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Novel tRNA Gene Organization in the 16S-23S Intergenic Spacer of the *Streptococcus pneumoniae* rRNA Gene Cluster

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Isoleucine and alanine tRNAs are encoded tandemly within the 16S-23S intergenic spacer of some eubacterial rRNA gene clusters. Southern hybridization analysis and DNA sequence analysis demonstrated a novel gene organization for an rRNA gene cluster on the *Streptococcus pneumoniae* chromosome. A sequence specifying an alanine tRNA was found within the intergenic spacer, but no sequence specifying an isoleucine tRNA was found there. Southern hybridization analysis indicated that the location of the isoleucine tRNA gene was near the 5S rRNA gene in two of four rRNA gene clusters.

Typically, the gene organization of eubacterial rRNA gene clusters is 5′-16S-23S-5S-3′ (2 and references within; 20), although several exceptions have been observed (12, 21, 23). Between the 16S and 23S rRNA genes and downstream of the 5S gene are regions known as the intergenic spacer and distal spacer, respectively. Both of these regions have been shown to encode tRNAs in some rRNA gene clusters. In the intergenic spacer, a glutamate tRNA or tandem isoleucine-alanine tRNAs may be encoded (5, 9, 13, 14, 22, 25). Some distal spacers have been shown to encode an aspartate and/or a tryptophan tRNA or a threonine tRNA (3, 9, 17). Here we report results from Southern hybridization analysis of the rRNA gene clusters of *Streptococcus pneumoniae* and DNA sequence analysis of the intergenic spacer.

Southern hybridization analysis of rRNA and spacer tRNA genes. Total DNA was extracted (11) and purified from late-log-phase cultures of *S. pneumoniae* (ATCC 33400). The concentration of the DNA preparation was determined by the diphenylamine assay as previously described (6). In separate reactions, 1 μg of total DNA was digested with either *Hin*dIII or *Pst*I. Conditions for the digestions were those specified by the supplier of the restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The restriction digests and lambda *Hin*dIII fragments were electrophoresed in a 1% agarose gel. The fractionated DNA was transferred to a solid support membrane (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) as previously described (19). The membrane-bound DNA fragments were sequentially probed with 5′-end-labeled [γ-^{32}P] ATP oligodeoxyribonucleotides specific for the three rRNA genes and the tRNA^{Ile} and tRNA^{Ala} genes. Probes are described in Table 1. The 5S rDNA probe was mixed, in that the sixth nucleotide was synthesized with equimolar ratios of all four deoxyribonucleotides, because of the variability at that position in eubacterial 5S rRNAs. Conditions for prehybridizations, hybridizations, and probe removal were those specified by the supplier of the hybridization membrane (Schleicher & Schuell).

Figure 1 shows the results of the hybridization experiments. Estimated sizes of the DNA fragments that hybridized with the probes are presented in Table 2. Patterns (*Hin*dIII or *Pst*I) were identical for hybridizations with the 16S rDNA and tDNA^{Ala} probes, suggesting that the two genes are linked. A single signal corresponding to 4.0 kb was detected when the 16S or 23S rDNA probe or the tDNA^{Ala} probe was hybridized to the *Hin*dIII-digested DNA. This result strongly suggests a linkage between the 16S and 23S rRNA genes and the tRNA^{Ala} gene.

Most striking of our observations was the lack of similarity between the hybridization patterns generated from the tDNA^{Ile} and tDNA^{Ala} probes. Two signals corresponding to 6.1 and 3.5 kb in the *Hin*dIII-digested DNA were observed from the hybridization with the tDNA^{Ile} probe. Four signals corresponding to 6.1, 3.5, 3.2, and 2.5 kb were observed when the 5S rDNA probe was hybridized against the *Hin*dIII-digested DNA. Two signals corresponding to 8.9 and 7.1 kb in the *Pst*I-digested DNA were observed from the hybridization with the tDNA^{Ile} probe. Four signals corresponding to 20.0, 8.9, 7.8, and 7.1 kb were observed following hybridization of the 5S rDNA probe to *Pst*I-digested DNA. Thus, the two signals from the tDNA^{Ile} hybridization correspond to two of the four signals from the 5S rDNA hybridization (*Hin*dIII- or *Pst*I-digested DNA). These results strongly suggest a linkage between the tRNA^{Ile} and 5S rRNA genes. On the basis of our observations from the Southern hybridization analyses, we propose that there are at least four copies each of the 16S, 23S, and 5S rRNA genes on the *S. pneumoniae* chromosome. There appear to be four copies of the tRNA^{Ala} gene, each probably located between a 16S and a 23S rRNA gene. Probably only two copies of the tRNA^{Ile} gene are present, and they appear to be linked to two different 5S rRNA genes.

