JOURNAL OF BACTERIOLOGY, May 1993, p. 3213–3219
0021-9193/93/103213-07$02.00/0

Vol. 175, No. 10

Characterization of Intergenic Spacers in Two *rrn* Operons of *Enterococcus hirae* ATCC 9790

LEONARDO A. SECHI† AND LOLITA DANEO-MOORE*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received 21 December 1992/Accepted 8 March 1993

Two DNA restriction enzyme fragments coding for the 3′ termini of 16S rRNA, the 5′ termini of 23S rRNA, and the intergenic spaces between them in *Enterococcus hirae* ATCC 9790 were cloned and sequenced. The intergenic space of one of these genes contains a $tRNA^{Ala}$ sequence, whereas the other does not. Nevertheless, the intergenic spaces contain several regions that exhibit high levels of sequence homology and are capable of forming structures with similar base pairs. An analysis of Southern blots of chromosomal DNA cut with one and two restriction enzymes indicated that *E. hirae* has a total of six *rrn* operons.

The study of sequences in the genes for rRNA is a powerful tool in bacterial taxonomy (23). Moreover, since related bacteria exhibit more similarities in their rRNA sequences than unrelated bacteria do, rRNA sequences are useful in studies of bacterial evolution (4, 22, 23). Less is known about homologies in the intergenic spaces between the 16S and 23S rRNA genes. Bott and Hollis found sequence homology in three *rrn* operons of *Bacillus subtilis* that contained no tRNA sequences (3). In *Escherichia coli*, in which rRNA processing has been studied in some detail, highly conserved stem structures flank the mature 16S and 23S rRNA sequences, and glutamate tRNA or in-tandem isoleucine and alanine tRNAs are found in the intergenic spacer between the 16S and 23S rRNAs (14, 21). The flanking stem structures and the 5′ termini of tRNAs are thought to be important in rRNA processing (20, 21). In this study we analyzed homologies in the intergenic spaces of two *rrn* genes of *Enterococcus hirae*. One contains a single $tRNA^{Ala}$ gene, in agreement with a recent report on *Streptococcus pneumoniae* (2), and the other has no tRNA genes. In addition, we estimated the number of *rrn* operons in *E. hirae*.

When *Apa*I (5′-GGGCC\C) was used to cut the DNA of *Enterococcus hirae* ATCC 9790, we obtained approximately 20 DNA fragments, ranging in size from 340 to 40 kbp as determined by pulsed-field gel electrophoresis (24). However, *Apa*I-cut DNA also produced a very distinct, broad, single band at 1.8 kb (Fig. 1, lane A). *Sma*I-cut DNA (5′-CCC\GGG) produced 16 bands detectable by pulsed-field gel electrophoresis, ranging in size from 300 to 46 kbp (24), and, in addition, a 4.6-kb band barely detectable in agarose gels (not clearly visible in Fig. 1, lane D). *Apa*I and *Sma*I are rare cutters for enterococcal DNAs, whose G+C contents range from 37 to 38 mol% (18). The same amounts of DNA digested with other restriction enzymes gave very large numbers of bands on agarose gels (Fig. 1, lanes B, C, and E to I).

Cloning and sequencing of the 1.8-kb *Apa*I fragment. *Enterococcus hirae* cells were grown in 3% (wt/vol) Todd-Hewitt broth (Difco) supplemented with 2% (wt/vol) glucose and 0.1% (wt/vol) L-cysteine, and chromosomal DNA was extracted and purified from mid-exponential-phase cultures (12, 16). A 1-μg portion of total DNA was digested overnight with *Apa*I under conditions specified by the manufacturer (Promega). Gel electrophoresis was performed in a 0.7% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8). The 1.8-kb *Apa*I band was purified by using a Gene-clean kit (Promega) and was ligated into the *Apa*I site of Bluescript± (Stratagene). *Escherichia coli* DH5α was transformed with the ligation mixture and plated on Luria broth agar supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (United States Biochemicals). Several white colonies were obtained. Plasmid preparation (15) showed that all of the colonies carried a 1.8-kb *Apa*I insert. The sequence of one clone was determined by the dideoxy chain termination method (17). A search of the GenBank data base (1) indicated that the *Apa*I fragment contained sequences complementary to the 3′ terminus of 16S rRNA, to alanyl-tRNA, and to the 5′ terminus of 23S rRNA. At the 3′ terminus of the 16S rRNA the levels of homology were highest with *Enterococcus sulfureus* and various lactobacilli, bacilli, and clostridia. There was also homology with the 5′ termini of the 23S rRNAs of bacilli and *S. pneumoniae*. In the intergenic space between the 16S and the 23S genes the levels of homology were highest with *S. pneumoniae*, with various lactobacilli, with the *rrnB* gene of *B. subtilis*, with *Lactococcus lactis*, and specifically with the alanyl-tRNA sequences of several bacteria. The sequence has been deposited in the GenBank data base, and portions of this sequence are discussed below.

