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The Actinomycete *Thermobispora bispora* Contains Two Distinct Types of Transcriptionally Active 16S rRNA Genes

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Here we present the first description of the presence of two distinct types of 16S rRNA genes in the genome of a (eu)bacterium, *Thermobispora bispora*. We cloned and determined the nucleotide sequences of all four rRNA operons of *T. bispora*. Sequence comparisons revealed that the genome of *T. bispora* contains two distinct types of 16S rRNA genes, each type consisting of two identical or nearly identical copies, and three identical copies of the 23S RNA gene. The nucleotide sequences of the two types of 16S rRNA genes differ at 98 nucleotide positions (6.4% of total nucleotides) together with six regions of deletion-insertions. None of the base substitutions or insertion-deletions corresponds to any of the approximately 600 evolutionarily invariable or rarely variable nucleotides, indicating that both genes are functional. Both types of 16S rRNA genes are transcribed and processed as determined by Northern (RNA) hybridization and reverse transcriptase-mediated PCR.

Most organisms have multiple copies of rRNA genes. It is generally believed that all the copies of rRNA genes of an organism are identical or nearly identical in nucleotide sequence (18, 37). The homogeneity of rRNA genes is thought to occur by concerted evolution (13) of the repeated genes and stringent selection pressure on the primary sequences of rRNA molecules to maintain their precise interactions with components of the complex protein-synthesizing machinery (37). For the same reason, rRNA genes are thought unlikely to be horizontally transferred between organisms. The homogeneity of rRNA genes, together with some other properties, makes them the most widely used molecular chronometers for inferring phylogenetic relationships between organisms (1, 16, 37, 38.) However, several recent reports described considerable differences in nucleotide sequences between copies of rRNA genes in a single organism. The first reports came from the studies of 5S rRNA genes of the amphibian Xenopus laevis (33) and the loach Misgurnus fossilis (21). Both organisms have two classes of 5S rRNAs that are specific to either somatic or oocyte ribosomes. The genome of the eucaryotic parasite Plasmodium berghei contains two types of 18S rRNA genes which differ at 3.5% of the nucleotide positions and demonstrate life cycle stage-specific expression (11, 23, 32), and the metazoan Dugesia mediterranea possesses two types of 18S rRNA genes with 8% dissimilarity (5). An archaebacterium, Haloarcula marismortui (24, 25), was also reported to contain two distinct types of transcriptionally active 16S RNA genes. Although the number of such cases is small, they may represent a fairly common phenomenon considering the limited number of cases in which nucleotide sequences are available for all copies of an rRNA gene of an organism. Thus, the possession of different types of an rRNA gene in an organism may serve some unknown but essential biological functions.

In a previous study of the phylogenetic position of the actinomycete *Thermobispora bispora* (31), we obtained complete nucleotide sequences from several 16S rRNA clones retrieved from the genomic DNA by PCR and found two distinct sequences. This observation prompted us to conduct an extensive investigation of the organization and nucleotide sequence composition of the rRNA operons (*rrn*) of this organism. Here we report the cloning, sequencing, and characterization of the four *rrn* operons of *T. bispora* and discuss the significance and implications of our results.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *T. bispora* ATCC 19993^T (previously defined as *Microbispora bispora*) was purchased from the American Type Culture Collection (ATCC), Rockville, Md., and *T. bispora* JCM3082 was obtained from the Japan Collection of Microorganisms, Wako. *T. bispora* ATCC 15737 and DSM43038 (equivalent to JCM3082) were purchased from the ATCC and DSM-German Collection of Microorganisms and Cell Cultures, respectively. *T. bispora* IFO14880 (equivalent to ATCC 19993) was ordered from the Institute for Fermentation, Osaka, Japan. Mycelia were obtained by growing a single colony of each strain in media and under conditions recommended by the suppliers.

Preparation of genomic DNA. Genomic DNA was prepared as described previously (31).

Slot blot and Southern hybridization. For slot blot hybridization, DNA samples were blotted onto a Hybond-N membrane (Amersham, Aylesbury, Buckinghamshire, England) with a slot hybridization apparatus made by Hoefer Scientific Instruments (San Francisco, Calif.). Southern blotting, labelling of probes, and hybridizations were carried out by following standard protocols (20).

Cloning and sequence analysis of rRNA gene operons. Ten micrograms of genomic DNA was cleaved by a mixture of restriction enzymes BamHI and XhoI and then electrophoresed on a 0.8% low-melting-point agarose (GIBCO BRL, Gaithersburg, Md.) gel. The fractions of the gel corresponding to the positions of the rRNA gene operons were excised (see Fig. 1). The gel slices were melted by heating at 65°C, and the DNA was purified by extraction, twice with 1 volume of phenol and once with 1 volume of chloroform-isoamyl alcohol (24:1), followed by ethanol precipitation. The DNAs corresponding to different operons were spliced separately into Bluescript SK (Stratagene, La Jolla, Calif.) at appropriate restriction sites, and Escherichia coli DH5a cells (GIBCO BRL) were transformed. About 50 recombinant E. coli colonies from each transformation were picked and grown in 100 µl of LB with 100 µg of ampicillin per ml in 96-well microtiter plates for 16 h. Fifty microliters of each culture was transferred to a new microtiter plate, and the cells were lysed and DNA was denatured in the wells by the addition of an equal volume of a solution containing 0.1% sodium dodecyl sulfate and 1 M NaOH. Twenty microliters of each lysate was slot blotted onto a Hybond-N membrane (Amersham), and the presence of an rRNA gene in a recombinant plasmid was determined by hybridization. The complete nucleotide sequences of 16S and 23S rRNA genes were determined in both orientations by use of a set of oligonucleotide primers corresponding to conserved motifs within the genes (17, 31). Based on the results obtained from earlier rounds of sequencing analyses, new primers were designed for analyzing the sequences flanking the 16S and 23S rRNA coding regions. DNA sequencing reactions were conducted with the Amersham Sequenase kit (version 2).

Nucleotide sequence analyses. The alignment of nucleotide sequences and calculations of sequence similarity were performed with the DNASTAR (Mad-

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Α



FIG. 1. Southern hybridization analysis of the *rm* operons of *T. bispora*. (A) Genomic DNAs of strains ATCC 19993 and JCM3082 were cleaved by either a mixture of *Bam*HI and *XhoI* (B+X) or *XhoI* alone (X). Two duplicate membranes were prepared. One was hybridized with a 16S rRNA-specific probe (left), and the other was probed with a 23S rRNA-specific probe (right). The 16S rRNA probe was prepared by PCR amplification of the complete coding region from the genomic DNA with a pair of oligonucleotide primers targeting the conserved ends of the gene (17). The probe specific for nt 24 to 255 of 23S rRNA (*E. coli* number [17]) was prepared similarly with a pair of primers targeting two internal conserved sequences. (B) The four *Bam*HI-*XhoI* DNA fragments containing the *rm* operons were cloned into pBluescript. The recombinant plasmids were restricted with *Bam*HI and *XhoI*. The hybridization was conducted as described for panel A.

ison, Wis.) program. The secondary structure analysis was carried out with the MFOLD and PLOTFOLD programs in the Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, Wis.).

