ala} genes respectively (data not shown).

By RNase T₁ fingerprinting and RNase T₂ redigestion we concluded that the third tRNA was tRNA^{Val} which was recently sequenced (33). Presumably this tRNA hybridized to pLS-P as a result of its extensive homology with tRNA^{Ile}; comparison of the tRNA sequences revealed two regions of homology which extended for 12 out of 12 and 16 out of 18 nucleotides.

DISCUSSION

The Number of rRNA Gene Sets

This study indicates that there are at least nine and probably ten rRNA gene sets in *B. subtilis*, and that two of them contain spacer tRNA genes. These results differ from those of other reports which concluded that none of the rRNA gene sets contained spacer tRNA genes (5,7,9).

The restriction enzyme map of the rRNA genes (Figure 2) was generated primarily by analysis of cloned rDNA fragments and is completely consistent with the hybridization patterns observed in Southern blots of genomic DNA.

However, interpretation of the genomic DNA blots using rRNA probes is complicated by the existence of closely spaced tandem repeats of rRNA gene sets in B. subtilis (7). Thus the map cannot be considered as invariant amongst all the rRNA gene sets until the existence of each restriction enzyme site within each gene set has been verified. It is also unknown whether any of the rRNA gene sets contain a duplication of the 5S rRNA gene, as is found in E. coli (34; E. Lund, unpublished results).

We have recently isolated a variant of B. subtilis 168 in which one non-spacer tRNA containing gene set has been deleted. Further analysis to determine the precise nature of this deletion is now in progress. A deletion of this particular rRNA gene set cannot account for the smaller number of gene sets reported by others (4,5) as their published hybridization data clearly indicate the presence of this gene set.

Genes for Spacer tRNAs

A comparison of the rDNA organization in B. subtilis and in E. coli reveals both similarities and differences. All seven of the rRNA operons in E. coli contain spacer tRNA genes; three of these operons each contain both tRNA^{Ile}₁ and tRNA^{Ala}_{1B} genes and the other four operons each contain a tRNA^{Glu}₂ gene (1,2,3). It has been suggested that the spacer tRNAs could play a role in rRNA precursor processing or in the regulation of rRNA transcription. The finding that only two of the nine or ten rRNA gene sets in B. subtilis contain spacer tRNAs implies that spacer tRNAs are not necessary for rRNA precursor processing, at least in this species.

As is generally the case for the tRNAs of E. coli, the 3' CCA ends of both the tRNA^{Ala} and tRNA^{Ile} are encoded by the tRNA genes. The tRNA^{Ala} sequence which could be transcribed from the spacer tRNA gene reported here differs by one nucleotide (an A to a G change at position 3) from the sequence of a B. subtilis tRNA^{Ala} recently reported (33). The fingerprint analysis described above is consistent with either or both tRNA sequences being present in our tRNA^{Ala} preparations. Although tRNAs which could have been transcribed from the genes in the spacer regions appear in B. subtilis cells, it should be noted that genes for tRNA^{Ala} and tRNA^{Ile} are found elsewhere in the genome. Therefore the expression of the spacer tRNA genes has not been established.

DNA sequencing of the large spacer fragment represented by pLS-P demonstrates that genes for both tRNA^{Ile} and tRNA^{Ala} are present in at least one spacer region. From the results of the hybridization analysis of tRNA^{Ile} and tRNA^{Ala} sequences in the B. subtilis genome, it is likely that there are two

large spacer regions which contain tRNA^{Ile} and the tRNA^{Ala} genes. It is unlikely that any of the small spacer fragments contain tRNA genes since no such genes have been detected in the three or four small spacer regions which have been sequenced (this work, 32). In addition, when B. subtilis 4S RNA was hybridized to EcoRI cut genomic DNA, no tRNAs hybridized to 1.2 kb sized fragments whereas 4S RNA did hybridize to 1.4 kb sized fragments (data not shown).

It is intriguing that the two tRNA genes which are present in the spacer region of some B. subtilis rRNA gene sets are also found in some of the rRNA operons of E. coli as well as between the 16S and 23S rRNA genes of the chloroplast DNAs in Euglena gracilis (35) and Zea mays (36). It remains unclear whether these similarities actually result from a functional aspect of the spacer tRNA location.

The regions of the 16S and 23S rRNA genes and the sequence of the tRNA^{Ile} and tRNA^{Ala} genes which are reported here are highly conserved between B. subtilis, E. coli, and the chloroplasts. A computer analysis (18,19) performed to compare the spacer tRNA gene sequences reveals 70-90% homology. Likewise, the rDNA sequences show very long stretches of complete homology. In contrast the DNA sequences of the spacer regions of B. subtilis appear to be quite divergent from the spacer regions of E. coli rrnB (37), rrnE (38), rrnD' and rrnX (39), as well as from the spacer regions of the chloroplasts (35,36).

The spacer tRNA containing rDNA fragments which we were able to clone extend from the PstI site in the 16S rDNA to the PstI site in the 23S rDNA. Attempts to clone the 1.4 kb EcoRI rDNA fragments which contain spacer tRNA genes were unsuccessful. However, the 1.2 kb EcoRI small spacer fragments which lack the tRNA^{Ile} and tRNA^{Ala} genes are readily clonable. Both the 1.4 kb and the 1.2 kb EcoRI fragments contain between 200 and 300 base pairs of DNA which are not present in the cloned PstI spacer fragments. This DNA extends from the EcoRI site to the PstI site in the 16S rDNA (see Figure 2). Taylor and Burgess (40) have reported that an RNA polymerase binding site which functions in vitro is located just downstream from an EcoRI site in the E. coli 16S rRNA gene. Perhaps a homologous promoter in the 16S rRNA gene of B. subtilis allows transcription of B. subtilis tRNA genes in E. coli. If production of these tRNAs were lethal to E. coli cells, cloning of the 1.4 kb EcoRI fragment would be impossible.

rDNA Sequences

A comparison of the 16S rDNA sequences (and rDNA sequences predicted by

rRNA sequences) from *B. subtilis* (this work), *B. stearothermophilus* (41), *B. brevis* (42, C. Woese cited in ref. 32), and *E. coli* *rrnB* (37) is shown in Figure 7a. The 16S rRNA of *B. stearothermophilus* has an extra UCU near the 3' end (43) when compared to that of *E. coli* (37). These three additional nucleotides are also found in the *B. subtilis* 16S rDNA sequence and may also be found in its 16S RNA.