Cloning of the intergenic spacer. Total DNA from *S. pneumoniae* was digested with *Hin*dIII and electrophoresed in a 1% agarose gel. DNA fragments of ca. 3 to 5 kb were purified from the gel as previously described (24) and ligated into the *Hin*dIII site of pUC9. *Escherichia coli* JM 83 was transformed with DNA from the ligation reaction. The resulting transformants were screened with the tDNA^{Ala} probe. The colony hybridization method we employed has been previously described (15). Plasmid DNA was prepared from one of the transformants that strongly hybridized with the probe. This recombinant plasmid contained an insert of ca. 4.0 kb that hybridized to the 16S and 23S rDNA probes and the tDNA^{Ala} probe and was designated pSP1. The insert of pSP1 was mapped with six restriction endonucleases (data not shown), and an internal *Sma*I fragment (ca. 900 bp) was ligated into the *Sma*I site of pUC18. The resulting recombinant was designated pSP4. Unidirectional deletions of the

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TABLE 1. Oligodeoxyribonucleotides used to probe for rDNAs and tDNA^{Ile} and tDNA^{Ala} genes in *S. pneumoniae*

Probe	Region of complementarity to RNA of *E. coli*	Sequence (5'→3')
16S	1392–1406	ACG GGC GGT GTG TAC
23S	241–255	TCG CTC GCC GCT ACT
5S	41–55	CCC ATN CCG AAC TCA
Ile[a]	17–40	TGG TTA GAG CGC ACC CCT GAT AAG
Ala[a]	23–37	AGC GCC TGC TTT GCA

[a] Sequence is the DNA analog to the RNA; thus, it is not complementary to the RNA.

TABLE 2. rDNA and tDNA Southern hybridization analysis of total *S. pneumoniae* DNA

Oligonucleotide probe	Restriction endonuclease	Size of fragment (kb) corresponding to signal
16S	*Hin*dIII	4.0
	*Pst*I	5.0, 4.8, 3.8, 3.2
23S	*Hin*dIII	4.0
	*Pst*I	2.1
5S	*Hin*dIII	6.1, 3.5, 3.2, 2.5
	*Pst*I	20.0, 8.9, 7.8, 7.1
tDNA^{Ala}	*Hin*dIII	4.0
	*Pst*I	5.0, 4.8, 3.8, 3.2
tDNA^{Ile}	*Hin*dIII	6.1, 3.5
	*Pst*I	8.9, 7.1

pSP4 insert were generated with exonuclease III as previously described (7). The insert of pSP4 and its derivatives were used as templates to determine the nucleotide sequence of the putative intergenic spacer. Double-stranded templates were sequenced by the dideoxy chain termination method as previously described (18). The sequence of the putative intergenic spacer and portions of the flanking 16S and 23S rRNA genes is presented in Fig. 2.

Nucleotides 1 to 160 shared 86% positional identity with the 3' end of the *Bacillus subtilis* 16S rRNA gene. Nucleotides 411 to 932 shared 68% positional identity with the 5' end of the *B. subtilis* 23S rRNA gene. Nucleotides 214 to 286 were 100% identical to 73 of 76 nucleotides of the *B. subtilis* intergenic spacer tRNA^{Ala} gene in *rrnO* (14). Both noncoding regions (nucleotides 161 to 213 and 287 to 410) flanking the tRNA^{Ala} gene were searched for the presence of tRNA genes by sequence homology to eubacterial tRNA genes in the DNA sequence data bank (GenBank). In addition, these regions were analyzed for any tRNA-like secondary structure (PCGene). No tRNA or tRNA-like sequences were found in these regions.

Curiously, we noted numerous direct repeats (5 nucleotides or larger) in the noncoding portions of the intergenic spacer. These observations included one 8-base repeat (GGTCTTGT) at nucleotides 185 to 192 and 206 to 213 and one 9-base repeat (AGAAAATAA) at nucleotides 348 to 356 and 402 to 410. Sequence analysis also revealed one 6-base tandem repeat (TAAGGA, nucleotides 162 to 173) within the region of the intergenic spacer where the tRNA^{Ile} is typically encoded. Short direct repeats appear to play an integral role in some forms of illegitimate recombination (1, 4).

