The Journal of Microbiology, December 2008, p. 697-703 Vol. 46, No. 6
DOI 10.1007/s12275-008-0193-0

Organization of Three rRNA (*rrn*) Operons from *Sphingobium chungbukense* DJ77

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(Received August 5, 2008 / Accepted October 2, 2008)

The nucleotide sequences of all three rRNA operons (*rrnA*, *rrnB*, and *rrnC*) of *Sphingobium chungbukense* DJ77 were determined. The three *rrn* operons have the same gene order (16S rRNA-tRNA^{Ile}-tRNA^{Ala}-23S rRNA-5S rRNA-tRNA^{fMet}). The nucleotide sequences were identical over a 5,468 bp region spanning the 16S rRNA gene to the 5S rRNA gene. Variability was observed in the 5S rRNA-tRNA^{fMet} spacer sequence of *rrnB*. The tRNA^{fMet} gene sequences were identical except for two bases (T_{5794} and A_{5871} in *rrnB*, T_{5942} and A_{5956} in *rrnA*, but C_{5942} and G_{5956} in *rrnC*). Comparative sequence analyses of ribosomal RNA operons from DJ77 with those of the class *Alphaproteobacteria*, to which the genus *Sphingobium* belongs, reveal close evolutionary relationships with other members of the order *Sphingomonadales*.

***Keywords*:** 5S rRNA, 16S rRNA, 23S rRNA, tRNA, *rrn* operons, *Sphingobium chungbukense* DJ77

Sphingobium chungbukense DJ77 is an unusual and interesting bacterium. It degrades monocyclic and polycyclic aromatic compounds (Shin *et al*., 1997), it synthesizes cell envelope glycosphingolipids (Burenjargal *et al*., 2007) and it produces extracellular (Song *et al*., 2006). This strain, which was originally classified as *Sphingomonas chungbukensis* (Kim *et al*., 2000), has recently been reclassified as *Sphingobium chungbukense* based on the phylogenetic analyses of 16S rRNA gene sequences (Pal *et al*., 2005).

Within the order *Sphingomonadales*, three whole-genome sequencing projects are currently in progress *Sphingobium chungbukense* DJ77 (Chungbuk National University, Korea), *Sphingomonas* sp. SKA58 (J. Craig Venter Institute) and *Sphingomonas elodea* (Hiram College) and five whole-genomes have been sequenced [*Novosphingobium aromaticivorans* DSM11244 (Joint Genome Institute, JGI: http://genome.jgi-psf.org/finished_microbes/novar/novar.home.html), *Sphingopyxis alaskensis* RB2256 (JGI: http://genome.jgi-psf.org/finished_microbes/sphal/sphal.home.html), *Sphingomonas wittichii* RW1 (JGI: http://genome.jgi-psf.org/finished_microbes/sphwi/sphwi.home.html), *Zymomonas mobilis* ZM4 (Seo *et al.*, 2005), and *Erythrobacter litoralis* HTCC2594 (J. Craig Venter Institute)]. Among the five completed whole genome, the organization of the ribosomal RNA transcription units (16S rRNA, tRNA^{Ile}, tRNA^{Ala}, 23S rRNA, 5S rRNA, and the tRNA^{fMet} gene) was conserved. There was one unit in *S. alaskensis* and *E. litoralis*, two units in *S. wittichii*, and three units in *N. aromaticivorans* and *Z. mobilis*. All of these ribosomal RNA transcription units contained the same sequences in terms of both the genes themselves and the intergenic regions.

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We located the rRNA operons in *Sphingobium chungbukense* DJ77, but we cannot obtain the full sequences because of difficulties in the assembly of repetitive sequences. Therefore, we amplified *rrn* operons using specific primers, then sequenced amplified products. Their primary and secondary structures and phylogenetic relationships within the order *Sphingomonadales* are reported in this paper.

Materials and Methods

Bacterial strains and cultures

S. chungbukense DJ77 was grown in culture medium [500 ml water with Bacto-trypton (1%), NaCl (0.5%), yeast extract (0.5%), and streptomycin (0.01%)] at 30°C for 24 h in a J-SWB2 shaker (Jisco Co., Korea). For the main culture, 1 ml of seed culture was inoculated into 19 ml medium. *S. chungbukense* DJ77 pellets were harvested by centrifugation and stored at -20°C until use.

Preparation of genomic DNA

Chromosomal and plasmid DNA were extracted using standard methods (Silhavy *et al.*, 1984; Sambrook *et al*., 1989).

