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Fragmentations of the Large-Subunit rRNA in the Family *Rhizobiaceae*

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A 130-nucleotide-long rRNA species corresponding to the 5′ end of the 23S rRNA gene was found in 96 strains belonging to different *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* species. Additional fragmentation in the central region of the large-subunit rRNA occurred in all agrobacteria, except *Agrobacterium vitis*, and in most *Rhizobium leguminosarum* and *Rhizobium etli* strains but did not occur in any of the other rhizobia and bradyrhizobia studied.

The structural organization of large-subunit (23S and 23S-like) rRNA molecules is known for only a limited number of bacteria (8). It has been published that in some strains of different species from a few genera, the 23S rRNA genes (rDNAs) contain in the 5′ half highly variable extra stem-loop structures in which the cleavage and fragmentation of 23S rRNA occur (4, 6, 8–10, 12, 16, 18). Such stem-loop structures, also called intervening sequences (IVSs), and the phenomenon of 23S rRNA fragmentation have not been shown for *Escherichia coli* (3). Recently, the *Agrobacterium* 23S rRNA was also shown to be fragmented (10). In Fig. 1 the cleavage sites in 23S rRNA which were mentioned above are presented. In order to analyze the distribution of the phenomenon of 23S rRNA fragmentation in members of the family *Rhizobiaceae*, the rRNA patterns of 96 strains belonging to different *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* species were investigated (Table 1).

Bacteria were grown on TY medium (1). Total RNAs were isolated from bacterial cultures at early and middle exponential growth phases by using the RNeasy Kit (Qiagen, Hilden, Germany) and analyzed electrophoretically on denaturing gels (17). Northern (RNA) transfer onto a nylon membrane (Qiagen) was done by the method of Sambrook et al. (17). The following three DNA probes were used for hybridization: $23S_{24\text{-}130}$, $23S_{115\text{-}1623}$, and $23S_{1059\text{-}2759}$. They correspond to the 23S rDNA sequences from 24 to 130, 115 to 1,623, and 1,059 to 2,759 nucleotides (*E. coli* numeration), respectively, from the 5′ end of the gene (3). Probes were prepared by PCR amplification in total DNA, which was extracted by the method of Masterson et al. (15). A PCR DIG labelling mix (2) was used. The PCR primers were downstream primer 24 (DP_{24}), 5′-(CT)G(AG)(CT)GGATGCCTTGG-3′, and upstream primer 130 (UP_{130}), 5′-GGGTT(AGCT)CCCCATTCGG-3′; DP_{115}, 5′-CCGAATGGGG(ACG)AACCC-3′, and UP_{1623}, 5′-C(TC)ACCTGTGTCGGTTT-3′; and DP_{1059}, 5′-CTTGGCTT(AG)GA(AG)GCAGC-3′, and UP_{2759}, 5′-CTTAGATGC(CT)TTCAGC-3′, with each pair flanking probes $23S_{24\text{-}130}$, $23S_{115\text{-}1623}$, and $23S_{1059\text{-}2759}$, respectively (3). Amplifications were carried out on a Hybaid Thermocycler (MWG-Biotech, Munich, Germany). Initial denaturation at 95°C for 5 min was followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Thereafter, a final extension for 10 min at 72°C was performed. Northern hybridization, stripping, and reprobing were performed according to the manufacturer's instructions (2). DP_{115} (see above) and UP_{388} [5′-CACGTGT(CT)CCGCCC(GCT)TACT(CT)T-3′] were used together for PCR analysis of the region $23S_{115\text{-}388}$, in which 5′ end fragmentation occurred. For analysis of the central $23S_{1059\text{-}1956}$ region of the 23S rDNA, DP_{1059} (mentioned above) and UP_{1956} (5′-AATTACCCGGCAAGG-3′) were used. Amplification mixtures had volumes of 50 μl, to which 100 ng of total DNA, 1.5 U of EuroTaq polymerase in the corresponding buffer (Eurogentec, Belgium GA, Seraing, Belgium), 20 pmol of each primer, and 200 μM (each) deoxynucleoside triphosphates (Gibco-BRL Life Technologies, Eggenstein, Germany) were added. Cycling was performed as described above. Both kinds of amplification products were analyzed electrophoretically on 3% small-DNA agarose (FMC-Biozym Diagnostik GmbH, Hameln, Germany). The products corresponding to the 5′ end of the 23S rDNA flanked by DP_{115} and UP_{388} were also analyzed by direct nonradioactive cycle sequencing with DP_{115} labelled at its 5′ end with digoxygenin (DIG-DP_{115}). All primers were obtained from MWG-Biotech. The program for the cycle sequencing was initial denaturation for 3 min at 94°C and 25 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. A GATC sequence direct blotting apparatus (MWG-Biotech) and a DIG nonradioactive kit (Boehringer Mannheim GmbH, Mannheim, Germany) were used.