Our experimental observations were consistent with those for other eubacterial rRNA gene clusters with the exception of the absence of the tRNA^{Ile} gene from the intergenic spacer. Therefore, we tentatively propose the gene organization of 5'-16S rDNA-tDNA^{Ala}-23S rDNA-5S rDNA-tDNA^{Ile}-3' for two of the gene clusters and 5'-16S rDNA-tDNA^{Ala}-23S rDNA-5S rDNA-3' for the remaining two rRNA gene clusters on the *S. pneumoniae* chromosome, even though we have not yet demonstrated a direct linkage

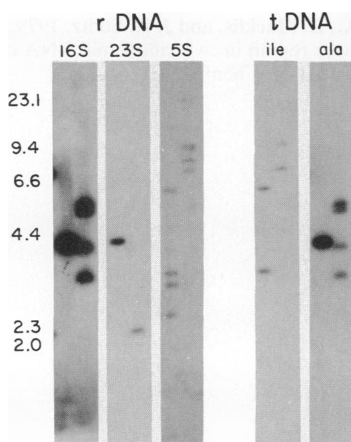

FIG. 1. Southern hybridization patterns of *S. pneumoniae* rDNAs and tDNA^{Ile} and tDNA^{Ala}. Total DNA was digested with *Hin*dIII or *Pst*I (left and right lanes, respectively). Numbers on left show size in kilobases.

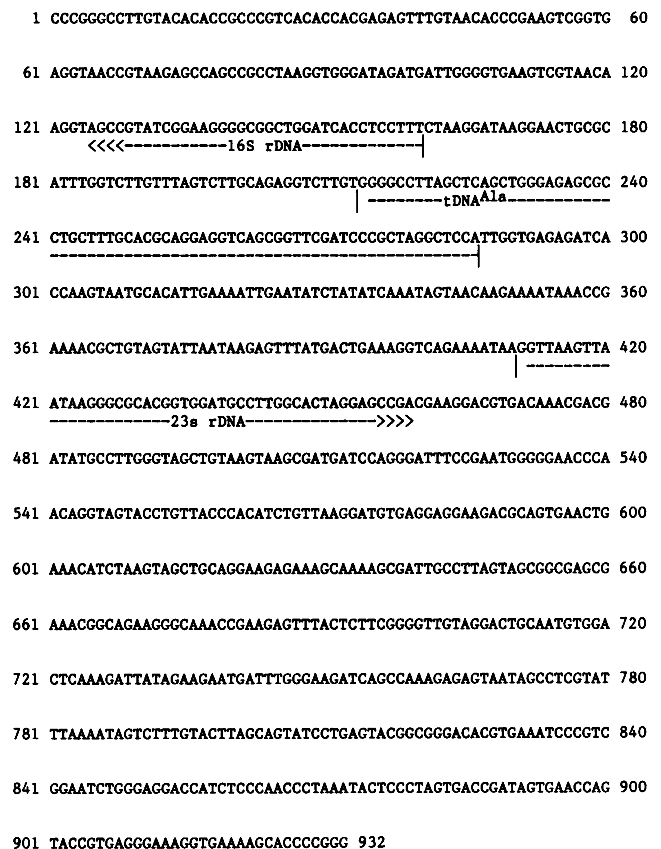

FIG. 2. Nucleotide sequence of the cloned intergenic spacer from *S. pneumoniae*.

between the 5S gene and the other rRNA genes. It is interesting that the archaebacteria *Halobacterium halobium*, *H. cutirubrum*, and *Methanococcus vannielii* have the same intergenic spacer tRNA gene organization as *S. pneumoniae* (8, 10, 16).

In summary, Southern hybridization analysis of total DNA indicates that there are four copies of the rRNA gene clusters on the *S. pneumoniae* chromosome. DNA sequence analysis of the cloned intergenic spacer demonstrates the presence of a $tRNA^{Ala}$ gene but the absence of the $tRNA^{Ile}$ gene typically encoded in tandem with the $tRNA^{Ala}$ gene in eubacterial rRNA gene clusters. In addition, Southern hybridization analysis indicates that two of the gene clusters contain a $tDNA^{Ile}$ linked to a 5S rDNA. Direct evidence for the linkage between these two genes awaits the cloning and sequencing of the 3.5-kb *Hin*dIII fragment (Table 2). The GenBank accession number for the 932-nucleotide sequence of the *S. pneumoniae* intergenic spacer is M60763.