Preparation of RNA. Glasswares and solutions for the preparation of RNA were first treated to remove RNase as described previously (20). Mycelia of the organism were harvested as described above. An approximately 300-µl volume of wet mycelia was resuspended in a 2-ml screw-cap plastic tube in 400 μ l of homogenization buffer containing 4 M guanidinium thiocyanate, 0.1 M Tris-Cl (pH 7.5), and 1% β-mercaptoethanol. A 300-µl volume of acid-washed glass beads (400- μ m diameter; Sigma) and 400 μ l of a phenol-chloroform-isoamyl alcohol (25:24:1) mixture were added. The cells were broken in a sonicator (Ultrasonic Processor XL; Heat Systems, Farmingdale, N.Y.) at full speed for three cycles of 2 min of beating at 4°C and 2 min of chilling on ice. The mixture was centrifuged at $14,000 \times g$ for 5 min in an Eppendorf microcentrifuge. The aqueous phase was transferred to a new RNase-free 1.5-ml Eppendorf tube and extracted once more with the phenol-chloroform-isoamyl alcohol mixture. The nucleic acid was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.3) and 3 volumes of cold 100% ethanol at -80°C for 30 min. The nucleic acid was centrifuged at $14,000 \times g$ in an Eppendorf microcentrifuge for 10 min at 4°C, and the pellet was washed once with 1 ml of 70% ice-cold ethanol. The nucleic acid was air dried and resuspended in 100 µl of H2O. DNA was then removed by treatment with RNase-free DNase I (Boehringer Mannheim) as described by Huang et al. (14).

Northern hybridization and reverse transcriptase-mediated PCR (RT-PCR). Northern hybridization was carried out by following standard procedures (20). For reverse transcription, an oligonucleotide hybridizing to the 3' end of 16S rRNA was used to prime the synthesis of cDNA. One microgram of the total RNA was used as the template, and reverse transcription was carried out as described in the instructions of the supplier of the RT (New England Biolabs, Inc., Beverly, Ma.). One microliter of the reverse transcription mix was used directly as the template in subsequent PCRs. A 50-µl PCR mixture contains 10 pmol of each of two primers, 200 μ M each dATP, dGTP, dCTP, and dTTP, 1 U of *Taq* DNA polymerase (Amersham), and 1× buffer provided with the *Taq* polymerase. The PCR program consisted of 20 cycles of 95°C for 40 s, 50°C for 30 s, and 72°C for 20 s. The PCR products were examined on a 1.2% agarose gel. **Nucleotide sequence accession numbers.** The GenBank accession numbers for the sequences of the four rRNA operons are U83909 to U83912.

RESULTS

Southern blot analysis and cloning of the rrn operons. To preclude the possibility that our previous observation of two types of 16S rRNAs was an artifact due to contamination, the only two strains of T. bispora that had been described previously (31) were purchased from four different sources. Single colonies were picked to inoculate liquid cultures, and genomic DNA was prepared from each culture. Southern blot analyses of all the independently prepared genomic DNAs invariably generated identical hybridization patterns with bands of equal intensity. Figure 1A shows the results of a Southern blot analysis of the genomic DNAs of the T. bispora strains ATCC 19993 and JCM3082, which were cleaved by either XhoI alone or by a mixture of BamHI and XhoI. Neither of the two enzymes would cut within the 16S rRNA genes according to the sequences determined previously (31). A DNA probe containing the entire 16S rRNA gene coding sequence detected four bands with equal intensities from the products of both restriction reactions, and the genomic DNAs of the two strains produced identical hybridization patterns. The hybridization result demonstrates the presence of four rrn operons in each genome. The four BamHI-XhoI DNA fragments were 8.2, 5.7, 5.5, and 5.4 kb in length, and the respective operons were designated rrnA, rrnB, rrnC, and rrnD. The sizes of the four XhoI DNA fragments were 11.5, 8.3, 6.3, and 5.6 kb. When a DNA probe specific for the region of nucleotides (nt) 24 to 255 (E. coli numbering [41]) of the 23S rRNA gene was used to hybridize the product of the BamHI-XhoI cleavage, only three bands corresponding to rrnA, rrnC, and rrnD were detectable and no new band appeared at another position. From the XhoI restriction product, only the 8.3-, 6.3-, and 5.6-kb DNA fragments were detected. A second probe specific for the region of nt 1930 to 2563 of 23S rRNA detected an identical hybridization pattern, suggesting that the BamHI-XhoI fragments corresponding to rrnA, rrnC, and rrnD contain 16S and at least most of the 23S rRNA genes but the fragment corresponding to rmB does not contain a 23S rRNA gene. The four BamHI-XhoI DNA fragments from the genome of the type strain ATCC 19993 were separately cloned into a Bluescript SK plasmid (Fig. 1B).

Nucleotide sequence analysis of 16S and 23S rRNA genes. The 5.5- and 5.4-kb BamHI-XhoI fragments corresponding to *rrnC* and *rrnD* operons, respectively, were sequenced. Both fragments contain one gene each for 16S and 23S rRNA; the 5.5- but not the 5.4-kb fragment also contains a 5S rRNA gene. Sequence comparison showed that the two 16S rRNA genes differed substantially, with about 92% sequence similarity, while the two 23S rRNA genes are identical. This result confirmed our previous observation of two types of 16S rRNA in the genome of T. bispora and also inspired us to obtain the 16S and 23S rRNA sequences from rrnA and rrnB. The 16S rRNA sequence of *rmA* was found to be nearly identical to that of rrnC, with only two base substitutions, and the 16S rRNA sequence of *rrnB* was the same as that of *rrnD*. Operons *rrnA*, rrnC, and rrnD have identical 23S rRNA sequences. In agreement with the result of the Southern hybridization described above, no 23S rRNA gene was found in *rrnB*. These sequence comparisons reveal that the genome of T. bispora contains two distinct types of 16S rRNA genes with two copies each but only