Amplification of *rrn* operons

Amplification and determination the complete nucleotide sequences of *rrn* operons were done using the primers in Table 1.

For PCR, 1 μl of purified DNA was used as a template in a 50 μl PCR mixture containing 2.5 mM of deoxynucleoside triphosphates dNTPs, 0.4 mM (each) primer, 1× TaKaRa Ex reaction buffer and 3 U of TaKaRa Ex *Taq* polymerase (TaKaRa, TaKaRa Korea). The PCR procedure included an initial denaturation step at 95°C for 9 min and 37 cycles of amplification (95°C for 39 sec, 52°C for 35 sec, and 72°C

Table 1. Sequences and locations of primers that were used to amplify and sequence three *rrn* operons (*rrnA*, *rrnB*, and *rrnC*) in *S. chungbukense* DJ77

Primer		Length (bp)	Template	Start	End
rrnA-F1	5'-AGGGGGATATAGGGCGATTAAG-3'	22	*rrnA*	14	35
rrnA-R1	3'-ACTTAAAATCCTGCCTTTCGAACC-5'	24	*rrnA*	6113	6136
rrnA-F2	5'-AGAGGGGGATATAGGGCGATTA-3'	22	*rrnA*	12	33
rrnA-R2	3'-GTCAGCACGGATCAGGACTC-5'	20	*rrnA*	6155	6174
rrnB-F1	5'-CTGATCCTTGTGCCAAAACC-3'	20	*rrnB*	6	25
rrnB-R1	3'-ATGGCTAGCCGACGTCCT-5'	18	*rrnB*	6164	6181
rrnB-F2	5'-CTTCTGATCCTTGTGCCAAAAC-3'	22	*rrnB*	3	24
rrnB-R2	3'-CAGAACCGTCTCCATGAACTG-5'	21	*rrnB*	6121	6141
rrnC-F1	5'-AGATCGACACCGAAACATGG-3'	20	*rrnC*	34	53
rrnC-R1	3'-GTCAGAAATGTGCGTCATGCTAT-5'	23	*rrnC*	6159	6181
rrnC-F2	5'-GCAGATCGACACCGAAACAT-3'	20	*rrnC*	32	51
rrnC-R2	3'-AGGGGAGCACGTCAGAAAT-5'	19	*rrnC*	6173	6191
1-F	5'-GATTTATCGCCCAAGGATGA-3'	20	*rrnA, B, C*	691	710
1-R	3'-CCCGTCAATTCCTTTGAGTT-5'	20	*rrnA, B, C*	1362	1381
2-F	5'-GTGCATGGCATTTCAGTGG-3'	19	*rrnA, B, C*	1292	1310
2-R	3'-TCCCCTACGGCTACCTTGTT-5'	20	*rrnA, B, C*	1957	1976
3-F	5'-GTTGAGCTAACCCGCAAGG-3'	19	*rrnA, B, C*	1894	1912
3-R	3'-ACGTTGCTCAGCTTCTTGGT-5'	20	*rrnA, B, C*	2558	2577
4-F	5'-TTGACATTGTGAATGGGTTTTT-3'	22	*rrnA, B, C*	2493	2514
4-R	3'-ACTGGTTCGCTATCGGTCAT-5'	20	*rrnA, B, C*	3158	3177
5-F	5'-CGGAGCACGTGAAACTCTGT-3'	20	*rrnA, B, C*	3096	3115
5-R	3'-CAACCTCCTGGTTGTTTTGG-5'	20	*rrnA, B, C*	3760	3779
6-F	5'-AAAGGGAAACAGCCCTAACC-3'	20	*rrnA, B, C*	3690	3709
6-R	3'-CCCCACAAAAAGTTGCCTTA-5'	20	*rrnA, B, C*	4337	4356
7-F	5'-CAAATTGCCTCCGTACCTTC-3'	20	*rrnA, B, C*	4297	4316
7-R	3'-GACCGACTTTCGTCTCTGCT-5'	20	*rrnA, B, C*	4967	4986
8-F	5'-GCTCAGGACGGTTGGAAAC-3'	19	*rrnA, B, C*	4894	4912
8-R	3'-TGCCGTGCATTTTACGGTAG-5'	20	*rrnA, B, C*	5564	5583

for 5 min).

