Note

Paenibacillus macerans Possesses Two Types of 16S rDNA Copies in a Genome with a Length Difference of Twelve Base Pairs

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Two Paenibacillus macerans strains, JCM 2500T and MCRI 12, exhibited two types of 16S rDNA copies in their genomes, accompanied by a length difference of 12 bp at positions 203 to 214 (Escherichia coli numbering). The long-type sequences were newly identified for P. macerans 16S rDNA, and the copy numbers were different between the two strains. Both types of 16S rDNA were expressed in each strain, and it was predicted that the polymorphism at this position is located in helix H10, based on a comparison with the E. coli 16S rRNA secondary structure model.

Key words: Paenibacillus macerans; 16S rDNA; 16S rRNA; polymorphism

Acceptance of 16S rRNA as an appropriate tool for the identification and classification of bacteria has been based on the fact that multiple copies of rRNA genes (rDNA) present in a single organism are identical or nearly identical in nucleotide sequences. Most strains possess multiple 16S rDNA showing no intra-genomic heterogeneity. When more than a single 16S rDNA from one bacterial genome has been analyzed, the sequences determined have generally been found to be identical, or to differ from each other by less than 1% of their nucleotide positions.1) Although mutations such as transition or transversion are introduced by misincorporation or misrepair by DNA polymerase during DNA replication,2) certain cases of intra-genomic heterogeneity cannot be fully explained by these phenomena. For example, Ochrobactrum intermedium,3) Streptomyces spp.,4) Thermobispora bispora,4) and Thermomonospora chromogena5) show heterogeneity, differing from those introduced by misincorporation or misrepair.

In the process of identification of strain MCRI 12 (culture collection of the Marudai Central Research Institute), isolated from a commercially available pouch “convenience” food containing Chinese-style food, we unexpectedly discovered a new intra-genomic heterogeneity of Paenibacillus macerans. Paenibacillus spp. are gram-positive, spore-forming rods belonging to a class of bacilli of the phylum Firmicutes.6) These bacteria have been isolated from a variety of sources, including soil, water, the plant rhizosphere, food, diseased insect larvae, and clinical specimens.7) Some Paenibacillus spp. have shown intra-genomic 16S rDNA heterogeneity,1,7) but it has not previously been found in P. macerans.

We tried to identify MCRI 12 based on 16S rDNA sequencing. The strain was grown on nutrient agar (Difco Laboratories, Detroit, MI) for 48 h at 30°C, and in nutrient broth (Difco) for 24 h at 30°C. Bacterial DNA samples for sequencing were isolated from a single colony of the bacteria. Bacterial DNA extracts were prepared using PrepMan ultra reagent (Applied Biosystems, Foster City, CA). Partial fragments of 16S rDNA were selectively amplified by PCR using universal primers: 5F (5′-TGTGAGATTTGTACTCGGTGCAG-3′) as the forward primer and 531R (5′-TACCGCGGTGTGCACGAC-3′) as the reverse primer.8) PCR was performed in 50 μl of reaction mixture containing 1 μl bacterial DNA extract as the template, 2.5 mM MgCl2, 0.2 mM dNTP, 1 μM of each primer, and 1.25 U of AmpliTaq Gold DNA polymerase in GeneAmp gold buffer (Applied Biosystems). PCR conditions were consistent with the instructions provided with the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems), and sequencing was carried out with an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Direct sequencing gave an ambiguous result. In forward sequencing, the sequence at positions 5 to 202 (E. coli numbering) was determined, but the rest indicated double sequencing signals. Similarly, the sequence at positions 215 to 531 (E. coli numbering) was determined, but the rest indicated double sequencing signals. Similarly, the sequence at positions 215 to 531 (E. coli numbering) was determined, but the rest indicated double sequencing signals.