rrnA rrnB	CTCGACTGG <u>A</u> A G-	GAGTTTGATC	CTGGCTCAGG	ACG <u>AACG</u> CTG	GCGGCGTGCT	TAACACATGC	AAGTCGAGCG	AGGACCGCAC	CTTCGGGŤ TCT T	GTGGAGCCGA	98 99
rrnA rrnB	GC <mark>GGC</mark> GAADG	GGITGAGTAAC	ACGTGAGCAA	CCTGCCCTG	GCACCGGAT T T	AGCCCCGG <u>GA</u> T	AACTGGGATT C	AATACCGGAT	ACGACCACTT T G G	CCCGCATGGG T	198 199
rrnA rrnB	ATGGTGGTGG -	<u>AAA</u> GGTTCCC	CCTTGTGGGG	TTCCGGCCAG G	GGATGGGCTC	GCGGCCC <u>AT</u> C T	AGCTAGITGG T	CGG <u>GGTAA</u> CG T G	GCCCACCAAG T	GCGATGACGG	298 298
rrnA rrnB	GTACCOGCC	TGAGAGCGCG T	GTCGGCCACA	CT <u>GG</u> G <u>ACTG</u> A	GACACAGOCC	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	ATTGCGCAAT	CGCCGCAADC	398 398
rrnA rrnB	CTGACGCAGC	GACCCCCCT	<u>G</u> GGG <mark>GA</mark> II <u>GA</u> A	GGCCTTCG <mark>G</mark> G	TT <u>GTAAA</u> CCT	CITTCAGCAG	GGACGAAGTT	GACGTGTACC	tgcagaAgaA	Geccera	498 498
rrnA rrnB	ACTACGIGCC	AGCAGCCGCG	<u> </u>	GGGCGCGAGC	<u>GTT</u> GTC <u>CGG</u> A	GTINACITEGEC TCT	G <u>TAAAG</u> GCT A	CONAGECCEC	ттотсос©тс с с	GGCTGTGAAA T C	598 598
rrnA rrnB	GCCCACGGCT	T <u>AAC</u> CGTGGA C G	ATTGCAGTCG TC G CG	at <u>AC</u> gggcag g	G <u>CTAGA</u> GGGC T CT	GGCAGAG <mark>G</mark> GG T G CA	AGTG <u>GAA</u> IT <u>T</u> C C	CCGGIGIAGC T	GGTGAAATGC	GCAGATATCG	698 698
rrnA rrnB	GGABGAACAC	<u>C</u> GGTGGC <u>GAA</u>	GGCGGCTCCC TG	TGGGCCGTCC AGT	CTGACGCTCA	GCAGCGAAAG	CGTC	GAACAGGATT	AGATACCCI	<u>GTAGTC</u> CACG	798 798
rrnA rrnB	<u>C</u> CG <u>TAAAC</u> GT	TGGGCGCTAG G	GTGTGGGGGAT GC	CTTTTGTGAT T CCAC G	CTCCGTGCCG TC	TAGCTAACGC	AT <u>TAA</u> GCGCC G	CCCCTGGGG	AGTACGGCCG	CAAGGCTAAA	898 898
rrnA rrnB	ACTCAAABGA	<u>AC</u> TGACGGGG) T	GCCCCCCACAA	CCCCCCCACC	ATCTTGOTTA	ATTCGA IGCA	ACCCGAACAA	CCTTACCAGG A	GCTTGACATG T	CTGGGAAATC C	998 998
rrnA rrnB	CCGCA <u>GA</u> GAT	GCGGGGTGCC	TTCGGGCGTC C	CAGCAC <u>AGGT</u> TG	GRIGCARGC	TGTCGTCAGC	TCGTGTCGTG	AGATGTIGGG	TTAAGTCCCG	C <u>AACGAGCG</u> C T	1098 1098
rrnA rrnB	AACCCCCACC T	CTATG <u>TT</u> GCC G	ABCBGGTT AC CCCC	ATGC TTGTGGG G	OGEGCACTICA T G	TGGGGA <u>ACTG</u> AG	CCCGGGGTCA	ACCCGGAGGA	AGGCGCGAC T	GACGICAAGT	1190 1198
rrnA rrnB	CATCATGCCC	C <u>TTA</u> IGCCCT T	GGGCITGCAAA	<u>C</u> ATGC <u>TACA</u> A	TGGCCGGT <u>AC</u> T	AATGGGCTGC C TC	GATCCCGTCA	gggtgag <mark>C</mark> ga C	ATCCCCAA TTAG	ABCCGGTCCC	1288 1298
rrnA rrnB	AGTICAGAIC T	Geggt <u>Ctg</u> e <u>A</u>	ACICGACCCC	GT <u>GAAG</u> IC <mark>GG</mark> A	ACTOCOTAGT	AATCGCGGAT	CAGCAATGCC	GCGGTGAATA	C <u>GTTC</u> CCGGG	CT <u>TTGIAC</u> AC C	1388 1398
rrnA rrnB	ACCGCCCGTC	<u>A</u> cgt <u>CC</u> igaa	AGTCGGCAAC	ACCCGAAGCC	GGTGGCCTAA C	СССССТТТТТ Т	TGGGGGAGGG 	AGCCGTCGAA	GGTGGGGCTG TC	gtc <u>A</u> rt <u>Ccc</u> a	1488 1492
1177A 1777B	CGAAGTCGTA	ACAAGGTAGC	CGTACCGGAA	GGTGCGCCTC	GATCACCTCC	TTTCT					1543 1547

FIG. 2. Alignment of the nucleotide sequences of 16S rRNA genes of mA and mB. The 16S rRNA sequence of mA is presented, and the nucleotides that are different in mB are indicated. The boxed regions show the phylogenetically invariable or rarely variable nucleotides (17, 30).

one type of 23S rRNA gene consisting of three identical copies. The 16S rRNA genes of rrnA and rrnC are designated type I, and those of rrnB and rrnD are designated type II. An alignment (Fig. 2) of rrnA and rrnB 16S rRNA sequences revealed 98 nucleotide substitutions, about 6.4% of the total number of nucleotides of the gene, and six insertion-deletion gaps involving 20 bases. To exclude the possibility that one of the two types of genes might represent a pseudogene, the distribution of base substitutions and insertion-deletions was inspected. The base substitutions were found to be dispersed in a nonrandom fashion along the gene. Two examples of this include the following. First, in the 262-base region from nt 286 to 548, there is not a single base substitution, while within the next 120 bases, there are 24 base substitutions. Second, none of the base variations and deletion-insertions coincides with any of the 600 nt which are either evolutionarily invariable or present in the 16S rRNA molecules of more than 90% of the bacterial species whose 16S rRNA sequences have been determined (17, 30). If either of the two types of genes was a pseudogene, the nearly 100 base substitutions would be randomly distributed throughout the gene. The positions of the sequence variations were also examined against a consensus secondary structure model of the small-subunit rRNA (30). Within helical regions, compensating covariations of paired bases were readily identified in most cases (data not shown). The positions of the two major deletion-insertions reside in two highly volatile stem-loops of the small-subunit rRNA (17, 30). The nonrandom distribution of the base substitutions and compensating covariation of

paired bases strongly suggest that both types of 16S genes are functional.

Detection of the two types of 16S rRNAs by Northern hybridization and RT-PCR. To examine whether both types of 16S rRNA genes are transcribed, total RNA was prepared from a culture of strain ATCC 19993 and analyzed by Northern hybridization. Two oligonucleotide probes, each specific for one type of 16S rRNA, were designed, and their specificities were tested by hybridizing to the four cloned *rm* operons (Fig. 3). Each probe recognized only its correspondent 16S rRNA. Under identical conditions, both probes detected, in separate hybridizations, a single band at the same position as a band recognized by a universal 16S rRNA probe, indicating that both types of 16S rRNA genes were transcribed and correctly processed.

To confirm the results of Northern hybridization, RT-PCR was carried out. cDNAs were synthesized from the total RNA by using a primer that would hybridize to the 3' end of both types of 16S rRNA. Two pairs of PCR primers were designed so that each pair would amplify a section from only one type of 16S cDNA. The hybridization positions and the orientations of the primers are shown in Fig. 4A, and the results of RT-PCR are shown in Fig. 4B. Again, the specificities of each pair of primers were examined in PCRs by using the cloned *rmA* and *rmB* as templates. Each pair of primers was shown to amplify a fragment of the correct size only from its correspondent 16S rRNA gene. When the cDNA was used as the template, both pairs of primers amplified fragments of the correct size. A pair



FIG. 3. Detection of the two types of 16S rRNAs by Northern hybridization. (A) Total RNA of strain ATCC 19993 was electrophoresed on a 1% formaldehyde-agarose gel, and the 16S and 23S rRNAs were visualized by ethidium bromide staining. The three lanes contain replicate samples. (B) The specificity of the two gene-specific probes was tested by hybridization to the four cloned *rm* operons slot blotted onto a nylon membrane. The names of the operons are given on the left, and those of the probes are shown at the tops of the lanes. Probe T1 is type I 16S rRNA specifically targeting the region of nt 1436 to 1453 (see Fig. 2), and T2 is type II 16S rRNA specifically targeting nt 1446 to 1462. U3' is n universal probe for 16S rRNA that hybridizes to the 3' end sequence 5'GGCT GGATCACCTCCTT3'. All three probes were synthesized complementary to the target sequences. (C) The RNAs resolved on the agarose gel shown in panel A were blotted onto a membrane. The tree lanes were separated and hybridized to the different probes as shown at the top of the lanes.

of primers specific for the 23S rRNA sequence did not amplify fragments from the cDNA, although it produced a fragment of the correct size from the cloned *rmA* operon (but not from *rmB*, which does not contain a 23S rRNA gene). None of the three pairs of primers could amplify products from the mock cDNA reaction where RT was not added, which excluded the possibility of PCR amplification from residual genomic DNA. Together with the Northern hybridization results, those of the RT-PCR experiment clearly demonstrated that both types of 16S rRNA genes were transcribed in the cell. Although these experiments did not quantify the expression of each type of 16S rRNA gene, the amounts of the two types of 16S rRNAs appeared comparable as judged by the intensities of hybridization signals and bands of PCR-amplified fragments.