Sequence determination and analysis

Nucleotide sequences were determined using ABI 377, 3700, 3730, and GS-FLX (Genome Sequencer FLX) automatic sequencers (Koumi *et al*., 2004; Goldberg *et al.*, 2006). By masking the 454 Life Sciences PicoTiterPlate into 4 regions, pyrosequencing recovered 490,251 sequence reads, with average read length ~240 bp. The assembly results have a quality of over 40 and the reliability is 99.9%.

DNA sequences were assembled using the PHRED-PHRAP-CONSED contig assembly program (http://www.phrap.org). RNA secondary structure modeling was performed using *Mfold* (version 3.2; http://bioinfo.rpi.edu/applications/mfold/) (Zuker *et al.*, 2003). The $tRNA^{fMet}$ gene was identified using ARAGORN software (Laslett and Canback, 2004).

The sequences of rRNA operons of alphaproteobacteria used in the phylogenetic study were obtained from the NCBI GenBank sequence database. They were aligned using the CLUSTAL W multiple sequence alignment program (Chenna *et al*., 2003).

Nucleotide sequence accession numbers

The complete nucleotide sequences of the three *rrn* operons (*rrnA*, *rrnB*, and *rrnC*) from *S. chungbukense* DJ77 were deposited in the GenBank database under accession numbers EU679658, EU679659, and EU679660 (Table 2).

Results and Discussion

Complete nucleotide sequences of three *rrn* operons (*rrnA*, *rrnB*, and *rrnC*)

Sequenced clones were retrieved from the shotgun library of *S. chungbukense* DJ77 and contig assembly was performed to construct a consensus contig. The sequences for *rrn* operons were initially obtained from the Sanger sequencing data and pyrosequencing-based 454 sequencing data. To get