Some examples of the rRNA contents of strains studied are presented in Fig. 2A. The 23S rRNA of *E. coli* W 2438 (13) has a length of about 2.9 kb (Fig. 2A, lane 9), which corresponds to the size estimated for this molecule (3). All representatives of the family *Rhizobiaceae* studied in this work (Table 1) contained a smaller molecule, with a size of 2.6 kb, instead of this molecule (Fig. 2A, all lanes except lane 9). It has already been published that *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, which along with rhizobia belong to the alpha subclass of *Proteobacteria*, possess two fragments, 14S and 16S, of rRNA (instead of one molecule of 23S rRNA) in their mature 50S ribosomes (6, 12). In these cases, the presence of an IVS of variable size (between 50 and 100 bp) was demonstrated at a position of 1.2 kb, at which processing of the 23S rRNA molecule occurs. In addition, a large deletion within domain III of the 23S rDNA of these two bacterial species, which was proposed to be a typical feature for members of the alpha subclass of *Proteobacteria*, was demonstrated (14). By these two facts, it is possible to explain the postulated smaller size of the 23S-like rRNA of *Rhodobacter* spp. (2.74 kb) in comparison to the size (2.9 kb) estimated for *E. coli* 23S rRNA (14). In contrast to the

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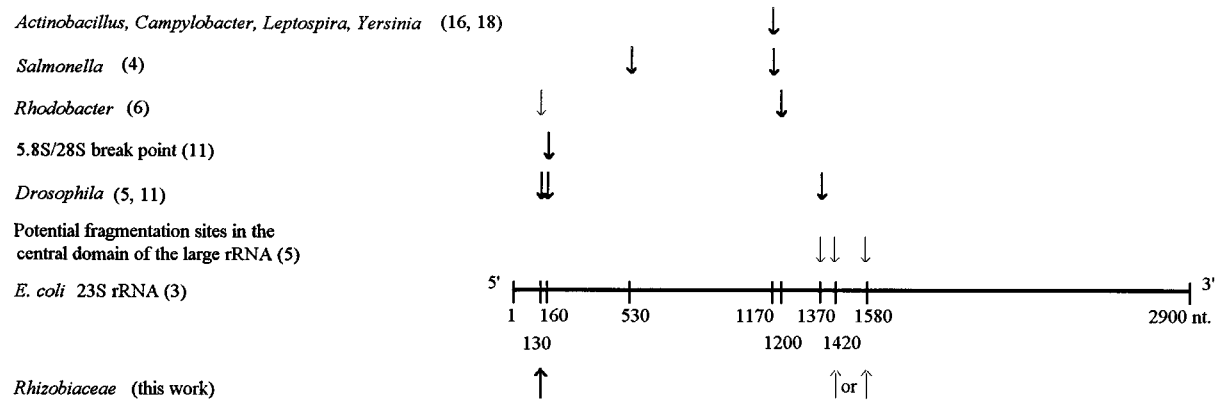

FIG. 1. Fragmentations of 23S rRNA. Boldface arrows, fragmentation positions already demonstrated; lightface arrows, positions of potential fragmentations. References are given parenthetically.