Analyses of the sequences upstream of the 16S rRNA coding region. To examine whether the difference between the 16S rRNA genes extends into their upstream regions, the nucleotide sequence from the beginning of the 16S rRNA coding sequence to the end of the cloned DNA fragment was determined for all four of the rrn operons (Fig. 5A). When rrnB and rrnD were compared, their sequences were found to be almost identical from nt -1 to -348, differing at only five positions, while the corresponding regions of *rrnA* and *rrnC* had much shorter identical sequences extending from nt - 1 to -43. The sequences further upstream do not show appreciable similarity between the two operons with the same type of 16S rRNA genes. When all four sequences were aligned, little similarity was observed except for a stretch of 29 bp (Fig. 5A) which is located 14 and 152 bp upstream from the first base of the 16S rRNA coding region in the operons coding for type I and type II 16S rRNAs, respectively. Using the 29-base sequence to search the GenBank, we identified a nearly identical sequence block in the external transcribed spacer (ETS) of rrn operons in several other actinomycete species (Fig. 5B). This sequence block also resides in the putative ETS in the *rrn* operons of *T*. bispora because it is located downstream of the putative promoter (3, 22) nearest to the 16S rRNA coding sequence. The function of this conserved sequence is unknown, but it is likely involved in the processing of the primary rRNA transcripts (15).

The RNA sequences surrounding the mature 16S and 23S rRNAs can normally form several stem-loop structures necessary for the processing of primary rRNA transcripts (15). A computer analysis identified stable stem-loops at expected positions of all four operons of *T. bispora* (data not shown). However, their roles in rRNA processing are only speculative.



FIG. 4. Detection of the two types of 16S rRNAs by RT-PCR. (A) Diagram showing the position and orientation of the oligonucleotide primers for RT-PCR. The bold arrow represents the oligonucleotide U3' (as described in the legend to Fig. 3) for priming reverse transcription. The target sequences of other primers are defined by the numbers in parentheses, and orientation is indicated by arrows. See Fig. 2 for the sequence of each primer. (B) Five pairs of primers applied to different templates for PCRs. The templates are indicated below the figure. The primer pairs were T1L and T1R1 (lanes 1), T1L and T1R2 (lanes 2), T2L and T2R1 (lanes 3), and T2L and T2R2 (lanes 4). A pair of primers specific for the 23S rRNA sequence was also used (lanes 5). The target regions of the two primers are nt 1930 to 1947 and nt 2545 to 2563, respectively (*E. coli* numbering [17]). M, molecular mass markers.

A TTRA

-403	CTCGAGTACC	TCGGCTCCCA	CTGGTACCGC	CGGGCCTGGG	AGCTCGCGCC
-353	GGTGCGCTCG	GCGCGGCTCG	CGGTGCTGCC	CGAGGTGGCC	GAGGGCTTCC
-303	GGCCCGCCCG	GCGCGTCCTC	AACTACGTGG	CGGGCCGCTA	CCTGTTCGCC
-253	GCGTCCGGCC	GCTGAGCCGT	GACACACGGT	GCCGGCGTTT	CCCCAGGTCA
-203	TCGAGGGAAT	CGCCGCGACG	CGCCCGGC <u>TC</u>	GATTTGCATC	TTGCGCGGAG
-153	ATGATTACCC	TCTTCTACTG	ACAGCGCGCG	GGAACGCGCG	GAGGAACCCC
-103	CAAGGAGATC	GATCCGAGAG	GGTGTTCCGC	ACGGCCTGGC	GGGCGACGAG
~53	GCGAGAGCCT	CCCGCACCTT	GAGAACTCAA	CAGCGTGCCC	TGTGTTACGG
-3	AACCTCGACT →165 rR	GGAGAGTTTG NA	ATCCTGGCTC	AG	

rrnC

-321 CTCGAGGGTA TCCGCGGACC GCCGATGAGG TAGGATGCCG GAACCCGCCT

- -271 TGCCACGCGT GAGACATCGC CCCACGGACG ATGTCAGGGA AACGCGAGAG
- -221 ACGAGGTAAC ATACCTCGCG CGGATCACCG GAGTACGCGA AACGGACACA
- -171 CCGCAACGGT GTGCACCCGCG GAGCGGACCC GATGAGAAGC GCGCCGGTGG -121 CAAAAGCAGC CGCCGGGCCG GGATAGATA CCTCCCCGGA ACCGGGCCTC
- -71 GGAAGAGGCG ACGGGGACGG CGGAGACGCC CGCAGCTTGA GAACTCAACA
- -21 GCGTGCCCTG TGTTACGGAA CCTCGACTGG AGAGTTTGAT CCTGGCTCAG

rrnB&D

В

CTCGAGGTCC GCTGAAGA<u>TC GGTTTG</u>CATG ACCGGCACCC AGTGGT<u>TATA</u> -430 GTCGAGGGGT TGCCTCGGGC AAGGTTCGGC CGTTTGATTC CTGCCGGTGT -380 CGGGGTTGGT GTCAAGTGCC GCCGCGGTTG GGTCGGTTTG ACACCGGTTT -330 CGGGTCTGGT GAGAATCGGA GGGCTGTTTC GGGCCAGGGC GTGAAATCCC -280 GGTGGGTTCC AGGTTCGGGG CTTCGGGGTT TCGGGTCGTG GTGCCGGGGC -230 CCGTTTCTTG AGAACTCGAC AGTGTGTCAA AAGCCAGTGC ATTGGGCGCT -180 GTCGCGATGA CGCCGTGATC TGGTGTTGTC GTGGTGGGTG ATTGTGGCTG -130 CCATEGTECC CECTCECTE TETTEGTEGE CEGTECCETE ETEGTETCEC -80 CGGGATTTTC TTCCCGTGTG TTTGAGGTTT ATCGGTGGAG AGTTTGATCC -30 TGGCTCAG

	M. b: M. b: S. an S. co S. gi Fran	ispora nbifacie celicol riseus kia spee	rrnB&D ens or rrn A&D cies	CCCGTTECTTGAGAACTCGACAGIBTEIT CCCGTTCCTTGAGAACTCGACAGIGTEIT TCCGTTCCTTGAGAACTCAACAGCGTGCC CCCGTTCCTTGAGAACTCAACAGCGTGCC CCCGTCCTTGAGAACTCAACAGCGTGCC
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FIG. 5. Nucleotide sequences upstream from the 16S rRNA coding region. (A) Complete sequences for *rmA*, *rmC*, and *rmD* from the beginning of the 16S rRNA coding region to the *XbaI* site (CTCGAG) at the ends of the cloned operons. Operon *rmB* is nearly identical to *rmD* downstream from the position indicated by the vertical arrow, but its sequence upstream from this position has no homology with that of *rmD* and does not contain putative promoter. This region of *rmB* is therefore not shown. The nucleotides that are different in *rmB* are indicated by smaller letters under the corresponding nucleotides of *rmD*. Putative promoters are underlined. A region that is highly conserved in all four operons is double underlined. (B) Alignment of conserved sequences in the ETS of *rm* operons of several actinomycetes. The database accession numbers for the sequences are as follows: *Frankia* sp., GenBank M88466; *Streptomyces coelicolor* EMBL X60514 and Y00411; *Streptomyces ambifaciens*, GenBank M26245; and *Streptomyces griseus*, GenBank M76388.