Determination of the number of *rrn* operons. The cloned 1.8-kb insert was labeled with ^{32}P (ICN Radiochemicals) by using the random hexanucleotide primer labeling method (5) (Prime-a-gene labeling system; Promega) and was used as a probe of a Southern blot (19) of chromosomal DNA digested overnight with a number of restriction endonucleases. λ DNA was labeled in the same way and mixed with the probe to provide a size marker. Hybridizations were carried out at 68°C, and the blots were washed at 68°C under high-stringency conditions (15). Figure 2 (an autoradiograph of a blot from a gel similar to the gel in Fig. 1) shows that *Apa*I-cut DNA hybridizes with the probe only in the 1.8-kb region,

* Corresponding author.
† Permanent address: Istituto di Microbiologia & Virologia, Università di Sassari, Sassari, Italy.

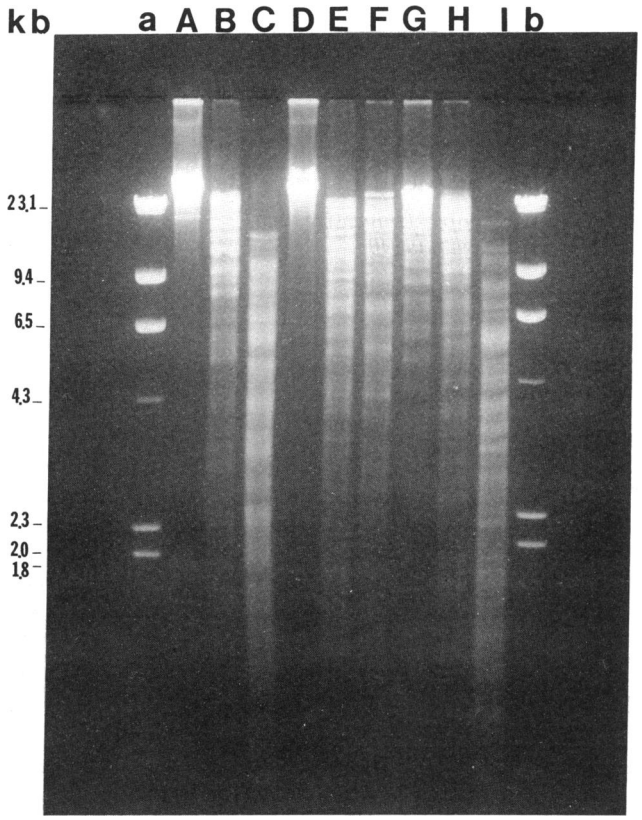

FIG. 1. Agarose gel containing ethidium bromide-stained *Enterococcus hirae* DNA (1 μg per lane) cut with *Apa*I (lane A), *Xba*I (lane B), *Hin*dIII (lane C), *Sma*I (lane D), *Bgl*II (lane E), *Pst*I (lane F), *Bam*HI (lane G), *Eco*RI (lane H), and *Cla*I (lane I). Lanes a and b contained *Hin*dIII-cut λ DNA markers (Promega). Note the band visible just below the 2.0-kb λ marker in lane A.

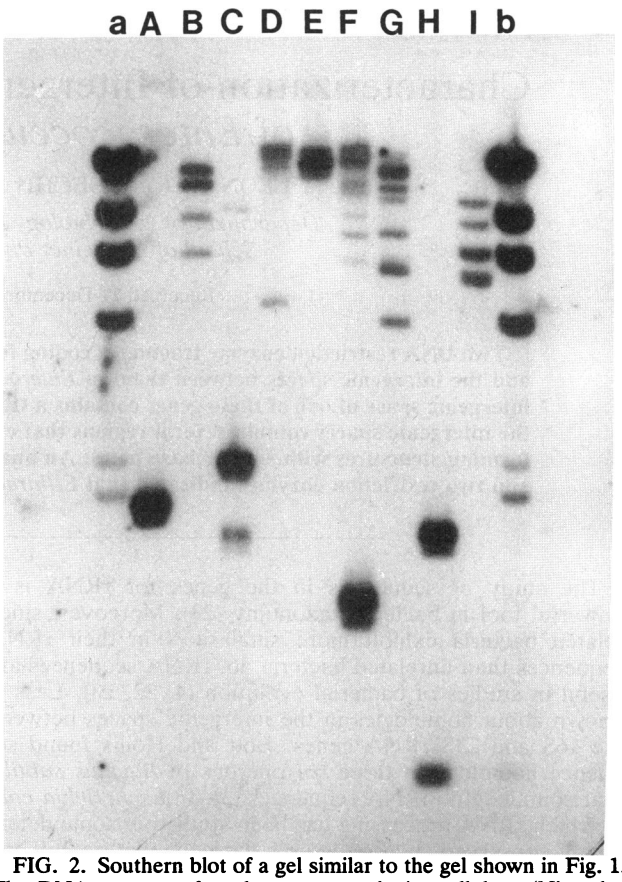

FIG. 2. Southern blot of a gel similar to the gel shown in Fig. 1. The DNA was transferred to supported nitrocellulose (Nitroplus 2000; MSI, Westboro, Mass.) by using a vacuum transfer device (ABN, Emeryville, Calif.) and a modification of the method of Southern (19). The blot was hybridized with the cloned 1.8-kb *Apa*I fragment and with λ DNA. For lane contents see the legend to Fig. 1.