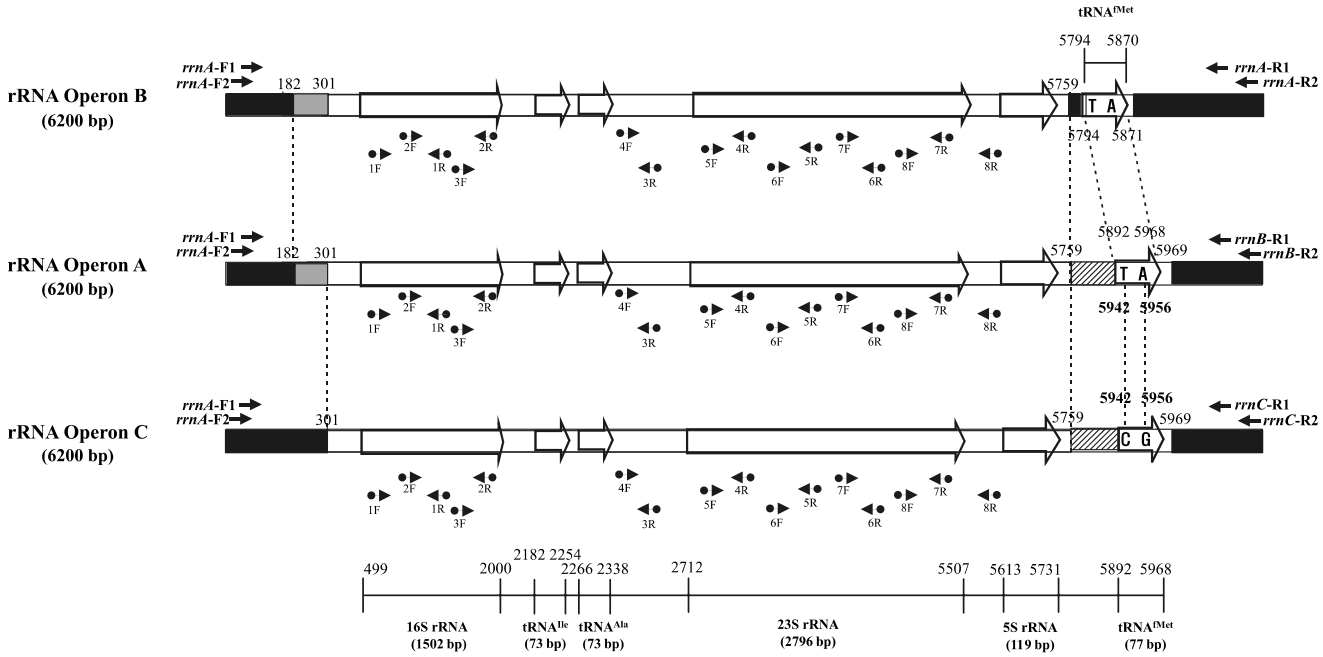


Fig. 1. Organization of three *rrn* operons (*rrnA*, *rrnB*, and *rrnC*) in *S. chungbukense* DJ77. Sizes and orientations of genes found in *rrn* operons were indicated. represents regions with no sequence similarity among *rrnA*, *rrnB* and *rrnC*. represents regions with identical sequences between *rrnA* and *rrnB*, but not with *rrnC*. represents regions with identical sequences between *rrnA* and *rrnC*, but not with *rrnB*. represents regions with identical sequences among *rrnA*, *rrnB*, and *rrnC* with minor exceptions. In $tRNA^{fMet}$ genes, there are two locations with dissimilar bases (T_{5794} and A_{5871} in *rrnB*, T_{5942} and A_{5956} in *rrnA*, but C_{5942} and G_{5956} in *rrnC*).

the complete sequence of each operon two primer sets were designed and synthesized (Table 1). One set was for specific amplification of each *rrn* operon and the other set was for direct sequencing of the amplified operons. We obtained PCR products of the expected sizes using two primer sets per operon, and eight primer sets were used to verify the final sequences of three operons (Fig. 1).

We analyzed a 6,200 bp long sequence of each operon. The total number of nucleotides was 5,470 bp for the *rrnA* and *rrnC* operons and 5,371 bp for the *rrnB* operon. The 16S ribosomal RNA, $tRNA^{Ile}$, $tRNA^{Ala}$, 23S ribosomal RNA, 5S ribosomal RNA, and $tRNA^{fMet}$ genes were in the same order in all three operons. The gene for $tRNA^{fMet}$, which is lacking a base pair between the 5'-terminal residue and the fifth nucleotide from the 3'-end of tRNA, was identified using ARAGORN (Laslett and Canback, 2004). The 16S ribosomal RNA gene was 1,502 bp, the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes were 73 bp, the 23S ribosomal RNA gene was 2,796 bp, the 5S ribosomal RNA gene was 119 bp and the $tRNA^{fMet}$ gene was 77 bp. The nucleotide sequences were identical over a 5,468 bp region, which spanned the 16S rRNA gene to the 5S rRNA gene. We confirmed the previously reported sequences of the 16S rRNA and 5S rRNA genes (Lee *et al*., 2005; Kwon and Kim, 2007). As shown in Fig. 1, the sequences of three *rrn* operons