Sequence comparison of the internal transcribed spacers (ITS) separating 16S and 23S rRNA genes. The sequences of 16S-23S spacers of rrnA and rrnC are both 169 bp long and have about 50% nucleotide similarity. The spacer of rrnD is much longer, containing 320 bp. Although rrnB does not have a 23S rRNA gene, the sequence immediately downstream from the 16S rRNA gene, except for an insert of 30 bp, is nearly identical to the first 223 bases of the rmD spacer, but the sequence further downstream does not show any meaningful similarity to the other three spacer sequences. Figure 6 shows the alignment of all four spacer sequences. The spacers of the operons with the same type of 16S rRNA genes have high sequence similarities. However, other than a few conserved nucleotide blocks, little similarity is present between the spacers of the operons with different types of 16S rRNA genes. All the spacers start and end with conserved nucleotide blocks of AAGG and CGTGT, except for the *rmB* spacer, in which the 3' end is apparently deleted together with the 23S rRNA gene.

In the middle of the spacer, a 30-bp sequence was found highly conserved. The last 25 nt of the spacers of *rmD* and *rmC* are identical, which is a rather surprising observation since their upstream sequences extending to the promoter regions have, in phylogenetic terms, either very low or little similarity. No tRNA sequence was found in any of the three 16S-23S spacers.

DISCUSSION

The presence of two distinct types of a small-subunit rRNA gene in a single genome has been reported in organisms belonging to the domains *Eucarya* and *Archaea* (5, 11, 24, 25). Here we present the first description of two distinct types of 16S rRNA molecules in the genome of an actinomycete, T. bispora, of the domain Bacteria. Although the two rRNAs differ at 98 nt positions, none of the substitutions coincides with the nearly 600 nt which have been designated as either invariable or conserved in more than 90% of the bacterial species (17, 30). Such a highly biased localization of base substitutions cannot be a consequence of a random accumulation of mutations in a pseudogene. The nonrandom distribution of base substitutions is also characterized by the frequent occurrence of compensating covariations of interacting bases in the rRNA molecule. Thus, both rRNA molecules should retain all the important structural features necessary for protein synthesis. Therefore, we conclude that both types of rRNA molecules are functional. The conclusion gains further support from the following observations. First, both types of 16S rRNAs are present in the cells as shown by both Northern hybridization and RT-PCR experiments. Second, in a background of little sequence similarity, conserved sequence blocks are present in both the ETS and ITS regions, which have been shown to specify several crucial functions including the precise processing of precursor rRNAs and assembly of ribosomes (15).

In previous reports, the origin of distinct types of an rRNA gene in a single genome has been explained by either divergent evolution following gene duplication or by lateral gene transfer between different species (5, 11, 25). One piece of evidence for lateral gene transfer would be the demonstration of different phylogenetic affiliations of the two 16S rRNA sequences with those of other bacterial species (10, 11, 29). We conducted a phylogenetic analysis including 16S rRNA sequences from representative species of most genera of actinomycetes available from the public databases. The results showed that the two 16S rRNA sequences of T. bispora, despite their substantial difference, were still the most closely related species and formed a cluster distant from other groups of actinomycetes (data not shown) (see reference 31). Owing to the lack of closely related actinomycete species, the phylogenetic argument for lateral gene transfer is, therefore, inconclusive.

Another line of evidence for lateral gene transfer would be via the detection of mosaic genes or operons, which can often be reflected in different GC contents and biased codon usage associated with different DNA segments (7, 9, 29). A careful sequence comparison of various regions of the four operons did not detect any significant uneven distribution of GC content within or between operons. However, operon rrnD seems to possess features typical of a mosaic DNA. Operon rrnB is incomplete without a 23S rRNA gene, but it is nearly identical to rmD in sequence, extending from nt -350 upstream from the 16S rRNA coding sequence to 233 bases into the 16S-23S ITS. Over the same region, these two operons have low or little sequence similarity with rrnA and rrnC. Interestingly, from the last 25 nt of the ITS to the end of 23S rRNA gene, rmD suddenly becomes identical to rrnC. The alignment of the last 25 nt of the ITSs of *rrnA* and *rrnC*, which have the same type