where a broad band and a possible minor band of hybridization are visible (Fig. 2, lane A). The 1.8-kb probe did not hybridize with higher-molecular-weight *Apa*I-cut DNA (Fig. 2, lane A), indicating that all of the DNA homologous to the 1.8-kb fragment was located in this band. *Eco*RI-cut DNA produced two bands (one at 1.6 kb and one at 0.5 kb) that hybridized with the 1.8-kb probe (Fig. 2, lane H). Again, high-molecular-weight *Eco*RI-cut DNA did not contain a fragment that hybridized with the 1.8-kb probe. In contrast, *Sma*I-cut DNA hybridized with the probe at 4.6 kb (Fig. 2, lane D), where a single band was barely visible in ethidium bromide-stained gels (Fig. 1, lane D). However, higher-molecular-weight DNA also hybridized with the probe (Fig. 2, lane D). These results were reproducible with several DNA preparations.

Hybridization of the 1.8-kb *Apa*I probe with the *Xba*I and *Cla*I chromosomal digests of *Enterococcus hirae* DNA revealed four bands for each preparation (Fig. 2, lanes B and I). Since two of the bands in each digestion mixture hybridized repeatedly with the probe more intensely than the other two bands did, we analyzed the autoradiographs by scanner densitometry (13), using a Bio-Rad transmittance-resistance instrument. For the *Xba*I digest analysis (Fig. 2, lane B, starting from the top) the areas were 31, 29, 16, and 14 arbitrary units for bands 1 to 4, respectively. For the *Cla*I digest analysis (Fig. 2, lane I, starting from the top) the areas were 15, 13, 31, and 28 arbitrary units, respectively. Since the two smallest and most heavily labeled *Cla*I fragments were at about 5.8 and 7.0 kb, *Cla*I sites could flank each of two *rrn* operons, for a total of four operons. Two additional *rrn* operons would be contained in two higher-molecular-weight *Cla*I fragments. In this model, *Enterococcus hirae* contains six *rrn* operons. In an alternate model the less heavily (one-half) labeled fragments at 12.4 and 8.8 kb were due to an *rrn* operon which was cut into two fragments within a region homologous to the probe. This model would be generated by three *rrn* operons or by multiples of three *rrn* operons. *Cla*I-cut sites were not found within the cloned 1.8-kb *Apa*I sequence. However, in order to distinguish between the two models, the 1.8-kb *Apa*I probe was hybridized with *Apa*I-*Cla*I double digests of ATCC 9790 chromosomal DNA. The double digests gave the same pattern of hybridization as the *Apa*I digest did (data not shown), indicating that *Cla*I sites are absent in the region homologous to the probe in all *rrn* operons and that the first model is consistent with the data. *Xba*I does cut the cloned 1.8-kb *Apa*I sequence determined here, 98 bases from the 3′ end of the probe; *Apa*I-*Xba*I double digests of the ATCC 9790 chromosome gave a band at about 1.7 kb when they were hybridized with the 1.8-kb *Apa*I fragment (data not shown).

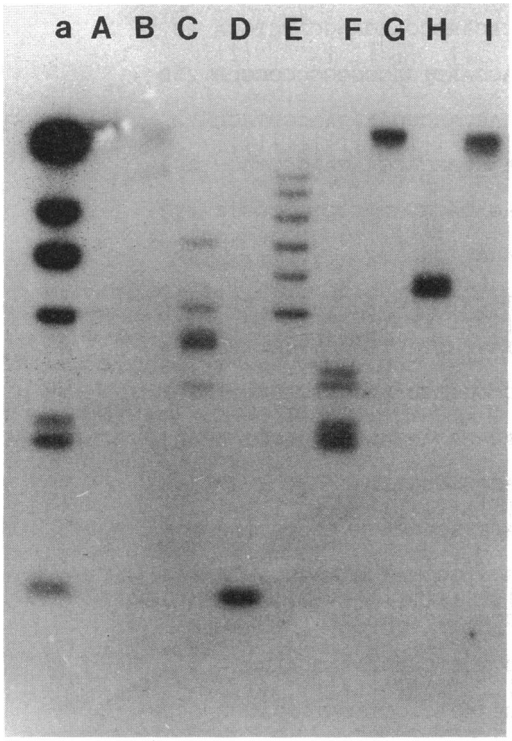

FIG. 3. Southern blot of an agarose gel containing *Enterococcus hirae* DNA (1 μg per lane) cut with *Csp*I (lane A), *Xho*I (lane B), *Ava*I (lane C), *Hpa*II (lane D), *Pvu*II (lane E), *Ssp*I (lane F), *Stu*I (lane G), *Hin*cII (lane H), and *Sac*I (lane I). Lane a contained *Hin*dIII-cut λ DNA markers (Promega). The blot was hybridized with a 450-bp fragment from the 5′ end of the cloned 1.8-kb *Apa*I fragment and with λ DNA.