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was determined in reverse sequencing, but the forward part showed double signals. This result suggested the existence of two types of 16S rDNA copies with local polymorphism containing a gap at positions 203 to 214 (E. coli numbering). The BLAST program was used to compare the present 16S rDNA sequences with sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/) and DDBJ (http://www.ddbj.nig.ac.jp/) databases. We carried out a homology search using the BLAST program with the sequences determined in forward and reverse sequencing, and discovered a similarity with *P. macerans* IAM 12467T (accession no. AB073196) and *Paenibacillus* sp. R-7487 (accession no. AY397771). These two sequences showed different sequences at position 203 to 214 (E. coli numbering) with a length difference of 12 base pairs, indicating identity with double sequencing signals in MCRI 12.

Based on these results, we designed other primers for confirmation of sequencing of the polymorphism points. The specific primers for sequencing of polymorphism points are shown in Fig. 1A and B. The target products were certainly obtained by PCR using the primer sets 5F-Pm232Ra, 5F-Pm232Rb, Pm191Fa1-531R, Pm191Fa2-531R, and Pm191Fb-531R. Direct sequencing with the primer sets showed two types of 16S rDNA, with different sequences at positions 203 to 214 (E. coli numbering), as expected (Fig. 1). Alignment was performed using the ClustalW program (http://www.ddbj.nig.ac.jp/). The DDBJ accession nos. for the 16S rDNA sequences of JCM 2500T are AB162431 and AB162432. The copy number of 16S rDNA was determined by a Southern blotting experiment using a 16S rDNA probe on genomic DNAs digested with HindIII and/or EcoRI (restriction sites for both HindIII and EcoRI are absent in *P. macerans* 16S rDNA) (Fig. 2A). Genomic DNA was purified with a QIAGEN DNA kit (Qiogene,

A BLAST analysis of the short-type sequence containing 6 bp at the polymorphism position showed identity of over 99% to *P. macerans* IAM 12467T without any single nucleotide polymorphisms. The cells of MCRI 12 were gram-positive, spore-forming rods. Its biochemical properties were consistent with those of *P. macerans* according to biochemical tests using the API 20E and API 50CHB systems (data not shown). Consequently, MCRI 12 was identified as *P. macerans*. The long-type sequence was quite similar to the short-type sequence except at the polymorphism position, while the particular 18 bp in the long-type sequence was absent from all *Paenibacillus* spp. 16S rDNA sequences available in data banks, except for a single sequence obtained from *Paenibacillus* sp. R-7487.

For the *P. macerans* type strain JCM 2500T (obtained from the Japan Collection of Microorganisms), direct sequencing after PCR amplification using primer 5F-531R did not show double sequencing signals. Nevertheless, PCR amplification products using primer Pm200F-531R, anchored in the particular 18 bp for the long-type sequence, were also obtained from JCM 2500T (data not shown). Moreover, two sequences similar to those of MCRI 12 were also identified in JCM 2500T, using the specific primer sets for the polymorphism points (Fig. 1). The DDBJ accession nos. for the 16S rDNA sequences of JCM 2500T are AB162431 and AB162432.

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DNAs digested with *Hind*III and/or *Eco*RI were subjected to electrophoresis for 1.5 h at 50 V in a 0.8% agarose gel in TBE using Mupid gel electrophoresis (Advance, Tokyo). Electrophoresis gels were transferred to Hybond N+ nylon membranes (Amersham Biosciences, Little Chalfont, UK) by alkaline blotting. 16S rDNA digoxigenin (DIG)-labeled probes were obtained by PCR using 5'-end DIG-labeled primers of 5F-531R (Kurabo Industries, Osaka, Japan). Hybridization of the probes was detected with a DIG nucleic acid detection kit (Roche Diagnostics, Mannheim, Germany). At least eight 16S rDNA hybridizing fragments (*rrs* operons) were found in each strain according to the fragment patterns of double digestion with *Hind*III and *Eco*RI for JCM 2500T, and of *Eco*RI digestion for MCRI 12. The copy number of the long-type sequence was determined using the oligonucleotide probe corresponding to the particular 18 nucleotides (Fig. 2B). A 5'-end DIG-labeled probe of Pm200F (Kurabo) was used as the oligonucleotide probe. MCRI 12 had five long-type copies based on the double digestion fragment patterns obtained with *Hind*III and *Eco*RI. JCM 2500T had two copies of the long type, according to the fragment patterns of *Hind*III and *Eco*RI digestion.