23S-like rRNA of the above mentioned species of alpha subclass proteobacteria, within the members of the family *Rhizobiaceae* studied, this molecule is centrally fragmented only in some strains (Fig. 2A, lanes 7 and 12 to 15, and 3A, lanes 1, 3 to 5, 7, and 8; Table 1) and the size of the intact 23S rRNA-like molecule is smaller (approximately 2.6 kb). One possible explanation for this observation is a cleavage event close to the 5′ end of 23S rDNA, as was predicted for *Rhodobacter sphaeroides* on the basis on the presence of a putative IVS found at position 130 of the gene (6). To prove this hypothesis, we performed Northern hybridization experiments with $23S_{24\text{-}130}$ probes derived from different bacteria (Fig. 2B through D). As seen in Fig. 2B, all of the *Rhizobium* and *Agrobacterium* strains presented contain an additional short rRNA species which hybridizes with the $23S_{24\text{-}130}$ probe derived from *Rhizobium leguminosarum* RSM 2001. By using this probe, no hybridization signals were obtained with *E. coli* W 2438 or a *Bradyrhizobium* sp. (lupinus) in this experiment (Fig. 2B, lanes 9 and 10, respectively). To confirm the presence of the short rRNA species in the bradyrhizobia studied, hybridization with a $23S_{24\text{-}130}$ probe derived from *Bradyrhizobium japonicum* TU 61A152 were performed (Fig. 2C). In the case of *E. coli*, in which (as mentioned above) the size of the 23S rRNA corresponds to the length of the gene (as estimated by sequencing), no shorter rRNA species occurs. By hybridization with a $23S_{24\text{-}130}$ probe derived from *E. coli* W 2438, it was demonstrated that this part of the molecule remains covalently linked to the 23S rRNA in *E. coli* (Fig. 2D).

As mentioned above, in some of the strains studied the 2.6-kb rRNA was additionally fragmented; in those cases, another class of molecules of 1.3 kb occurred (Fig. 2A and 3A). By Northern hybridizations with probes $23S_{115\text{-}1623}$ (Fig. 3B) and $23S_{1059\text{-}2759}$ (data not shown), the event of central fragmentation of the 2.6-kb rRNA was confirmed for those strains. The presence of two hybridization signals (Fig. 3B, lanes 1, 3 to 5, 7, and 8) which correspond to the 2.6- and 1.3-kb rRNA bands is evidence that in these cases each strain contains some *rrn* operons with central fragmentation and some without it. In contrast to 5′ end fragmentation, this kind of fragmentation was not a common feature of all members of the family *Rhizobiaceae*. As seen in Table 1, central fragmentation occurs in all *Agrobacterium* species except *Agrobacterium vitis*. It occurs in 16 of the 22 *R. leguminosarum* strains and 4 of the 5 *Rhizobium etli* strains studied but does not occur in any *Bradyrhizobium* strain, *Rhizobium ciceri*, *Rhizobium fredii*, *Rhizobium galegae*, *Rhizobium loti*, *Rhizobium meliloti*, or *Rhizobium* sp. nodulating *Hedysarum coronarium* L. (*R. hedysari*) studied in this work.

In order to check for the presence of variable IVSs at the 5′ end and in the central part of the 23S rRNA, we performed amplifications of regions $23S_{115\text{-}388}$ and $23S_{1059\text{-}1956}$, respectively, from a variety of representative strains. The amplification products of the $23S_{115\text{-}388}$ region of the members of the family *Rhizobiaceae* studied were larger than the corresponding product from *E. coli* (Fig. 4A). In addition, the amplification products for most of the strains from this family were individual in size (Fig. 4A; compare lanes 2 and 3, 4 through 6, 9 and 10, and 11 and 12). For some strains, more than one amplification product was obtained (Fig. 4A, lanes 6, 9, and 13). The latter indicates differences between the $23S_{115\text{-}388}$ regions in *rrn* operons in the same strain. By keeping in mind the conservative structure of ribosomes, which is related to their central role in protein synthesis, these observations can be explained by the presence of highly variable IVSs which are processed to maintain the functionality of the 23S-like rRNA.

To prove this statement, we sequenced the 5′ end regions (after position 130 of *E. coli*) of several *R. leguminosarum*, *R. etli*, and *R. ciceri* strains and compared them with the corresponding sequences of several other representatives of alpha subclass proteobacteria available in the literature and in the EMBL database (Fig. 5). As seen in this figure, all of the strains analyzed possess extra sequences in this region in comparison to that of the *E. coli* gene. These sequences are situated near the starting sequencing point and vary to different extents. Their 3′ ends are less variable and are located at a position corresponding to bp 131 of the *E. coli* gene, where limited homology with the latter occurs. It was possible to distinguish the following three groups of alpha subclass proteobacteria by differences among each other and with the *E. coli* sequence in this region (positions 131 to 175): group I consisting of the *Rhizobium* strains analyzed in this work, group II consisting of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, and group III consisting of *B. japonicum* and *Rhodopseudomonas palustris*. Very high homology with the *E. coli* gene starts at position 175 of the latter. By this analysis, the presence of IVSs at the 5′ ends of the 23S-like rDNAs of rhizobia analyzed in this work is clearly demonstrated. The sequences between positions 131 and 175, which obviously remain in the 23S-like rRNA molecule after its processing, are also of interest because they seem to be useful for a design of taxon-specific probes for these bacteria. It is also interesting that the IVSs presented in Fig. 5 were variable to different