Thus, *Xba*I also defines six *rrn* operons flanked by *Xba*I sites, two at 6.5 and 9.4 kb and four at higher molecular weights.

Several restriction enzyme digestions were performed in order to try to identify six *rrn* fragments of approximately equal density when preparations were hybridized with the 1.8-kb *Apa*I probe. The data obtained with *Hin*dIII, *Sma*I, *Bgl*II, and *Bam*HI are also shown in Fig. 2. The data obtained for *Hin*dIII (Fig. 2, lane C), *Sma*I (lane D), *Bgl*II (lane E), *Pst*I (lane F), and *Bam*HI (lane G) could be the result of partial digestions or restriction polymorphism in some of the *rrn* operons. Of the remaining restriction enzymes screened, *Pvu*II (Fig. 3, lane E) consistently gave six bands (5.0, 5.8, 7.1, 9.6, 12, and 16 kb). The sizes of these bands are consistent with cuts outside *rrn* operons. These bands had approximately equal autoradiographic densities in four repeated hybridizations with a 450-bp fragment from the 5′ end of the 1.8-kb *Apa*I probe. *Kpn*I, *Sac*I, *Stu*I, *Ssp*I, and *Xho*I gave multiple bands of hybridization that may have been the result of partial digestion (Fig. 3). In contrast, *Hin*cII and *Hpa*II each gave a single broad band of hybridization, at 5.6 and 0.6 kb, respectively (Fig. 3, lanes H and D).

Our tentative conclusion from the *Cla*I, *Xba*I, and *Pvu*II cleavage data that there are six *rrn* operons in *Enterococcus hirae* is in agreement with the finding of six *rrn* operons reported for the related gram-positive coccus *S. pneumoniae* (6).

Cloning and sequencing of the 1.6-kb *Eco*RI fragment. A DNA library for *Enterococcus hirae* was made by partially digesting the chromosomal DNA with *Eco*RI and cloning in the *Eco*RI site of Bluescript±. *Escherichia coli* DH5α was transformed with the ligation mixture, and the transformants were screened by colony hybridization (15), using the 1.8-kb *Apa*I probe. Several colonies which hybridized with the probe were isolated, and their DNAs were analyzed by digestion with restriction enzymes, followed by agarose gel electrophoresis. All contained an approximately 1.6-kb *Eco*RI insert. Sequencing of a clone was done in both directions by the dideoxy chain termination method (17), and the results have been deposited in the GenBank data base.

The 1.8-kb *Apa*I fragment and the 1.6-kb *Eco*RI fragment overlap each other (Fig. 4). The 1.6-kb fragment used as a probe with ATCC 9790 chromosomal DNA cut with *Eco*RI hybridized only with the 1.6-kb region (data not shown); the 1.8-kb probe hybridized with both the 1.6-kb *Eco*RI chromosomal DNA digest and the 0.5-kb *Eco*RI chromosomal DNA digest (Fig. 2, lane H).

Sequence comparison of the two cloned *rrn* fragments. The 1.8-kb *Apa*I and 1.6-kb *Eco*RI sequences were aligned for optimal homology (Fig. 4). The 3′ terminus of the 16S rRNA gene was identified by 16 nucleotides homologous to the 3′ ends of *S. pneumoniae* (2), *B. subtilis* (7), and *Escherichia coli* 16S rRNAs (14). The 1.6-kb *Eco*RI sequence contained an *Apa*I site at residue 240 (Fig. 4A, bottom line). The 110-bp residues downstream from the 16S rRNA are highly homologous in the two sequences.

Figure 4C shows what appear to be the 5′ termini of the two 23S rRNA genes. The 5′-terminal GGTTAA nucleotides are homologous to the 5′ termini of the 23S rRNAs of *B. subtilis rrnB* (7), *S. pneumoniae* (2), and *Escherichia coli* (14). A region upstream from the 23S rRNA sequence is virtually identical in the two *Enterococcus hirae* sequences (residues 1081 to 1146) (Fig. 4C, bottom line). An *Eco*RI restriction site was found 435 bases before the 5′ *Apa*I site (residue 1377) (Fig. 4C, top line), which is consistent with the observation that the *Eco*RI-cut DNA produces a 1.6-kb fragment and a 0.5-kb fragment that hybridize with the 1.8-kb *Apa*I probe (Fig. 2, lane H).