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REFERENCES

1. **Anderson, P.** 1987. Twenty years of illegitimate recombination. Genetics **115**:581–584.
2. **Baylis, H. A., and M. J. Bibb.** 1988. Organisation of the ribosomal RNA genes in *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. **211**:191–196.
3. **Duester, G. L., and W. M. Holmes.** 1980. The distal end of the ribosomal RNA operon *rrn*D of *Escherichia coli* contains a $tRNA_1^{thr}$ gene, two 5S rRNA genes and a transcription terminator. Nucleic Acids Res. **8**:3793–3807.
4. **Ehrlich, S. D.** 1989. Illegitimate recombination in bacteria, p. 799–832. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
5. **Feingold, J., V. Bellofatto, L. Shapiro, and K. Amemiya.** 1985. Organization and nucleotide sequence analysis of an rRNA and tRNA gene cluster from *Caulobacter crescentus*. J. Bacteriol. **163**:155–166.
6. **Giles, K. W., and A. Myers.** 1965. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature (London) **206**:93.
7. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
8. **Hui, I., and P. P. Dennis.** 1985. Characterization of the ribosomal RNA gene clusters in *Halobacterium cutirubrum*. J. Biol. Chem. **260**:899–906.
9. **Ikemura, T., and M. Nomura.** 1977. Expression of spacer tRNA genes in ribosomal RNA transcription units carried by hybrid ColE1 plasmids in *E. coli*. Cell **11**:779–793.
10. **Jarsch, M., and A. Bock.** 1983. DNA sequence of the 16S rRNA/23S rRNA intercistronic spacer of two rDNA operons of the archaebacterium *Methanococcus vannielii*. Nucleic Acids Res. **11**:7537–7544.
11. **Johnson, J. L.** 1981. Genetic characterization, p. 450–474. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
12. **Lamfrom, H., A. Sarabhai, and J. Abelson.** 1978. Cloning of *Benekea* genes in *Escherichia coli*. J. Bacteriol. **133**:354–363.
13. **Lehner, A. F., S. Harvey, and C. W. Hill.** 1984. Mapping and spacer identification of rRNA operons of *Salmonella typhimurium*. J. Bacteriol. **160**:682–686.
14. **Loughney, K., E. Lund, and J. E. Dahlberg.** 1982. tRNA genes are found between the 16S and 23S rRNA genes in *Bacillus subtilis*. Nucleic Acids Res. **10**:1607–1624.
15. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10**:296–298.
16. **Mankin, A. S., and V. K. Kagramanova.** 1986. Complete nucleotide sequence of the single ribosomal RNA operon of *Halobacterium halobium*: secondary structure of the archaebacterial 23S rRNA. Mol. Gen. Genet. **202**:152–161.
17. **Morgan, E. A., T. Ikemura, L. Lindahl, A. M. Fallon, and M. Nomura.** 1978. Some rRNA operons in *E. coli* have tRNA genes at their distal ends. Cell **13**:335–344.
18. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Srivastava, A. K., and D. Schlessinger.** 1990. Mechanism and regulation of ribosomal RNA processing. Annu. Rev. Microbiol. **44**:105–129.
21. **Taschke, C., M. Klinkert, J. Wolters, and R. Herrmann.** 1986. Organization of the ribosomal RNA genes in *Mycoplasma hyopneumoniae*: the 5S gene is separated from the 16S and 23S rRNA genes. Mol. Gen. Genet. **205**:428–433.
22. **Tomioka, N., and M. Sugiura.** 1984. Nucleotide sequence of the 16S-23S spacer region in the *rrn*A operon from a blue-green alga, *Anacystis nidulans*. Mol. Gen. Genet. **193**:427–430.
23. **Ulbrich, N., I. Kumagai, and V. A. Erdmann.** 1984. The number of ribosomal RNA genes in *Thermus thermophilus* HB8. Nucleic Acids Res. **12**:2055–2060.
24. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**:615–619.
25. **Young, R. A., R. Macklis, and J. A. Steitz.** 1979. Sequence of the 16S-23S spacer region in two ribosomal RNA operons of *Escherichia coli*. J. Biol. Chem. **254**:3264–3271.