TABLE 1. Bacterial strains used and central fragmentation of 2.6-kb rRNA into two 1.3-kb fragments

Species and strain	Source[a]	Central fragmentation of rRNA[b]
R. leguminosarum bv. viceae		
ATCC 10004^T	N. Amarger	+
L 113	N. Amarger	+
RSM 2001	P. R. Hirsch	+
T3	J. E. Beringer	+
KH 1159	J. E. Beringer	+
WS 1370	W. Ludwig	−
VF 39	W. Ludwig	+
B-10	W. Ludwig	+
R. leguminosarum bv. phaseoli		
H 132	N. Amarger	+
H 441	N. Amarger	+
TU 127K 12b	W. Ludwig	−
TU 127K 80e	W. Ludwig	−
TU 127K 44	W. Ludwig	−
TU 127K 89	W. Ludwig	+
TU 127K 105	W. Ludwig	−
TU 102K 102	W. Ludwig	+
TU 102K 81C-2	W. Ludwig	−
TU 102K 81-C	W. Ludwig	+
Brasil 5	W. Ludwig	+
R. leguminosarum bv. trifolii		
H 112	N. Amarger	+
WS 1376	W. Ludwig	+
USDA 2152	W. Ludwig	+
R. etli		
CFN 42^T	W. Ludwig	+
Viking I	W. Ludwig	−
F6	W. Ludwig	+
Bra5	W. Ludwig	+
Nitragin 8251	W. Ludwig	+
Rhizobium sp. related to *R. etli* Cli 80	W. Ludwig	+
R. tropici		
CIAT 899^T	W. Ludwig	−
CFN 299	W. Ludwig	−
BR 833	W. Ludwig	−
BR 864	W. Ludwig	−
BR 816	W. Ludwig	−
R. loti		
NZP 2037	W. Ludwig	−
MSDJ 865	N. Amarger	−
Zw 3	L. Raitcheva	−
R. meliloti		
41	W. Ludwig	−
114	L. Raitcheva	−
112	L. Raitcheva	−
122	L. Raitcheva	−
RCR 2011	W. Ludwig	−
WS 1373	W. Ludwig	−
R. ciceri		
IC-2091	S. Nour	−
IC-2018	S. Nour	−
UPM-Ca7^T	S. Nour	−
Rhizobium sp. (*Cicer*)		
IC-6	S. Nour	−
IC-60	S. Nour	−
UPM-Ca36	S. Nour	−
R. fredii		
NGR 234	W. Ludwig	−
MSDJ 1536	N. Amarger	−
MSDJ 1537	N. Amarger	−
R. galegae bv. officinalis		
2246	NBIMTC	−
2247	NBIMTC	−
2248	NBIMTC	−
2249	NBIMTC	−
2250	NBIMTC	−
2251	NBIMTC	−
Bg9	G. Radeva	−
12	G. Radeva	−
14	G. Radeva	−
1141	HAMBI	−
625	W. Ludwig	−
R. galegae bv. orientalis		
540^T	HAMBI	−
CIAM 0707	G. Radeva	−
842	G. Radeva	−
848	G. Radeva	−
843	G. Radeva	−
912	G. Radeva	−
R. hedysari		
A6	A. Squartini	−
IS 123	A. Squartini	−
RH 100	A. Squartini	−
RH44	A. Squartini	−
CC1335	A. Squartini	−
HCNT	A. Squartini	−
A. tumefaciens		
ATCC 23308^T	W. Ludwig	+
C58	W. Ludwig	+
281	W. Ludwig	+
4404	W. Ludwig	+
A4	J. Shell	+
GMI 9023	W. Ludwig	+
A. rhizogenes		
A8	J. Shell	+
DSM 30148	W. Ludwig	+
WS 1383	W. Ludwig	+
A. rubi WS 1384	W. Ludwig	+
A. radiobacter 3001	NCPPB	+
A. vitis 3554	NCPPB	−
Agrobacterium spp.		
KAg3	W. Ludwig	+
ChAg4	W. Ludwig	−
B. japonicum		
TU 61A118b	W. Ludwig	−
TU 61A152	W. Ludwig	−
TU 61A212	W. Ludwig	−
TU 61A 182	W. Ludwig	−
TU 61A 24	W. Ludwig	−
Bradyrhizobium sp. (soybean) S 273	L. Raitcheva	−
Bradyrhizobium sp. (peanut) F 303	L. Raitcheva	−
Bradyrhizobium sp. (lupinus) MSDJ 718	N. Amarger	−