and flanking regions differ in three regions. First, the sequences of *rrnA* and *rrnC* were identical except for two bases in the $tRNA^{fMet}$ gene (T_{5794} and A_{5871} in *rrnB*, T_{5942} and A_{5956} in *rrnA*, but C_{5942} and G_{5956} in *rrnC*). These two bases are located in the base paired TψC stem of the tRNA. Base changes from T and A to C and G did not disrupt the secondary structure of tRNA. Second, upstream sequence (nucleotide 182∼301) of *rrnA* was the same as that of *rrnB*, but deleted or displaced in that of *rrnC*. Third, variability was also observed in the 5S rRNA-$tRNA^{fMet}$ spacer sequence of *rrnB*, which is different and shorter than those of *rrnA* and *rrnC*.

Comparative sequence analysis of *rrn* operons

S. chungbukense DJ77 belongs to the alpha division of proteobacteria. Among the 185 alphaproteobacteria whose genome sequences were completely known and available with annotation at NCBI on July 2008, we chose 31 alphaproteobacteria which had the same *rrn* operon gene organization as DJ77. Selected genera were *Caulobacter*, *Brucella*, *Ochrobactrum*, *Parvibaculum*, *Methylobacterium*, *Beijerinckia*, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Dinoroseobacter*, *Rhodobacter*, *Silicibacter*, *Jannaschia*, *Paracoccus*, *Granulibacter*, *Gluconobacter*, *Acidiphilium*, *Rhodospirillum*, *Rhodobacter*, *Silicibacter*, *Sphingomonas*, *Sphingopyxis*, *Novosphingobium*, and *Zymomonas*. A survey of the number of *rrn* operons of 31 alphaproteobacteria revealed that *Parvibaculum lavamentivorans* DS-1, *Dinoroseobacter shibae* DFL 12, *Rhodobacter sphaeroides* ATCC 17029, *Silicibacter* sp. TM1040, *Jannaschia* sp. CCS1, *Mesorhizobium* sp. BNC1, *Rhodobacter sphaeroides* 2.4.1 and *Sphingopyxis alaskensis* RB2256 had one *rrn* operon that *Caulobacter* sp. K31, *Caulobacter crescentus* CB15, *Brucella melitensis biovar Abortus* 2308, *Paracoccus denitrificans* PD1222, *Silicibacter pomeroyi* DSS-3, *Acidiphilium cryptum* JF-5, and *Sphingomonas wittichii* RW1 had two *rrn* operons, that *Brucella ovis* ATCC 25840, *Brucella abortus bv.* 1 str. 9-941, *Methylobacterium radiotolerans* JCM 2831, *Beijerinckia*