The intergenic space regions of the two cloned fragments (Fig. 4B) differ the most. The *Apa*I fragment contains $tRNA^{Ala}$, whereas the *Eco*RI fragment does not. Nevertheless, there are 68 residues that are identical in the two sequences (residues 793 to 861) (Fig. 4B, top line).

In order to explore this homology further, the intergenic spaces of both *Enterococcus hirae rrn* fragments were subjected to a FoldRNA program (9, 10, 25). As shown in Fig. 5, the GAGAG sequence underlined in Fig. 4A formed a loop with a CTCTC pentanucleotide in both sequences (Fig. 4A and B, Fig. 5). In both sequences there was base pairing between the 68 bases that are identical for the two fragments (residues 793 to 861 in Fig. 4B, top line) and the regions of homology between the two sequences after the 16S rRNA sequence (Fig. 4A). From the results of the FoldRNA program it appears that the secondary structures of the intergenic spacers are similar and are independent of the presence or absence of $tRNA^{Ala}$ (Fig. 5). The folded structures shown in Fig. 5 suggest the possibility that a $tRNA^{Ala}$ gene may have been deleted or added to a common ancestral structure and/or that the proposed secondary configuration may be important in rRNA processing.

The organization of the cloned 1.8-kb *Apa*I intergenic space is similar to that of the *S. pneumoniae* intergenic space