Intra-genomic heterogeneity of 16S rDNA appears as double sequencing signals in direct sequencing, such as that found by Teyssier et al.\(^3\) and Marchandin et al.\(^10\) for *O. intermedium* and *Veillonella* spp. respectively. In the present study, double sequencing signals appeared in direct sequencing with universal primers for MCRI 12. At first, only single sequencing signals were found for JCM 2500T because of the small number of long-type copies in this strain; by contrast, MCRI 12 possesses many more of these genes. However, when the direct sequencing results for JCM 2500T were examined carefully, feeble double sequencing signals were identified. It is believed that the ratio of the numbers of the different copies in a 16S rDNA polymorphism is reflected in the signal intensity found in direct sequencing.

We amplified 16S rRNA molecules selectively by RT-PCR for short- and long-type sequences using primer sets 5F-Pm232Ra and 5F-Pm232Rb respectively on total-RNA extracts (Fig. 3). Total bacterial RNA was isolated and purified using the SV total RNA isolation system (Promega, Madison, WI), and reverse transcription-PCR (RT-PCR) was performed with the AccessQuick RT-PCR system (Promega). Non-reverse-transcribed RNA was used as the PCR template for the negative control. RT-PCR products for both short- and long-type 16S rRNAs were obtained from each strain, and we concluded that both types of 16S rRNA are expressed in each strain. Prediction of RNA secondary structure by energy minimization was performed with the on-line Mfold server at http://mfold.burnet.edu.au. The polymorphism position was predicted to be located in helix H10, based on comparison with the *E. coli* 16S rRNA secondary structure model. *Veillonella* spp. show a difference in sequence but not a difference in length at...
the same helix\(^{1)}\) and *Ochrobactrum intermedium* exhibits a polymorphism with a length difference of 42 nucleotides for helix H9, a near neighbor of H10.\(^{3)}\) Based on the *E. coli* 16S rRNA secondary structure model, a polymorphism accompanied with a long length difference was found for helix H10 in the present study, in contrast with previously detected 16S rRNA polymorphisms. In the Database of Ribosomal Cross-Links,\(^{11)}\) there are no known interactions with rRNA or ribosomal proteins listed for helix H10. Furthermore, H10 is located far from the active center of the 30S subunit, which is located on the intersubunit side, including the decoding center, and is instead positioned near the spur or foot of the 30S subunit on the solvent side.\(^{12)}\) We found no difference in certain phenotypical traits such as colony aspect, morphology, and growth properties between strains JCM 2500\(^{7)}\) and MCRI 12. For the reasons stated above, it is believed that extensions of H10 have little effect on ribosome function; this speculation fits with the low conservation within this area between 16S rRNA from all bacteria in the Gutell laboratory database (http://www.rna.icmb.utexas.edu/).

In bacterial type strains, large scale intra-genomic heterogeneity is generally recognized as an unusual property, though it has been found for *Thermobispora bispora*\(^{4)}\) and *Thermomonospora chromogena*\(^{5)}\). In the present study, two types of 16S rDNA copies were found from *P. macerans*, including a long-type sequence which was newly identified from the type strain. Since minority copies of 16S rDNA are not sequenced by direct sequencing using universal primers, 16S rDNA copies in a single bacterium might be more heterogeneous than recognized at present. For identification using 16S rDNA information, the possible existence of other bacteria possessing large-scale intra-genomic heterogeneities should receive further attention.

References


