Sequence of 16S rRNA Gene of Rat-Origin Cilia-Associated Respiratory (CAR) Bacillus SMR Strain

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ABSTRACT. The 16S rRNA gene of the SMR strain of cilia-associated respiratory (CAR) bacillus, which was isolated from a spontaneously infected rat at our institute, was sequenced. Its 1,521 nucleotides were determined. On the basis of the results of the sequence analysis, the SMR strain was found to be most closely related to members of the *Flavobacter/Flexibacter* group. This sequence was compared with the previously determined 16S rRNA gene sequences (rat-origin: three; mouse-origin: one; rabbit-origin: one) of CAR bacillus isolates. The SMR strain showed the highest sequence similarity (99.9%) to the rat-origin CARB-NIH strain (Schoeb *et al.*, 1993), and it was concluded that the strains are identical.

KEY WORDS: CAR bacillus, sequence similarity, 16S rRNA gene.

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Cilia-associated respiratory (CAR) bacillus is an unclassified, gram-negative, filamentous bacterium that was tentatively designated in 1985 as one of the agents that cause chronic respiratory diseases in laboratory rodents [7]. The bacilli cannot grow by conventional cultivation; infection was therefore evidenced by the bacilli colonizing in the affected airways by patho-morphological and ultrastructural methods. Similar bacilli have been seen in the respiratory tracts of rats [10, 12, 13], mice [8], rabbits [1, 11, 17], cattle [9], pigs [16] and goats [6, 18]. Experimental infection was successful in rats, mice, rabbits, guinea pigs and hamsters [4, 5, 15, 21, 22].

Recently, it has been suggested that strains of CAR bacillus isolated from rats and mice may be distinctly classified from those isolated from rabbits, based on the following observations. These strains have a different ability to develop pathological lesions in rabbits. In the experimental infection in rabbits, the rabbit-origin CAR bacillus caused respiratory lesions, such as peribronchial lymphoplasmocytic hyperplasia [5], that resembled those of natural cases [1, 11, 17]. In contrast, the rat- and mouse-origin CAR bacillus did not induce these lesions in rabbits [5, 22]. In serological examinations, antigens made from the rat- and mouse-origin CAR bacillus did not necessarily show positive reactions against sera obtained from infected rabbits [11, 15, 22]. Immunohistochemically, CAR bacilli that colonized the respiratory epithelium of pigs and rabbits reacted negatively or weakly positively to anti-rat CAR bacillus sera [16, 17]. CAR bacillus found in goats was also negative to anti-rat CAR bacillus sera, but was positive to anti-rabbit CAR bacillus sera [6, 18]. On the other hand, CAR bacillus found in cattle was positive to anti-rat CAR bacillus sera [9]. These data suggest that CAR bacilli are divided into two groups at least, one that has common antigens with the rat-origin CAR bacillus and another that has common antigens with the rabbit-origin CAR bacillus. In molecular biology, PCR primers, specific to the rat-origin CAR bacillus, were not functional with the DNA template obtained from infected pig tracheas [16]. Antigenic profiles

obtained by Western blot analysis and restriction enzyme analysis with *EcoRI* in chromosomal DNA did not show a great difference between the rat-origin and rabbit-origin isolates [4]. However, the existence of different patterns between both isolates using *HindIII*, *EcoRV* and *PstI* [20] suggests that each chromosomal DNA was different. More recently, 16S rRNA gene analysis has indicated that the rat- and mouse-origin CAR bacilli were most closely related to members of the *Flavobacter/Flexibacter* group [3, 20, 23], while the rabbit-origin CAR bacilli were most closely related to members of the genus *Helicobacter* [5].

The SMR strain of CAR bacillus was previously isolated from a naturally infected rat at our institute [13, 14]. Although this strain has been morphologically, pathogenically and serologically shown to be similar to the rat-origin CAR bacilli, Kurisu *et al.* have reported that the SMR strain positively reacted to sera from some naturally infected rabbits [11]. Therefore, we have no conclusive evidence that the SMR strain relates with the rat- and mouse-origin CAR bacillus. In the present study, the 16S rRNA gene sequence was analysed for the SMR strain to genetically clarify the taxonomic relationship with other CAR bacillus strains. Then, it was indicated that the SMR strain is most closely related to those of the *Flavobacter/Flexibacter* group, and that it is identical to the rat-origin CARB-NIH strain, originally isolated by Ganaway *et al.* and Schoeb *et al.* [7, 20].