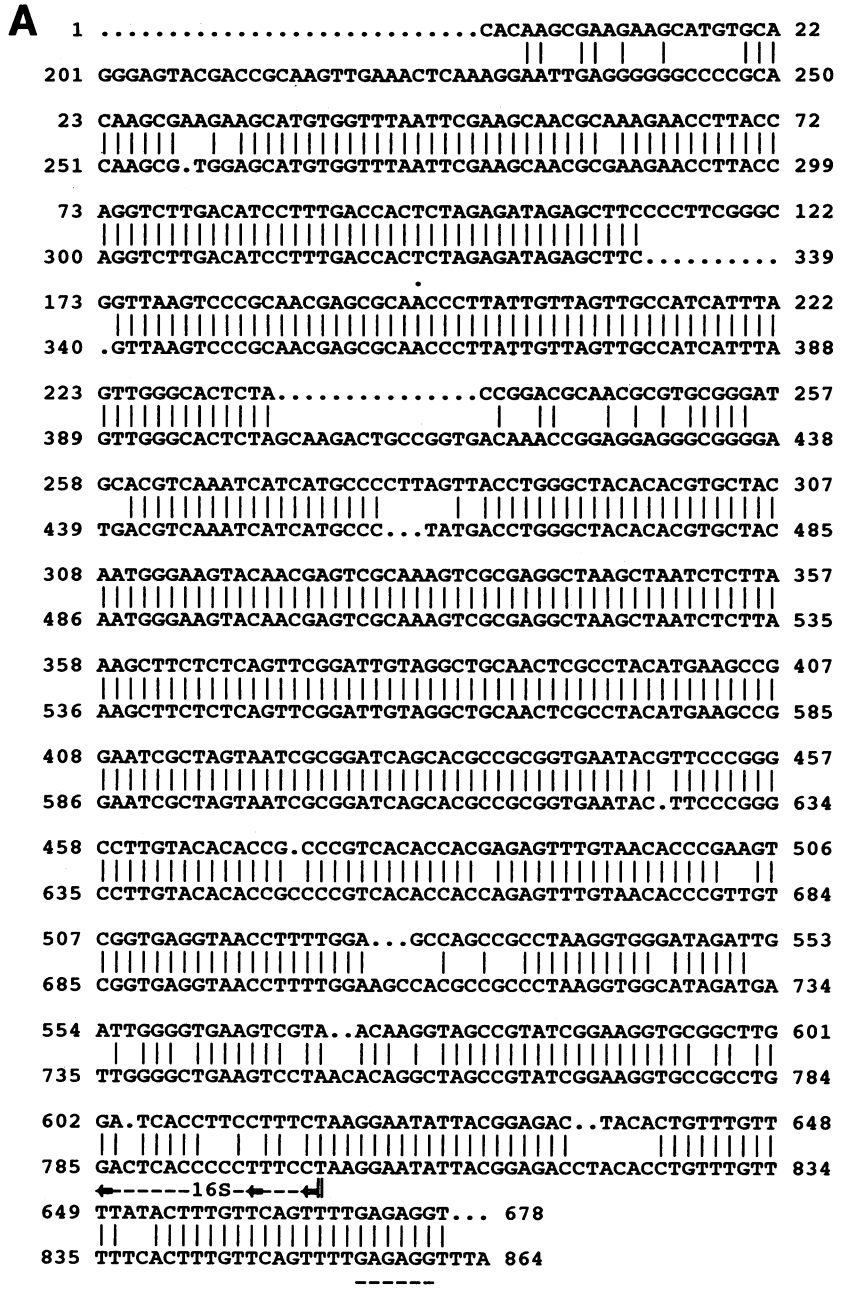

FIG. 4. Partial sequence comparison of the 1.8-kb *Apa*I-cloned fragment (top line) with the 1.6-kb *Eco*RI fragment (bottom line). Vertical lines indicate identical residues; dots indicate missing bases. (A) Presumptive 16S rRNA sequences aligned for homology. The 3′ terminus of 16S rRNA for both sequences is underlined; also underlined is a consensus GAGAG sequence. A sequence of about 110 bp 3′ of the 16S rRNA sequence is also shown. (B) Intergenic space realigned to show the identity of 68 bp in the two sequences. The $tRNA^{Ala}$ in the 1.8-kb *Apa*I fragment is overlined, as is the pentanucleotide CTCTC. (C) The 5′ terminus of the 23S rRNA in the two sequences is underlined. The highly homologous 95 bases 5′ of the 23S rRNA gene are included.

sequenced recently, which contains a single tRNA gene for alanine (2). This novel tRNA organization in the intergenic space is different from the organization of many eubacterial *rrn* operons (4, 11, 21, 22). However, a similar tRNA gene organization has been found in *Halobacterium cutirubrum* (8) and in *Methanococcus vannielii* (4).

The restriction maps of both cloned *Enterococcus hirae* fragments reveal restriction endonuclease sites which are highly conserved among different bacteria. For example, *Haemophilus influenzae* also has the *Apa*I and *Eco*RI sites conserved in comparable locations (13). A *Sma*I restriction site at the 3′ end of the 16S rRNA is conserved in the 1.8-kb *Apa*I fragment but not in the 1.6-kb *Eco*RI fragment. A *Sma*I site at the end of the 16S RNA gene reportedly is highly conserved in streptococci and enterococci (6, 23).