[a] NBIMTC, National Bank Industrial Microorganisms and Tissue Cultures, Sofia, Bulgaria; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpernden, United Kingdom.

[b] +, central fragmentation; −, no central fragmentation.

extents for strains within the three *Rhizobium* species analyzed. In the case of *R. etli*, the highest variability was demonstrated, whereas the structures of the IVSs of the two *R. leguminosarum* bv. viceae strains were almost identical. The nucleotides which differ in this region of these two strains are underlined in this figure. This is in agreement with the results presented in Fig. 4A, lanes 7 and 8, which also demonstrate the presence of IVSs of the same size in both strains. However, in many other *R.*

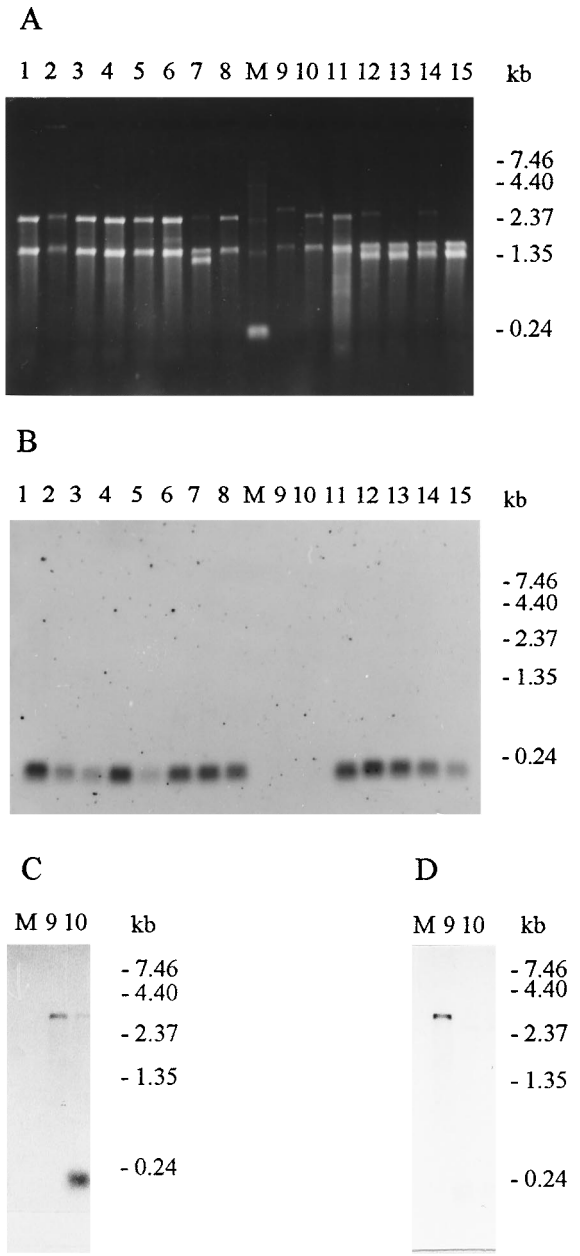

FIG. 2. Presence of a short-rRNA species in members of the family *Rhizobiaceae*. (A) rRNA contents. Lanes: 1, *R. fredii* MSDJ 1537; 2, *R. meliloti* 114; 3, *R. ciceri* UPM-Ca7^T; 4, *R. tropici* CIAT 899^T; 5, *R. loti* MSDJ 865; 6, *R. galegae* HAMBI 540^T; 7, *R. leguminosarum* ATCC 10004^T; 8, *R. leguminosarum* WS 1370; 9, *E. coli* W 2438; 10, *Bradyrhizobium* sp. strain (lupinus); 11, *Agrobacterium vitis* NCPPB 3554; 12, *Agrobacterium tumefaciens* ATCC 23308^T; 13, *Agrobacterium radiobacter* NCPPB 3001; 14, *Agrobacterium rhizogenes* DSM 30148; 15, *Agrobacterium rubi* WS 1384; M, 0.24- to 9.5-kb RNA ladder (Gibco-BRL). (B) Northern hybridization of panel A with the $23S_{24\text{-}130}$ probe derived from *R. leguminosarum* RSM 2001. (C) Northern hybridization of lanes 9 and 10 in panel A with the $23S_{24\text{-}130}$ probe derived from *B. japonicum* TU 61A152. (D) Northern hybridization of lanes 9 and 10 in panel A with the $23S_{24\text{-}130}$ probe derived from *E. coli* W 2438.