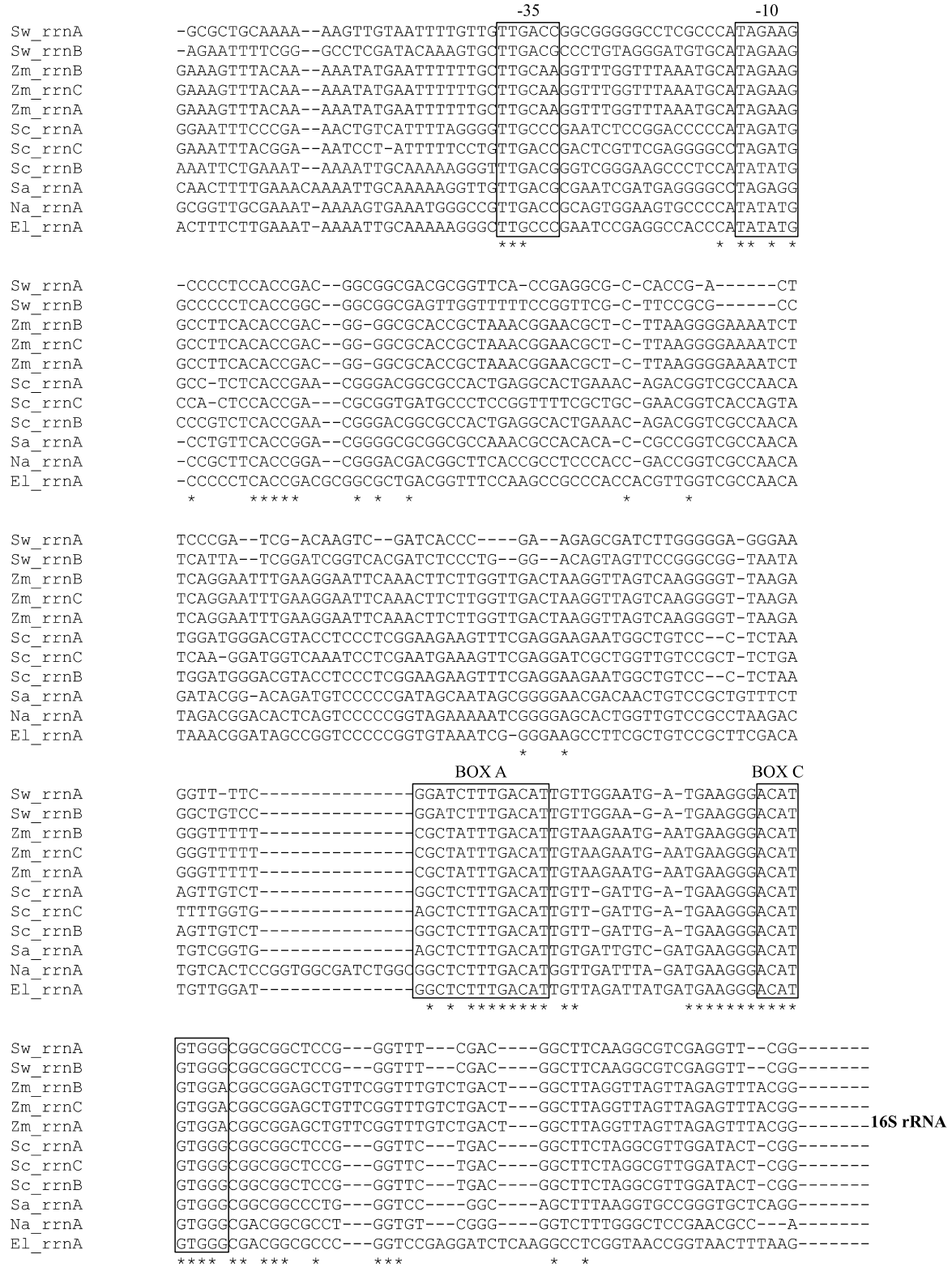
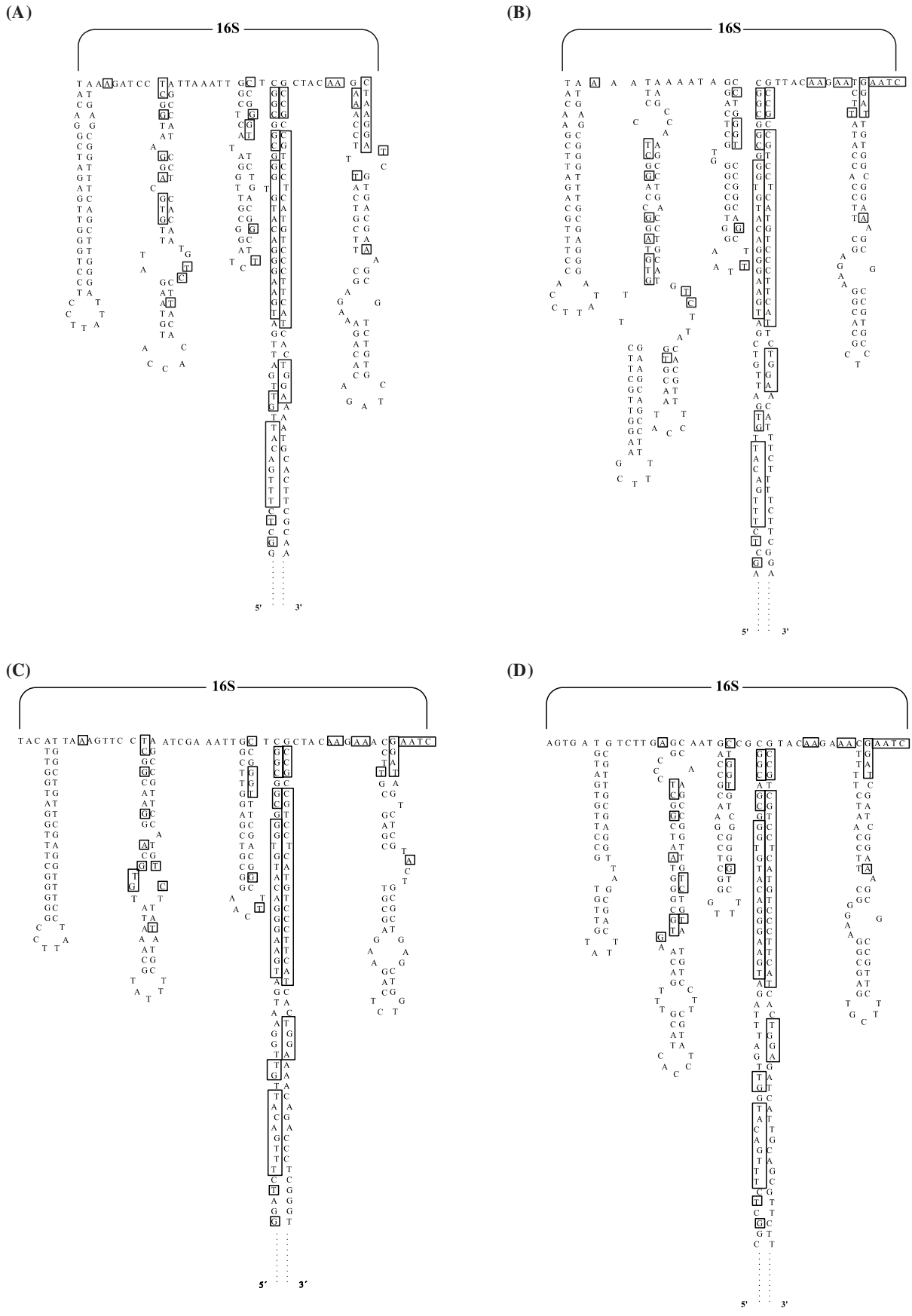