The bacilli were inoculated into the allantoic cavity of embryonated chicken eggs after several inoculations into mice [14]. After harvest, the bacilli were stored at -80°C until used. Chromosomal DNA of the SMR strain was extracted and purified according to the method described by Cundiff *et al.* [3], with some modifications. Briefly, 1 ml of the allantoic fluid containing the SMR strain was centrifuged at 14,000 rpm for 30 min, and the pellet was washed three times with PBS. The bacteria were lysed in 200 μl of a lysozyme solution (10 mg/ml) on ice for 45 min, followed by addition of 10 μl of a sodium dodecyl sulfate (10%, wt/vol) and 20 μl of a protein-

ase K solution (10 mg/ml). The lysate was then incubated overnight at 37°C. The DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE solution. The 16S rRNA gene was PCR-amplified using LA *Taq* DNA polymerase (Takara, Kyoto, Japan) and the primers 27f (5'-GAGTTTGTATCCTGGCTCAG-3') and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3') [19]. These primers are complementary to the conserved regions found at the 5' and 3' ends of most eubacterial 16S rRNA genes. The LA *Taq* was used to ensure high-fidelity amplification. PCR products were gel-purified with GeneClean II (BIO 101, Inc., La Jolla, CA, U.S.A.), and cloned with pCR2.1 vectors by using the Original TA Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). Sequencing reaction was carried out by the dye primer method using an ET primer kit (Reverse and Forward, Amersham, Buckinghamshire, England), and analysed with a model ABI 377 automatic DNA sequencer (Perkin-Elmer, Foster City, CA, U.S.A.). The obtained sequence of the SMR strain 16S rRNA gene was compared with eubacterial 16S rRNA sequences, available in the Genbank database, as well as those of other previously examined CAR bacillus isolates, including the rat-origin CARB-NIH strain [20], the CBR strain isolated from a rat [23], the R1, R2 and R3 (R) isolates obtained from rats [3], the CBM strain isolated from a mouse [23] and the B1-B6 (B) isolates obtained from rabbits [5]. Sequence alignment and similarity evaluation were carried out by DNASIS V3.5 (Hitachi Software Engineering, Yokohama, Japan).

We determined 1,521 nucleotides of the 16S rRNA gene of the SMR strain (Fig. 1). In comparison to the previously determined 16S rRNA gene sequence of CAR bacilli, this was the longest sequence found. By a homological analysis of the comparison of this sequence with those of eubacteria deposited in the database, it was revealed that the SMR strain was most closely related to *Flavobacterium ferrugineum* (83% similarity), *Chitinophaga pinensis* (83%), and *Flexibacter sancti* (81%) belonging to the *Flavobacter/Flexibacter* group. This result coincides with the previous taxonomic analysis of the rat- and mouse-origin CAR bacillus [3, 20, 23]. Sequence similarities among the 16S rRNA genes taken from the SMR strain, strains from rats (the CARB-NIH strain, the CBR strain and the R isolates), a strain from a mouse (the CBM strain) and strains from rabbits (the B isolates) are shown in Table 1. Between the SMR and the CARB-NIH strains, the sequence similarity was 99.9%; that is, only a single nucleotide, at position 1,485, was different among 1,489 overlapped nucleotides. Therefore, within the accuracy of sequencing technology, we conclude that these two strains, SMR and CARB-NIH, are genetically identical based on the

analysis of the 16S rRNA gene. This is supported by the fact that strains of the same species generally show sequence similarities >97% [2]. Additionally, a sequence similarity of 96.8% between the CBR strain and the CBM strain is thought to be attributable to strain variation [23]. Poor similarity of the rabbit-origin B isolates to the rat- and mouse-origin isolates clearly supports the previous idea that the rabbit-origin CAR bacillus is taxonomically distinct from those of the rat- and mouse-origin. A comparably low similarity of the R isolates with the other rat- and mouse-origin isolates raised the possibility that they might be different strains from the other rat- and mouse-origin strains. Figure 2 shows the comparison of the 16S rRNA gene sequences of the above-mentioned rat- and mouse-origin CAR bacillus strains. Almost identical sequences were recognized from positions 269 to 454. Different nucleotides appeared in only three bases, at positions 355, 417 and 440, in the CBR strain and in only one base, at position 410, in the R isolates. In the 16S rRNA gene sequence, it is known that there are conserved regions and variable regions. The former regions are those that are fully conserved over the species and genera in eubacteria, while the latter regions differ between species. Accordingly, it is suggested that positions 269 to 454 are peculiar to the rat- and mouse-origin CAR bacillus, and this region of the gene may therefore be useful in diagnosing rat- and mouse-origin CAR bacillus infection by the PCR method.