leguminosarum bv. viceae strains this region differs in size by PCR analysis (data not shown) (7). In general, the sizes of the amplification products of region $23S_{115\text{-}388}$ are highly variable and often individual to the strain, as demonstrated in Fig. 4A (see reference 7 also).

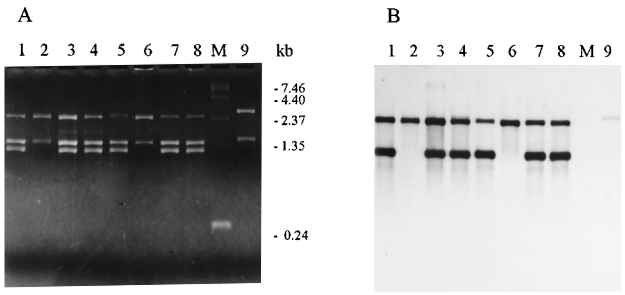

FIG. 3. Analysis of the central 23S-like rRNA fragmentation in members of the family *Rhizobiaceae*. (A) rRNA contents. Lanes: 1, *R. leguminosarum* TU 127K 89; 2, *R. leguminosarum* TU 127K 105; 3, *R. leguminosarum* TU 102K 102; 4, *R. leguminosarum* TU 102K 81-C; 5, *R. leguminosarum* WS 1376; 6, *R. leguminosarum* WS 1370; 7, *R. leguminosarum* VF 39; 8, *R. leguminosarum* B-10; 9, *E. coli* W 2438; M, 0.24- to 9.5-kb RNA ladder (Gibco-BRL). (B) Northern hybridization of panel A with the $23S_{115\text{-}1623}$ probe derived from *R. leguminosarum* RSM 2001.

The results presented confirm the predictions made by Dryden and Kaplan (6) concerning processing at the 5′ end of the 23S rRNA of *Rhodobacter sphaeroides* and the occurrence of a separate rRNA species, which remains noncovalently associ-