GGCTCACTGTGGACCGGTGGGCGGCTGGAG

rrnB AAGGTGCACC CCTTAAGCCC CTGFGTGATA AAAGBGCTT GGGGCTGAGT TTGTGGGGTGA 60 rrnD AAGGTGCACC CCTTAAGCCC CTGFGTGTGAA AAAGTCCTT GGGGCTGAGT TTGTGGGGTGAA rrnD AAGGATGAL- TTCATTCCAT CACCICG GTGATG-GAA CACCACTTCLA AACCACACGAC C rrnC AAGGATGALA TTCGTGCGC CASGCCGCAC GCCATGGGAA AGCACTTCLA AACCACACTCC C rrnC AAGGATGALA TTCGTGCGC CASGCCGGCAC ACGACCTGG GCCATGTGGTAGT AACCACACTCCC C rrnD ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCCATGTGTGAT ACCACTTCT I20 rrnD ATGTGGAGCG CTGGTTAGTC GCCCGGGCAC ACGAGCCTGG GCCATGTTTTTCT I20 rrnA GAGGCGGC GCGCTGGTTAGTC GCCGGGGCG GCGT TTGTGGGGGTCG ACGAGCCTGG I20 rrnA GAGGCGGGTCG TTATGCGGCC GCCGTGGCG GCGT TTGGGGCGGTC I30 I10 rrnA GGGCGGCACAC TGTCGGGCGCC GCCGCGGGGCG GGCACGC TTGCGGGCACCC					Ļ			
rrnb AAGGTGCACC CCTTAAGCCC CTGBTTTGCA AAAGTTCCTT GGGGCTGAGT TTGTGGGTGAGT TTGTGGGGGAGA rrnA AAGGATGAL- TTCATTCCTT CACCHCB GTGATG-GAA CACACTTC-A AAACGATGACT AAACGATGAA TTGTGGGTGAGT CACACTTC-A AAACGATGAA AAAGTCACTCTC CACACTTC-A CACACTTC-A AAACGATGAAA TTGTGGGTGAGC CACACTTC-A AAAGTCACTCTC ItoTGTGGAGC CACACTTC-A AAACGATGAAA AACGATGAGAC AACGATGAAA ATGTGGAGGCG CACACTTC-C ItoTGTGGGGGGAC ACGAGCCTGG GCGGGGGAA AAGGATGGAGC CACACTTCTC ItoT ItoT <t< td=""><td>rrnB</td><td>AAGGTGCACC</td><td>CCTTAAGCCC</td><td>CTGICGTGATA</td><td>AAAGGGCCTT</td><td>GGGGCTGAGT</td><td>TTGTGGGTGA</td><td>60</td></t<>	rrnB	AAGGTGCACC	CCTTAAGCCC	CTGICGTGATA	AAAGGGCCTT	GGGGCTGAGT	TTGTGGGTGA	60
rrnh AAGGATGAL- TTCATHCCAT CACCHCG GTGATG-GAA CACACTTC-A AAGCACAGAC rrnc AAGGATGAL- TTCGTGCCGC CAGGCCGCC GCCATGCGAA AACCACAGAC rrnd ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG AACCACAGAC rrnd ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG AACCACAGAC rrnd AAGGTGCGGC CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG AACCACHTTCT 120 rrnd AAGGTCCGGC CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGTTTCTT 120 rrnd GAGGTCGGC GTGCCGGCAC ACGAGCCGG CCGT TTGTGGGCGTCG TTTGTGGGCCGC GCGT rrnd GTGGCGGTCG TTATGCGGCC GCCCGCGGGC GCGCACGC TTTGTGGGTTC TGAGGGAACG GCC rrnd TCGGGCACAC TGTCGGGTTC TGAGGGAACG GCC GCC 180 rrnd TCGGCGGTCG TTATGCGGCC GCCGCGGGCG GCC GCC 240 rrnd GGGCACGC TGTGGGTTC TGAGGGTGCG	rrnD	AAGGTGCACC	CCTTAAGCCC	CTGGTTTGGA	AAAGTTCCTT	GGGGCTGAGT	TTGTGGGTGA	00
rrnc AAGGATGAAA TTCGTGCCGC CAGGCCGCCC GCCATGCGAA AAGCGTGGCGAA AAGCGTGGGGAA AAGCGTGGGGAAA AAGCGTGGGGAA AAGCGTGGGGGGAA AAGCGTGGGGGGGAA AAGCGTGGGGGGGGGGAA AAGCGTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	rrnA	AAGGATGA	TTCATTCCAT	CACCITCG	GTGATG-GAA	CACACTTCLA	AACCACAGAC	
rrnb ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAITTTCT 120 rrnb ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAITTTCT 120 rrnb ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAITTTCT 120 rrnb ATGTGGAGCG CTGGTTAGTC GCCGGGGCG CCGT TrtCT 120 rrnc GAA-TGCGG GCTGTTAGC GCCGCGCG CCGT TTGGGGGTCG TTGTGGGTCTG GCGGTCGG TTGTGGGTCTGGGTT TGTGGGGGCC GCGC TTTGGGGCACAC TGTGGGGTCG GGCAACC TTGTGGGCACAC TGTGGGGTCG GGCAACC GGCACGC TTGTGGGGTC TGAGGGAACG GGC GGC TTGAGGAACT TTGAGGGCACC TTGAGGGTCG GGCAACC GCCGGGGCC GCCGGGCGC GCCGGGGCC GCCGGGGCC GCCGGGGCG GCCGGGC GCCGGGCACC GCCGGGGGCC GCGCACC GCCGGGCC GCCGGGGCG GCCGGGC GCCGGGCACC GCCATA <	rrnC	AAGGATGAAA	TTCGTGCCGC	CAGGCGCACC	GCCATGOGAA	ACCTCCCA	AATCACTCTC	
rrmB ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCALTTTCT 120 rrmB ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCALTTTCT 120 rrmA GAGGTCGGG GATGCCGGG GCGTTAGTC GCCGGGCGC CCGGT rrmC rrmC ACGAGCCTGG TATGCGGCG CCGT rrmC 180 rrmB GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTCGGGGTCTGGC TTGGGGTCTGGGTT TGTGGGGTCGGGC 180 rrmB TCGGGGCACAC TGTCGGGGTTT TGAGGGAACG GGC TTGTGGGCGC 240 rrmA GGGCACAC TGTGGGGTC TGAGGGGTCG GGC GCGC 240 rrmA GGGCACGC TGTGGGGTC TGAGGGGGCG GGCAACCCC CCGCAGGAACC GC 240 rrmA GGGCACGC TGTGGGGTC TGAGGGTGCG GGCAACCCC CCGCAGGAACC GCATA 240 rrmB TCC GGGAACCG GGCAGGC GCCAGGC GCATA GCATA 320 rrmB TrmA GCGGGGCGGA GCACGCGG				000000000000000000000000000000000000000				
rrnB ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAITTTCT 120 rrnD ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAITTTCT 120 rrnD ATGTGGAGCG CTGGTTAGTC GCCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAITTTCT 120 rrnA GAGTCCGGG GTGGCGGGG GCGTGGTGG GCGGC GCGC GCGCC GCGC GCGCC								
rrnb ATGTGGAGCG CTGGTTAGTC GGCCGGGCAC ACGAGCCTGG GCTGGTGATG ACCAGTTTCT rrnb GABGTGCGGG GATGCCGTTG GCGTTCCGG CCGT rrnc GAA-TSCGAG GHTCCGGGGAC GCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGTCTGG 180 rrnb GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGGTCTGGGT 180 rrnb GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGGTCTGGG 180 rrnb TCGGGGACAC TGTCGGGGTT TGAGGGAACG GGC TTTAGGGCT 180 rrnc GGGCACAC TGTCGGGGTTT TGAGGGAACG GGC 180 rrnb TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC 180 rrnh GGGCACAC TGTCGGGTTT TGAGGGAACG GCC 180 rrnh TCGGGCACAC TGTCGGGTTT TGAGGGAACG GCC 180 rrnh GGGCACAC TGTCGGGTTT TGAGGGGGGC GCC 140 rrnh GGGCACGC TGTGGGGTC TGAGGGGGGGGGGGGGGG	rrnB	ATGTGGAGCG	CTGGTTAGTC	GGCCGGGCAC	ACGAGCCTGG	GCTGGTGATG	ACCAITTTCT	120
rrnA GABGTECCGG GATECLETTG GCGTTCCCGG GCGT rrnC GAA-TECCGG GATECLETTG GCGTTTCCCCGG GCGC rrnB GTGGCGGTCG TTATGCGGCC GCCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGGTCTCGG 180 rrnD GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGGTCTGGG 180 rrnD GTGGCGACAC TGTCGGGGTTG GCGGAGCCG TTTCGGGGCTCGGG GGC TTTAGGGGCACAC TGTCGGGGCC GCC 240 rrnD TCGGGCACAC TGTCGGGTTC TGAGGGAGCG GGC GGC CCCGGTGGGCT CCCGGTGGGCT CACTTAACGG 240 rrnA GGGCACGC TGTCGGGTCT TGAGGGTGCG GGC GCC CCGGTGGGCT CACTTGAGAACT 240 rrnA GGGCACGC TGTTGGGTTC TGAGGGGTGCG GCC GCGCACGC CCGGGAACC CCCGGAACC CCCGGGAACC CCCGGGAACC CCCGGGAACC CCCGGGAACC CCCGGAACC CCCGGGAACC GCCATA CCCGCATA CCCATA CCATA CCATA CCATA CCATA CCATA CCATA CCATA CCATA CCATA	rrnD	ATGTGGAGCG	CTGGTTAGTC	GGCCGGGCAC	ACGAGCCTGG	GCTGGTGATG	ACCACTTTCT	