Fig. 2. Comparison of *Sphingomonadales* sequences upstream of the 16S rRNA. Sequences include *rrnA* (Sw_rrnA) and *rrnB* (Sw_rrnB) of *Sphingomonas wittichii* RW1, *rrnA* (Zw_rrnA), *rrnB* (Zw_rrnA), and *rrnC* (Zw_rrnA) of *Zymomonas mobilis* subsp. *mobilis* ZM4, *rrnA* (Sc_rrnA), *rrnB* (Sc_rrnB), and *rrnC* (Sc_rrnC) of *Sphingobium chungbukense* DJ77, *rrnA* (Sa_rrnA) of *Sphingopyxis alaskensis* RB2256, *rrnA* (El_rrnA) of *Erythrobacter litoralis* ATCC 2594. The promoters of *rrn* operons have an identical sequence upstream of the 16S rRNA gene at the start of the operons and contain a 17 bp spacer between the -10 and -35 hexamers. The *box A* and *C* are anti-termination regions.


(A)
16S
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(C)
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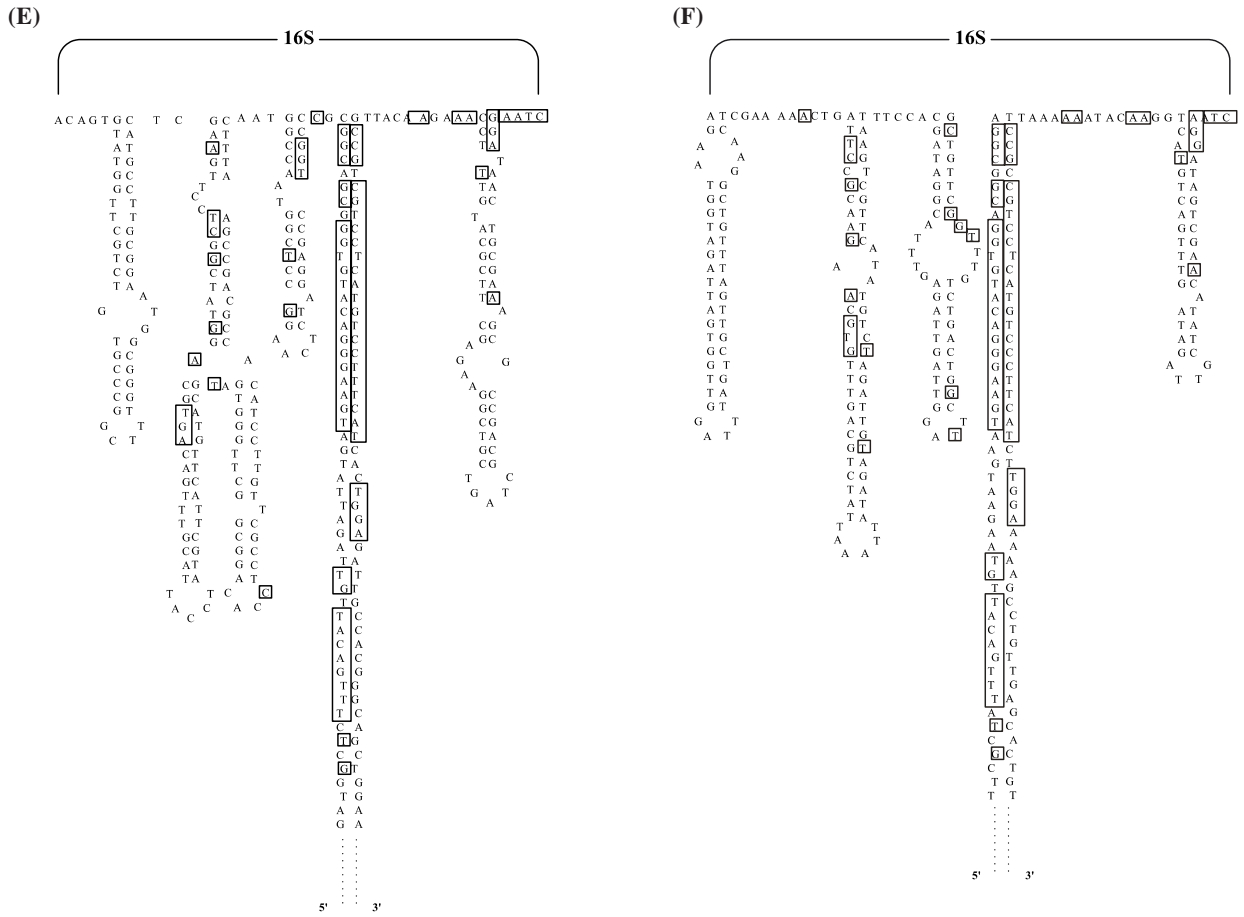