The nucleotide sequences of the ends of *B. subtilis* rRNAs have not been extensively studied. The 23S rRNA of *B. subtilis* appears to lack 2 nucleotides at the 5' end (44) when compared to the *E. coli* 23S rRNA sequence (37). However, these 2 nucleotides are found in the corresponding *B. subtilis* DNA sequence (Figures 4,7b).

The sequence of the portion of mature 16S rRNA which could be transcribed from pSS-P is identical to that which could be obtained from pLS-P. We have evidence for heterogeneity in the 23S rRNAs since the two cloned fragments differ by one base pair (position 117 in Figure 7b). A comparison of our DNA sequence of the small spacer region with those recently obtained

a. 16S rDNA

<i>B. subtilis</i>	0	CCC6G6CCTT	GTACACACCG	CCC6TCACAC	CACBAG6BT	T6TAACACCC	GAAGTC6BT6	60
<i>B. stearothermophilus</i>					-----G-----	-----C-----		
<i>B. brevis</i>					-T-G-----G	G-TG-AAA	-T-A--A	
<i>E. coli</i>								
	61	AG6TAACCTT	TTAGBAGCCA	6CC6CC6AAG	GT6GAACAGA	T6ATT666BT	GAAGTC6TAA	120
		-----GC	AA-----	-----TTA-C-TT	-----GT-----	-----C-----	-----	
		GCT-----	CGA-----GGC		-----TG-TTC-----			
	121	CAAGGTAGCC	GTATCG6AAG	GT6CG6CT66	ATCACCTCCT	TTCTA		165
		-----T-----	-----C-----	-----A-----	-----	-----		
		-----A-----	-----GG-----C	-----C-----T-----	-----	-----A-----		

b. 23S rDNA

<i>B. subtilis</i>	0	66TTAAGTTA	GAAG66C6C	AC66T6GAT6	CC6T66CACT	AG6ACCC6AT	GAAG6AC666	60
<i>E. coli</i>		-----CG-----	CT-----C-TAC		-----C-----G-----	CA-----G-----	-----T-----	
	61	AC6AACACCG	ATAT6CTTC6	666AGCT6TA	ASCAAGCTTT	GATCC66AGA	TTTCC6AT6	120
		CTA-T-TG--	---A--G-G--	-TA--G--AT	-TG--C-G--	ATA-----	(T) -----T-----	
	121	666AAACCCA	CCACTC6TAA	T66AGT66TA	TCCATATCT6	AATTCATAG6	ATAT6AGAG	180
		-----GTGTTT-CG	ACACACAA--	-----AT--A--	-----C-----	-----T-----	A	
	181	GCAGACCC66	G					191
		-----GA-----G-----						

Figure 7. Comparison of the Bacterial rDNA Sequences. (a) shows a comparison of the DNA sequences which correspond to the 3' ends of the mature 16S rRNAs of *B. subtilis*, *B. stearothermophilus*, *B. brevis* and *E. coli* *rrnB*. (b) is a comparison of the DNA sequences of the 5' ends of the 23S rRNA genes of *B. subtilis* and *E. coli*. Gaps in the sequences, indicated by triangles, were positioned to maximize homology; insertions are shown in the line below the sequence.

by Bott and Hollis (32) reveals that our sequence is identical to two of the small spacer regions which they sequenced. However, the sequenced portions of the 16S rRNA genes differ by one base pair. We note that this difference, at position 95 of our 16S rDNA sequence (see Figure 5) is in the region which is difficult to determine unambiguously. Their 23S rDNA sequence does not include the region which we find is heterogeneous.

The primary sequences of the sites recognized by B. subtilis rRNA processing enzymes are likely to be different from those of E. coli, since the sequences flanking the mature rRNA genes are very different in the two bacteria (45,46, Figure 4). The possibility that secondary structures in the regions surrounding the rRNAs are comparable must await analysis of the 5' end of the 16S rRNA gene and the 3' end of the 23S rRNA gene in B. subtilis.

The secondary structure presented in Figure 5b for the spacer tRNA containing transcript (from pLS-P) is very similar to the secondary structure which has been proposed for the transcript of the spacer region of rrnD (47). In both cases three double-stranded regions converge near the beginning of the tRNA^{Ile}, although the sequences and exact positioning of the regions differ. The significance of these double-stranded regions with regard to RNA processing remains unknown.

In E. coli, nucleotides 72 to 83 of 5S rRNA are complementary to nucleotides 143 to 154 of 23S rRNA (48). Likewise in B. subtilis nucleotides 119 to 128 of 5S rRNA are complementary to nucleotides 82 to 91 of 23S rRNA. If G-U pairing is included, the complementarity in B. subtilis for this region includes 21 of 26 consecutive nucleotides. The significance of this complementarity with respect to ribosome structure or protein synthesis has not been determined in either species.

It is unknown whether all rRNA gene sets of B. subtilis are expressed under all growth conditions and during sporulation. Therefore, the spacer tRNAs could serve as valuable markers in the analysis of expression of specific rRNA gene sets.

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