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1  GAGTTTGTATCCTGGCTCAGGATGAACGCTAGCGGACGGCTAACACATGCAAGTCGAGGGGACGGCGGTGATGCAATACACTGGCGGCGACGGCAACGGGTGCGTAACCGCTACACAACTTGCCTTTGAGTGGAGCATAACACCGAG
151  AAATTGGTGCTAATTCTCCATAGTATTCTGGAAGGAGTCTTTGGTAAATTAAGGCTCCGGCGCTCGAAGATGGGTGTGCTGCGGATTAGATAGTTCGGTGAGGTAACGGCTCACCAGCTACGATCGGTAACCTGGTGTGAGAGCAGAC
301  CAGTCACACGGACACTGAGACACGGGTCCGACTCTACGGAGGACGAGTGAAGATATGTTGCAATGGGCGCAAGCCGTGAACAGCCATGCCGTGTGAAGGATGAAGGCTCTCAGAGTTCGTAACCTCTTTATTTGGAATGAATCC
451  CCITTTCTAAGGGTTTGACAGTACCAATTGAATAGCAGCCGGCTAATCCGTCGACAGCCGGTAAATGAGAGGTCGAGGTCGAGCTTATCCGATTATTTGGTTTAAAGGGTGTGACGCGGATAGTGAATGCTGTAATGCC
601  CGAGCTTAACCTCGAATTCGATTCGATACTGCTATCTTGAATACCTGCGAGCTGAGTGAATATCTCATGATGCGTGAATGTCGAGATGACATAGAAATCCGATTCGGAAGGCCCTCAGTATACAGTATTTGAGCTCAGCA
751  CGAAGCGTGGGGATCAACAGGATTAGATACCTGGTATGCCACCCCTAAACGATGGATCTGACCTTAGCGATATCTGTTAGCGTCTGAGCGAAGCATTAAATATCCCACTGGGAAGTACCATCCGACGTTGAATCTCAAG
901  GAATTGACGGGGTCCGCAACAAGAGGTGGAACATGTGGTTAATTCGATGATACGGGAGGAACCTTACCTAGGCTAGAATGCGGTGGCGAGTCCCTGAAAGGGGGTCTGTAGCAATACACTGCCGCAAGGTGCTGATGGCTGTCGT
1051  CAGCTCGTGGCGTGAAGTGTGGTTAAGTCCCGACGAGCGCAACCCCAATCTTTTGTGCCAGCGAGTAATGTCGGGAACCTCGAGAAACTGCCCGCGCAAGGTGTGAGGAGGGAGGGGATGATGCAAGTCATATGCGCTTAT
1201  GCCTAGGGCTACACAGCTGTTACAATGGGTGCTACAGTGGGTCCGACATAGCAATATGAGCACTAATCCCAAAGCACTCTCAGTTTCGATTGTAGGCTGCAACTCGCTACATGAACCTGGAATCTCTAGTAATCGTATATCAGCAAT
1351  GATACGGTGAATACGTTCCCGACCTTGTACACACCCCGCTCAAGCCATGGAAATGGGGTGTATCTGAAGACGGTAGCGGAAAGGGGCTGTCTAGGGTAAACTCGTAATCGGGCTAAAGTCTGAACAGGTAGCGCTACGCAAGGTG
1501  TGCGTGGATCACCTCTTTCT

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Fig. 1. Nucleotide sequence of the 16S rRNA gene of the CAR bacillus SMR strain.

Table 1. Sequence similarity for six CAR bacillus 16S rRNA genes

Animal origin CAR bacillus strain/isolate	% similarity					
	SMR	CARB-NIH	CBR	R	CBM	B
Rat-origin						
SMR strain	—	99.9	95.7	91.2	96.2	73.6
CARB-NIH strain	99.9	—	95.7	91.2	96.2	73.6
CBR strain	95.7	95.7	—	89.0	95.5	71.6
R isolates ^{a)}	91.2	91.2	89.0	—	89.5	74.8
Mouse-origin						
CBM strain	96.2	96.2	95.5	89.5	—	72.9
Rabbit-origin						
B isolates ^{a)}	73.6	73.6	71.6	74.8	72.9	—

a) In comparison with the 16S rRNA genes from the R isolates and the B isolates, the consensus sequence of the R1, R2, R3 isolates [3] and that of the B1 to B6 isolates [5] are used, respectively.

Fig. 2. Comparison of the 16S rRNA gene sequences of CAR bacillus strains isolated from rats and a mouse. The identical nucleotides are indicated by dots (•), and gaps (-) are incorporated so as to obtain a relevant alignment. A nucleotide that differs between the SMR strain and the CARB-NIH strain is indicated by an arrowhead symbol (◀). Position number is based on the sequence of the SMR 16S rRNA gene. In comparison with the 16S rRNA gene from the R isolates (R), the consensus sequence is used.

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