Fig. 3. Secondary structure of precursor rRNA sequences surrounding 16S rRNA from (A) Sphingobium chungbukense DJ77, (B) *Sphingopyxis alaskensis* RB2256, (C) *Sphingomonas wittichii* RW1, (D) *Novosphingobium aromaticivorans* DSM 12444, (E) *Erythrobacter litoralis* HTCC2594, and (F) *Zymomonas mobilis* subsp. *mobilis* ZM4. The conserved regions are indicated in boxes.

indica subsp. *indica* ATCC 9039, *Rhizobium leguminosarum bv. viciae* 3841, *Rhizobium etli* CFN 42, *Sinorhizobium meliloti* 1021, *Sinorhizobium medicae* WSM419, *Granulibacter bethesdensis* CGDNIH1, *Gluconobacter oxydans* 621H, *Rhodospirillum rubrum* ATCC 11170, *Novosphingobium aromaticivorans* DSM 12444, and *Zymomonas mobilis* subsp. *mobilis* ZM4 had three *rrn* operons, that *Ochrobactrum anthropi* ATCC 49188 and *Methylobacterium* sp. 4-46 had four rrn operon, and *Methylobacterium extorquens* PA1 had five *rrn* operons. In summary, 8 species had one, 7 species had two, 13 species had three, 2 species had four and 1 species had five operons. The group with three operons had the largest number of species, 13 of 31. *S. chungbukense* DJ77 also had three *rrn* operons.

The *rrn* operon sequences published in GenBank were compared to the *rrn* operons from *S. chungbukense* DJ77. By comparing the DJ77 ribosomal RNA operon, sequence to the sequences of other alphaproteobacteria, we determined a close evolutionary relationship with other species of *Sphingomonadales*. The three DJ77 *rrn* operons were most similar to those in *Sphingopyxis alaskensis* RB2256. The similarities among the operons were in the order of *Sphingomonas wittichii* RW1, *Zymomonas mobilis* subsp. *mobilis* ZM4, and *Novosphingobium aromaticivorans* DSM 12444. These results disagree with a phylogeny that was constructed using the 16S rRNA gene sequences (Kim *et al*., 2000).

To identify motif sequences involved in transcription and antitermination, we compared the DJ77 sequences with other bacterial sequences. A putative promoter containing a 17 bp spacer between the -10 (TAG(T)ATG) and -35 (TTGC(A)CC) hexamers was found (Fig. 2). Although the sequences around the promoter had no homology between *rrn* operons, the promoter was found at a similar location relative to the 5' end of mature 16S rRNA.

The bacterial NusA (N-utilization substance A) protein is thought to be essential for the increased elongation rate associated with *rrn* transcription and to be a constituent of the *rrn* anti-termination complex (Arnvig *et al*., 2004). The *rrn* leader regions contain sequences with homology to the *E. coli rrn* anti-termination motifs (*boxB*, *boxA*, and *boxC*). The *rrn* leader regions also have similar characteristics and organization (Morgan, 1986). The region spanning *boxA* and *boxC* shows 100% sequence conservation of *rrn* leader regions in different species, suggesting an essential function. The *boxB* element, a stem-loop structure, has no strict se-

quence conservation between species or between *rrn* operons. Recently, Arnvig *et al*. (2004) demonstrated that the NusA-RNA interaction relies on the *boxC* region, a conserved region downstream of *boxA*, which demonstrates that this element has a key function. We found that the *rrn nut* site, and *boxA* and *boxC* in *Sphingomonadales* were highly conserved, although surrounding sequences were not homologous (Fig. 2 and 3). We did not find sequences with homology to *boxB* region.

Putative secondary structures of precursor 16S rRNA from *Sphingomonadales* strains

We determined a possible secondary structure of precursor rRNA sequences surrounding 16S rRNA in *rrn* operons by using *Mfold.* The secondary structures were very similar among *Sphingomonadales*, although there were few sequences that showed identity or homology with *Sphingopyxis alaskensis* RB2256, *Sphingomonas wittichii* RW1, *Novosphingobium aromaticivorans* DSM 12444, *Zymomonas mobilis*, or *Erythrobacter* (Fig. 3).

In conclusion, the sequence and organization of *rrn* operons of *S. chungbukense* were revealed in this paper for the first time. The fact that structure and organization of *rrn* operons are conserved perfectly, but each species have a different number of copies among *Sphingomonadales* is very interesting. In addition, the availability of these sequences may clarify the phylogenetic relationships within the alphaproteobacteria.

Acknowledgements

This work was supported by the research grant of the Chungbuk National University in 2006.

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