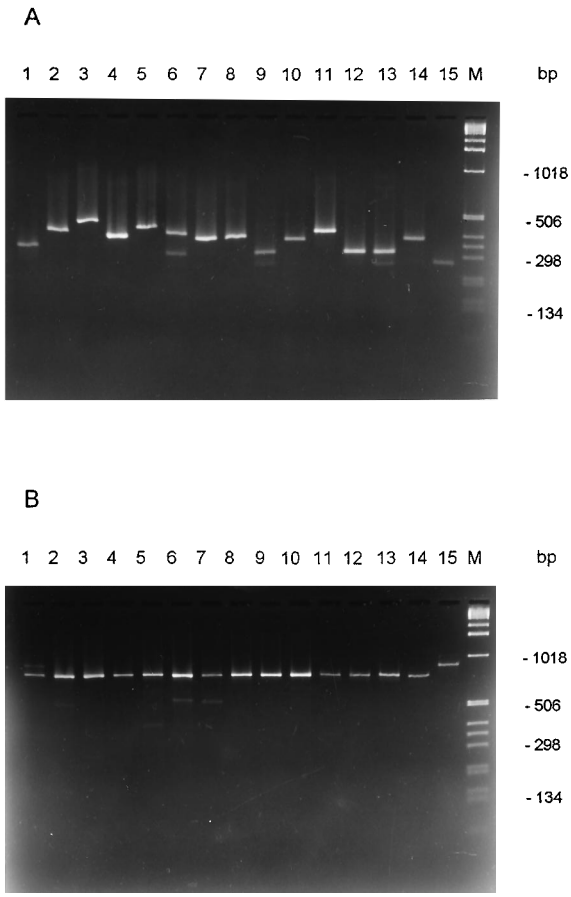

FIG. 4. Electrophoretic analysis of PCR products of regions $23S_{115\text{-}388}$ (A) and $23S_{1059\text{-}1959}$ (B). Lanes: 1, *R. ciceri* IC-2091; 2, *R. tropici* CIAT 899^T; 3, *R. tropici* CFN 299; 4, *R. etli* Viking I; 5, *R. etli* Nitragin 8251; 6, *Rhizobium* sp. related to *R. etli* Cli 80; 7, *R. leguminosarum* WS 1370; 8, *R. leguminosarum* ATCC 10004^T; 9, *R. fredii* MSDJ 1536; 10, *R. fredii* MSDJ 1537; 11, *R. galegae* HAMBI 540^T; 12, *R. galegae* 842; 13, *A. tumefaciens* C58; 14, *B. japonicum* TU 61A118b; 15, *E. coli* W 2438; M, 1-kb ladder (Gibco-BRL).