Nucleotide sequence accession numbers. The GenBank nu-

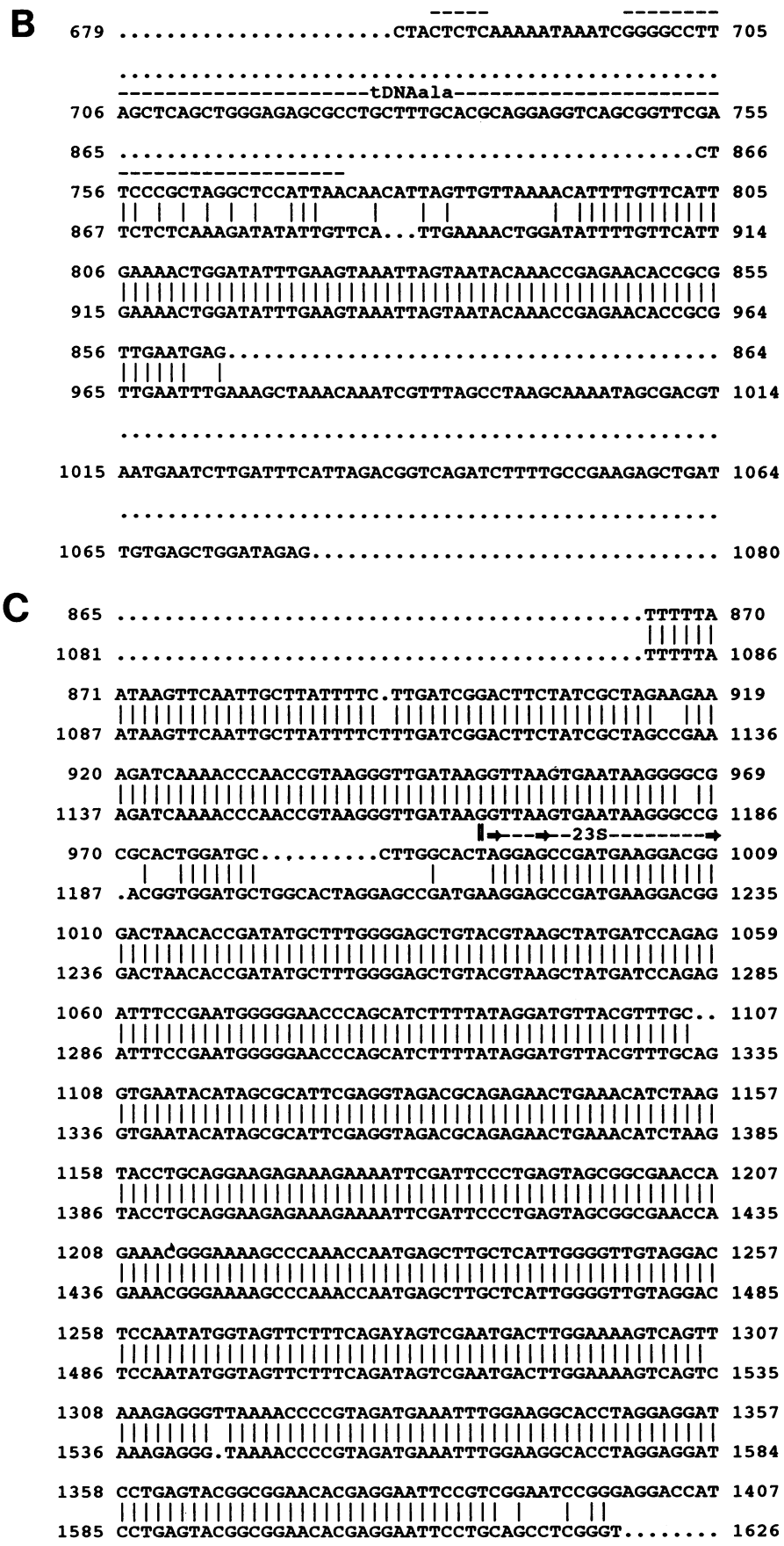

FIG. 4—*Continued.*

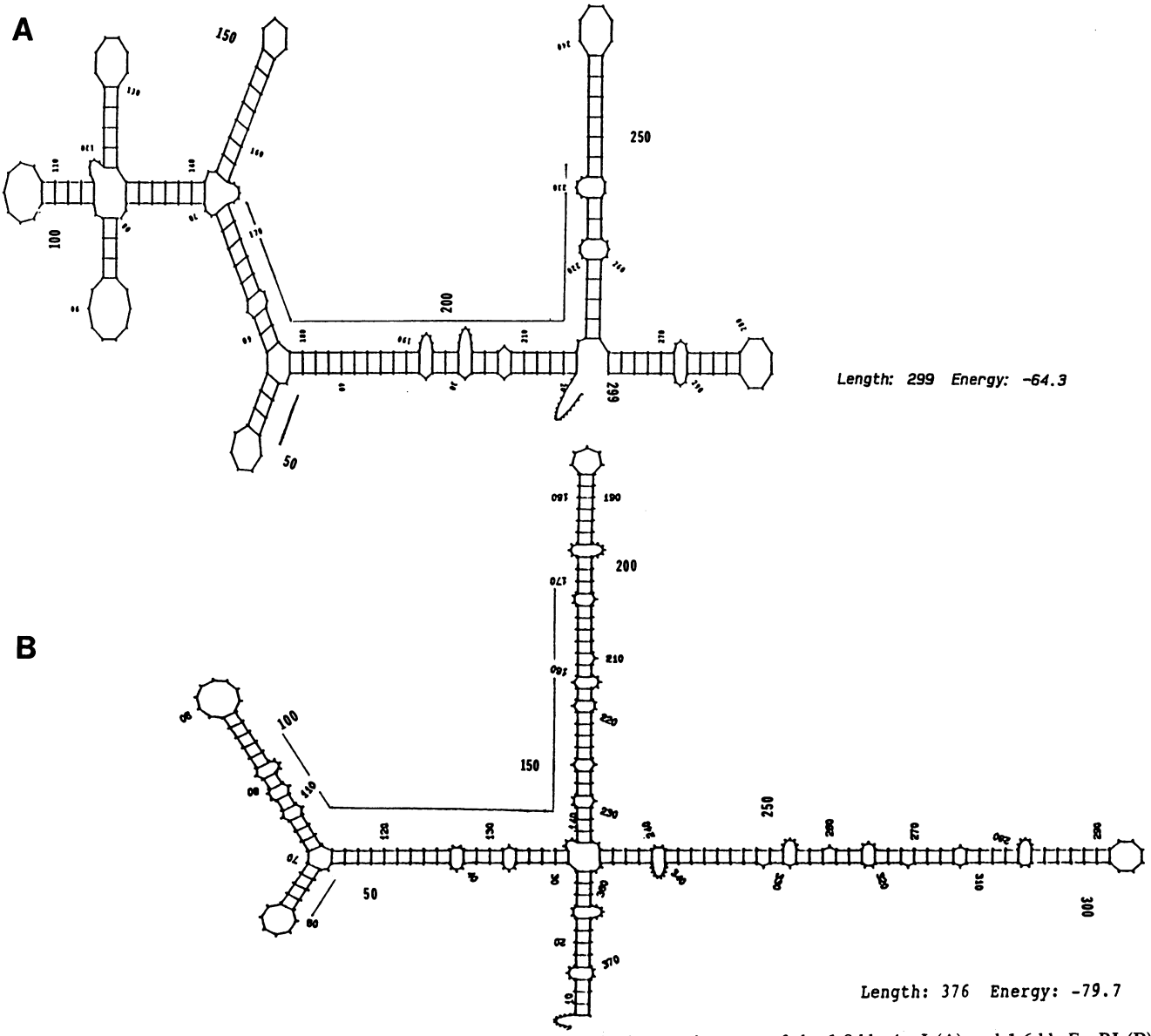

FIG. 5. Computer program folding of the RNA complementary to the intergenic space of the 1.8-kb *Apa*I (A) and 1.6-kb *Eco*RI (B) fragments. Note the $tRNA^{Ala}$ fold in the 1.8-kb sequence (70 to 140 bp) and the loops containing the GAGAG consensus sequence (45 to 50 bp in the 1.8-kb fragment and 55 to 60 bp in the 1.6-kb fragment). The 68-bp region of both fragments is also indicated (positions 167 to 232 in panel A and positions 100 to 168 in panel B). Each intergenic space sequence in the program was numbered; 1 corresponded to residue 799 in the sequence given in the bottom line in Fig. 3 and to residue 619 in the sequence given in the top line in Fig. 3. Because of the nature of the computer program, the two structures are not shown in the same scale.

cleotide sequence accession numbers are L00925 for the *Apa*I fragment and L00924 for the 1.6-kb *Eco*RI fragment.

We are grateful to G. D. Shockman, S. Fazari, F. M. Zuccon, O. Massidda, M. J. Hantman, and M. Whalen for discussions and to G. Harvey for editing and typing the manuscript.

This work was supported by Public Health Service grant DE08942 from the National Institutes of Health.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignement search tool. J. Mol. Biol. **215**:403–410.
2. **Bacot, M. C., and R. H. Reeves.** 1991. Novel tRNA gene organization in the 16S-23S intergenic spacer of the *Streptococcus pneumoniae* rRNA gene cluster. J. Bacteriol. **173**:4234–4236.
3. **Bott, F. K., and M. A. Hollis.** 1982. Nucleotide sequence of intergenic spacer DNA of three 16S-23S RNA genes from *B. subtilis*, p. 3–10. *In* Molecular cloning and gene regulation in bacilli. Academic Press, New York.
4. **Dennis, P. P.** 1986. Molecular biology of archaebacteria. J. Bacteriol. **168**:471–478.
5. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
6. **Gasc, A. M., L. Kauc, P. Barraille, M. Sicard, and S. Goodgal.** 1991. Gene localization, size, and physical map of the chromosome of *Streptococcus pneumoniae*. J. Bacteriol. **173**:7361–7367.

7. **Green, J. C., G. C. Stewart, M. A. Hollis, B. S. Vold, and K. F. Bott.** 1985. Nucleotide sequence of the *Bacillus subtilis* ribosomal RNA operon, rrnB. Gene **37**:261–266.