rrnc GAA-TGCGAG_GHTCGGGGGA TCHTCGGGGC GCGC rrnB GTGGCGGTCG_TTATGCGGCC_GCCGCTGGTG_GGGAAGCCGG_TTTCGGGGTT_TGTGGTCTGG rrnA GTGGCGGTCG_TTATGCGGCC_GCCGCTGGTG_GGGAAGCCGG_TTTCGGGGTT_TGTGGTCTGG rrnA TCGGGCACAC_TGTCGGGTTT_TGAGGGAACG_GGC rrnA TCCGGGCACAC_TGTCGGGTTT_TGAGGGAACG_GGC rrnA TCCGGGCACAC_TGTCGGGTTT_TGAGGGAACG_GGC rrnA TCCGGGCACAC_TGTCGGGTTT_TGAGGGAACG_GGC rrnA GGGCACGC_TGTTGGGTTC_TGAGGGTGCG_GGCGGCGCGCC rrnA GGGCACGC_TGTTGGGTTC_TGAGGGTGCG_GGCG-TCCAC_CGGAAGGCTGAGAACT rrnA GGGCACGC_TGTTGGGTTC_TGAGGGTGCG_GGCG-TCCAC_CGGAAGAGCCTGAAGAACT rrnA GGGCACGC_TGTTGGGGTC_TGAGGGTGCG_GGCG-TCCAC_CGGAAGAAG_CTTGAGAAACT rrnA GCCATA rrnA GCCATA rrnA GCATA rrnA GCATA rrnA GCATA rrnA GCATA rrnA GCATA	rrnA	GAGGTCCGGG	GATECCETTE	GCGTICCCGG	CCGT			
rrnB GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGTCTGG 180 rrnA GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGTCTGG 180 rrnA rrnA TTCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC 180 rrnA TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC 170 240 rrnA GGGCACGC TGTGGGTTC TGAGGGAACG GGC 180 180 rrnA GGGCACGC TGTGGGGTC GGGAAGC GGC 170 240 rrnA GGGCACGC TGTTGGGTTC TGAGGGTGCG GGTAACCCLC CCCGTAGAACT 240 rrnA GGGCACGC TGTTGGGTTC TGAGGGTGCG GGCAACCCLC CCCGTAGAACT 240 rrnB TAAAGTGAGT GACCGGAATC CGCCAGGCGC GGCATA 320 rrnA GCATA GCATA GCATA 340 rrnB FTGGGGTGCGA GACTCGTGT 340	rrnC	GAA-TGCGAG	<u>G</u> TCCGGGGA	TCITCCCGG	G <u>CG</u> C			
rrnB GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGTCTGG 180 rrnD GTGGCGGTCG TTATGCGGCC GCCGCTGGTG GGGAAGCCGG TTTCGGGGTT TGTGGTCTGGG 180 rrnD TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC TTTCGGGCACAC TGTCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC 240 rrnD TCGGGCACAC TGTCGGGTTC TGAGGGTGCG GGTAACCCCC CCCGGTGGGCT CACTTAACGG 240 rrnA GGGCACGC TGTTGGGTTC TGAGGGTGCG GGTAACCCCC CCCGGAGCC CACTTGAGAACT rrnB CGCACGC TGTTGGGTC TGAGGGTGCG GGCATGT GCCATA 320 rrnA GCCATA GCATA GCATA GCATA 340								
FIRB GIGGCGFICG THATGCGGCC GCCGCTGGIG GGGAAGCCGG THICGGGGTT TGTGGTCTGG 180 rrnb GIGGCGGTCG TTATGCGGCC GCCGCTGGTG GGAAGCCGG THICGGGGGTT TGTGGTCTGG 240 rrnb TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC GGCCGCTGGTGCG GGTAACCCCC CCCGAGAGCG CACTTAACGG 240 rrnb GGGCACGC TGTCGGGTTC TGAGGGTGCG GGTAACCCCC CCCCA-GAAG CTTGAGAACT Frnc GGGCACGC TGTTGGGGTTC TGAGGGTGCG GGCAACCCCC CCCGA-GAAG CTTGAGAACT rrnb GGGCACGC TGTTGGGGTTC TGAGGGTGCG GGCACCCCCGGATGAAG CTTGAGAACT Frnb GGGCACGC TGTTGGGGTC TGAGGGTGCG GGCATCACCCCGGATGAAG CTTGAGAACT rrnb rnn TAAAGTGAGT GACCGGAATC CGCCAGGTCC GTGACCTGGT TGCTGATTGT GAATTGCATA GCATA 320 rrnb rnn GGGGGTGCGA GCATCGTGT 340	-							
Frind GGGCGCTCC TTATGCGGCC GCCGCTGGTC GGGCAGCT TGTGGGCACAC TGTGGGCACAC TGTGGGGCACAC TGTGGGGCACAC TGCGGGCACAC TGTGGGGCACAC GGGCACGC GGCCGGGTTT TGAGGGAACG GGC GGC TGTGGGCACAC TGTGGGGTTT TGAGGGAACG GGC GGC TGTGGGGCACAC TGTGGGGTTT TGAGGGAACG GGC GGC TGTGAGGACG GGC GGC TGTGAGGACT GCATA GCATA GCATA GCATA GCATA GCATA GCATA GCATA GCATA 340 rrnb GTGGGGTGCGA GCATCGTGTT GTGGGGTGCGA GCATCGTGTT GCATCGTGTT GCATCGTGTT GCATCGTGTT GCATA <	rrnB	GTGGCGGTCG	TTATGCGGCC	GCCGCTGGTG	GGGAAGCCGG	TTTCGGGGTT	TGTGGTCTGG	180
rrnA rrnB TrnB rrnB rrnA rrnA <t< td=""><td>rrnD</td><td>GTGGCGGTCG</td><td>TTATGCGGCC</td><td>GCCGCTGGTG</td><td>GGGAAGCCGG</td><td>TTTCGGGGTT</td><td>TGTGGTCTGG</td><td></td></t<>	rrnD	GTGGCGGTCG	TTATGCGGCC	GCCGCTGGTG	GGGAAGCCGG	TTTCGGGGTT	TGTGGTCTGG	
rrnC rrnB TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC rrnD TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC GGCACGC TGTTGGGTTT TGAGGGAACG GGC GGCACGC CACTTAACGG 240 rrnA GGGCACGC TGTTGGGTTC TGAGGGTGCG GGCACCCCCCGATGAAG CTTGAGAACT rrnB rrnA rrnC GCATG rrnB GCATG rrnB GCATG rrnB GCATGTGGGTGCG rrnB GCATGTGGGTGCG rrnB GCATGTGGGTGCGA GGGGGGGGCGA GCATCGTGT 340	rrnA							
rrnB TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC rrnA TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC GGC rrnA GGGCACGC TGTGGGTTC TGAGGGTGCG GGCACGC CCTTGAGGAACT 240 rrnA GGGCACGC TGTGGGGTTC TGAGGGTGCG GGTAACCCC CCCGCALGAAG CTTGAGAACT rrnB rrnA GCATA GCATA GCATA 320 rrnB GGGGTGCGA GCATCGTGT 340	rrnC							
rrnB TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC rrnD TCGGGCACAC TGTCGGGTTT TGAGGGAACG GGC 240 rrnA GGGCACGC TGTGGGTTC TGAGGGACGC GGCAGGC CCTTAACGG 240 rrnA GGGCACGC TGTGGGTTC TGAGGGTGCG GGTAACCCC CCTCAL GAAACT rrnA GGGCACGC TGTTGGGTTC TGAGGGTGCG GGCACGC CCTGAGAACT 320 rrnA GCATA GCATA GCATA GCATA 340 rrnB								
rrnd TCGGGGACAC TGTCGGGTTT TGAGGGACGC GGCCACGC CACTTAACGG 240 rrnd GGGCACGC TGTTGGGTTC TGAGGGTGCG GGTAACCCC CCCCCAAGAC 240 rrnd GGGCACGC TGTTGGGTTC TGAGGGTGCG GGTAACCCC CCCCCAAGAC TTGAGGAACT rrnd GGGCACGC TGTTGGGTTC TGAGGGTGCG GGCATC CCCGGATGAAG CTTGAGAACT rrnd TAAAGTGAGT GACCGGAATC CGCCAGGTCC GTGACCTGGT TGCTGATTGT GAATTGCATA 320 rrnd	rrnB	TCGGGCACAC	TGTCGGGTTT	TGAGGGAACG	GGC			
rrnh GGGCACGC TGTTGGGTTC TGAGGGTGCG GGTAACOCCC CChCALGAAG CTTGAGAACT rrnC GGGCACGC TGTTGGGTTC TGAGGGTGCG GGCG-TGCAC CCGGATGAAG CTTGAGAACT rrnB TRND rrnB GCATG rrnB GCATG GCGTGCG GCATGTGGTGCG GCATA GCATA GCATA GCATA GCATA GCATA SCATA GCATA GCGGTGCGG GCATCGTGT	rrnD	TCGGGCACAC	TGTCGGGTTT	TGAGGGAACG	GGCCGGTTTG	CCGGTGGGCT	CACTTAACGG	240
rrnC CGGCACGC TGTTGGGTTC TGAGGGTGCG GGCG-TGCAC CCGCATGAAG CTTGAGAACT rrnB rrnC GCATA 320 rrnB	rrnA	GGGCACGC	TGTTGGGTTC	TGAGGGTGCG	GGTAACCCGC	CCTCA-GAAG	CTTGAGAACT	510
rrnB rrnD TAAAGTGAGT GACCGGAATC CGCCAGGTCC GTGACCTGGT TGCTGATTGT GAATTGCATA 320 rrnA rrnC cCATA GCATA GCATA GCATA GCATA GCATA GCATA GCATA GCATA GCATA GCATA	rrnC	GGGCACGC	TGTTGGGTTC	TGAGGGTGCG	GGCG-TGCAC	CCGGATGAAG	CTTGAGAACT	
rrnB rrnD TAAAGTGAGT GACCGGAATC CGCCAGGTCC GTGACCTGGT TGCTGATTGT GAATTGCATA rrnA rrnC rrnB							011011012101	
rrnB rrnD TAAAGTGAGT GACCGGAATC CGCCAGGTCC GTGACCTGGT TGCTGATTGT GAATTGCATA GCATA rrnC rrnB rrnB GTGGGTGCGA GCATCGTGT 340								
rrnd TAAAGTGAGT GACCGGAATC CGCCAGGTCC GTGACCTGGT TGCTGATTGT GAATTGCATA 320 rrnA rrnC GCATA crnB	rrnB							
rrnA GCATA	rrnD	TAAAGTGAGT	GACCGGAATC	CGCCAGGTCC	GTGACCTGGT	TGCTGATTGT	GAATTGCATA	320
rrnD GCATA rrnB 340	rrnA						GCATA	
rrnB 340	rrnC						GCATA	
rrnB 340								
rrnb GTGGGTGCGA GCATCGTGT 340								240
rrnd GTGGGTGCGA GCATCGTGT	rrnB		001000000					340
	rrnD	GIGGGIGCGA	GCATCGTGT					
rrna <u>GUSG-IIGICG</u> CGTGT	rrnA	GCGG-FGTCG	CGTGT					
rrnc <u>GTGGGTGCGA GCATCGTGT</u>	rrnÇ	GTGGGTGCGA	GCATCGTGT					
rrna <u>GCGG_RGTCG</u> CGTGT rrnc GTGGGTGCGA GCATCGTGT	rrnA rrnC	GCGG-ICGTCG GTGGGTGCGA	CGTGT GCATCGTGT					