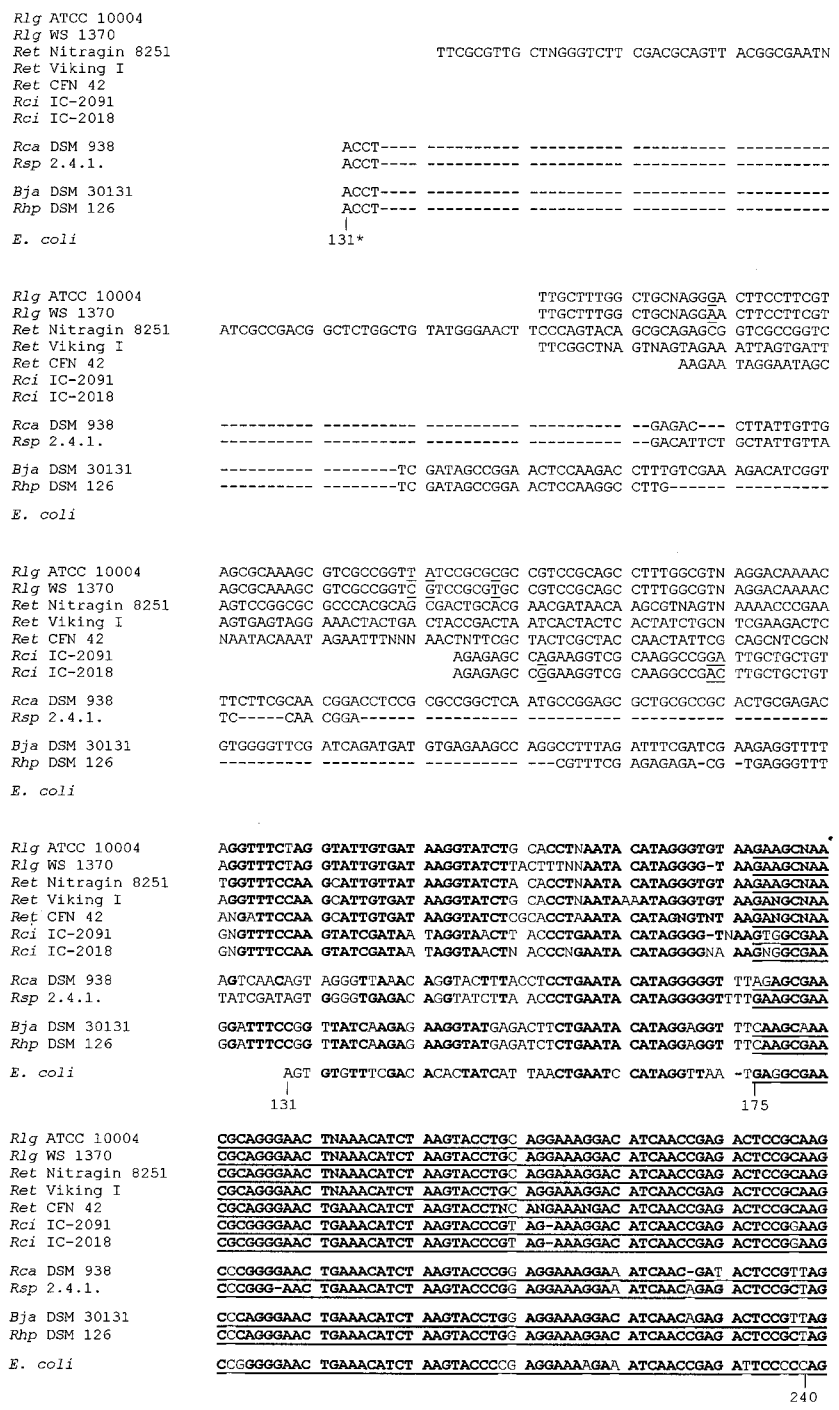

FIG. 5. Comparative sequence analysis of the 5′ ends of 23S rDNAs of representatives of alpha subclass proteobacteria. *Rlg*, *R. leguminosarum*; *Ret*, *R. etli*; *Rci*, *R. ciceri*; *Bja*, *B. japonicum*; *Rhp*, *Rhodopseudomonas palustris*; *Rca*, *Rhodobacter capsulatus*; *Rsp*, *Rhodobacter sphaeroides*. Homologous nucleotides are in bold letters. The region of high homology with *E. coli* is underlined. ∗, the first nucleotide in the IVS located after position 130 of *E. coli.*

ated with the large-subunit rRNA, similar to the 5.8S rRNA in eucaryotes. It has been demonstrated that the size of the latter in cytoplasmic ribosomes of all plants and animals investigated is fairly constant (156 to 162 nucleotides). The probability of the occurrence of a 5.8S rRNA equivalent in procaryotes (possibly smaller) was hypothesized much earlier (in 1981) by Jacq (11). Interestingly, the size of the short rRNA species in members of the family *Rhizobiaceae* (about 130 bases) corresponds to those shown for the 5.8S-like rRNA in *Drosophila melanogaster* and *Sciara corpophila* (11). In these two cases, the 5.8S rRNA is divided in two pieces, one of 123 nucleotides and another of 30 nucleotides, which is homologous to the 3′ part of the 5.8S rRNA (11).

No differences were detected in the sizes of the amplification products of the central $23S_{1059\text{-}1956}$ region of strains possessing an intact 2.6-kb rRNA or of those that were processed in this

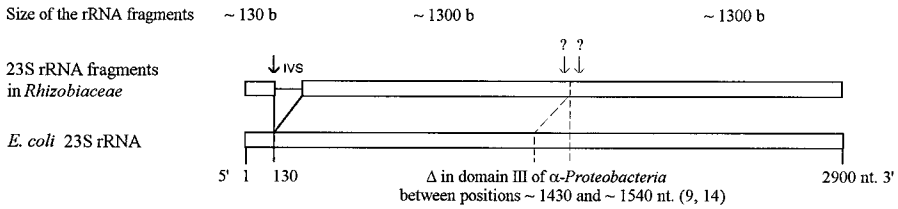

FIG. 6. Model for the fragmentation of 23S rRNA in members of the family *Rhizobiaceae*. Bold arrow, the 5′ end fragmentation position; lightface arrows, possible (?) central fragmentation positions. b, bases; nt., nucleotides.

region (Fig. 4B). From these results, it follows that the processing within the central part of the 2.6-kb rRNA which occurs in some members of the family *Rhizobiaceae*, in contrast to the situation in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, is not caused by the presence of large IVSs. Notice that differences in the sizes of PCR products smaller than 20 bp were not detectable in the experiment whose results are presented in Fig. 4B. It cannot be ruled out that in those cases short IVSs are present. For example, a break in the central domain of the *Drosophila* 26S rRNA, which is connected with the presence of an 18-bp IVS, was demonstrated (5). There are several described cases of fragmentation in the middle region of the 23S rDNA for procaryotes (4, 8, 9, 12, 18) (Fig. 1). On the basis of our results and the fact that the 23S rDNA in representatives of the alpha subclass proteobacteria is shortened by a large deletion within domain III, a model for fragmentations of the 23S-like rRNAs in rhizobia and agrobacteria studied can be proposed (Fig. 6). The central fragmentation of the 23S-like rRNA in rhizobia may occur at one of the two sites shown in Fig. 6. In both cases, the resulting molecules are about equal in size, approximately 1.3 kb. Both sites correspond to the cleavage sites in the universal model for central domain fragmentations in the large-subunit rRNAs of procaryotes and eucaryotes proposed earlier (Fig. 1). The nature and exact site of central fragmentation of the 23S-like rRNA in rhizobia and agrobacteria are under investigation in our laboratory.

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