8. **Hui, I., and P. Dennis.** 1985. Characterization of the ribosomal RNA gene cluster in *Halobacterium cutirubrum*. J. Biol. Chem. **260**:899–906.
9. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1989. Improved predictions of secondary structure for RNA. Proc. Natl. Acad. Sci. USA **86**:7706–7710.
10. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1990. Predicting optimal and suboptimal secondary structure for RNA. Methods Enzymol. **183**:281–306.
11. **Krawiec, S., and M. Riley.** 1990. Organization of the bacterial chromosome. Microbiol. Rev. **54**:502–539.
12. **Le Bouguenec, C., and T. Horodniceau.** 1982. Conjugative plasmids in *Streptococcus faecium* (group D). Antimicrob. Agents Chemother. **21**:698–705.
13. **Lee, J. J., H. O. Smith, and R. J. Redfield.** 1989. Organization of the *Haemophilus influenzae* Rd genome. J. Bacteriol. **171**:3016–3024.
14. **Lund, E., and E. J. Dahlberg.** 1977. Transfer RNA genes between 16S and 23S rRNA genes in rRNA transcription units of *E. coli*. Cell **7**:165–177.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Rodriguez, R. L., and R. C. Tait.** 1983. Recombinant DNA techniques. The Benjamin/Cumming Publishing Co., Menlo, Calif.
17. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
18. **Schleifer, K. H., and R. Kilpper-Balz.** 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci. Syst. Appl. Microbiol. **10**:1–19.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Srivastava, K. A., and D. Schlessinger.** 1989. *Escherichia coli* 16S rRNA 3'-end formation requires a distal transfer RNA sequence at a proper distance. EMBO J. **8**:3159–3166.
21. **Srivastava, K. A., and D. Schlessinger.** 1990. Mechanism and regulation of bacterial ribosomal RNA processing. Annu. Rev. Microbiol. **44**:105–129.
22. **Srivastava, K. A., and D. Schlessinger.** 1991. Structure and organization of ribosomal DNA. Biochemie **73**:631–638.
23. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
24. **Zuccon, F. M., L. A. Sechi, and L. Daneo-Moore.** 1992. Preliminary physical map of *Enterococcus hirae* ATCC 9790. Annu. Meet. Am. Soc. Microbiol. 1992, poster H 181.
25. **Zuker, M.** 1989. On finding all suboptimal foldings of an RNA molecule. Science **244**:48–52.