FIG. 6. Alignment of nucleotide sequences of 16S-23S spacers. Since there is low overall homology between the spacers of operons with different 16S rRNA genes, they are aligned separately and identical nucleotides are boxed. Two breaks were introduced in the alignment of rmA and rmC spacers to demonstrate the regions (underlined) that are conserved in all spacers. The spacer of rmB has an insert of 30 bp, which is shown above the rmB spacer sequence, and the arrow points to the site of insertion.

of 16S rRNA genes, contains four base substitutions and two gaps, implying the lack of functional constraint over the 25-nt sequence. Therefore, possession of this sequence by both *rmD* and *rmC* is highly unlikely a chance event of independent evolution of the two operons but rather likely a consequence of an intrachromosomal recombination that generated *rmD* by joining, within the 16S-23S spacer, a segment of a *rmB*-like operon with a segment of a *rmC*-like operon. Based on the observations described above, it is quite plausible that the organism recently (in evolutionary terms) acquired a partial *rmB*-like operon duplicated once and then recombined one copy with an existent *rmC*-like operon. However, we cannot exclude the possibility that the two types of 16S rRNA genes could also result from divergent evolution.

Regardless of the origin of different types of an rRNA gene in a single organism, it is apparent that some unknown mechanism(s) operates to maintain the interoperon differences. Thus, it is of fundamental importance to understand whether the phenomenon merely represents some sporadic events with little biological significance or points to some rather general mechanisms, such as regulation of gene expression at the translational level, in many organisms. To date, there have been at least six reports of the occurrence of distinct types of one rRNA species in a single organism in all three domains of life (5, 11, 21, 25, 33; present study) in addition to many reports of the presence of low levels of interoperon differences in rRNA genes (8, 12, 19, 26, 36). Clayton et al. (6) recently analyzed all of the available multiple sequences of 16S rRNA from one bacterial strain or different strains of the same species and found that of 250 pairs of sequences retrieved from GenBank, 26% showed from 1 to 5% nucleotide variations. There is an obvious discrepancy between these observations and the general assumption that multicopies of an rRNA gene of an organism have identical or nearly identical sequences. The concept of homogeneous rRNA genes originated mainly from the studies of rRNA genes of eucaryotes in the late 1970s and early 1980s. A review of some of the frequently cited papers that propose homogeneous rRNA operons revealed that their conclusions were largely based either on techniques which would not reveal relatively low levels of sequence variations (2, 16, 18, 27, 34, 35) or on analyses of a fraction of a gene or a few genes of an organism (18, 27). For example, endonuclease restriction analysis of genomic DNA or cloned rDNA would fail to detect nucleotide substitutions that did not destroy or create restriction sites for the enzymes in use (2, 18, 34); electron microscopic observation of heteroduplex DNA formation is limited to detection of large unpaired regions or loops (34); oligonucleotide cataloguing would reveal only a maximum of 45% of the information of a 16S rRNA (28); and the reverse transcription-based sequencing of rRNAs suffers from quite extensive ambiguities in sequence results (16, 28), which could be, at least in some cases, due to the presence of different rRNA species. Taken together, the presence of distinct types of an rRNA species in a single organism could be a common rather than unusual phenomenon in nature. Such a view will be proved or disproved when the nucleotide sequences of multiple rrn operons of more organisms are available for comparison.

The results of this and other studies also have significant implications for the reliability of the rRNA sequence-based phylogenetic analyses of organisms. Although the reported levels of heterogeneity may not pose a serious problem in establishing relatively distant relationships, the potential effect of the heterogeneity has to be considered in resolving relationships at species or subspecies levels or between species from closely related genera.

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