Assignment of the Bacterial Agent of Urinary Calculus in Young Rats by the Comparative Sequence Analysis of the 16S rRNA Genes of Corynebacteria

Tatsufumi TAKAHASHI, Masayoshi TSUJI[1)], Naoya KIKUCHI, Chiaki ISHIHARA[1)], Tsutomu OSANAI[2)], Noriyuki KASAI[2)], Ryo YANAGAWA, and Takashi HIRAMUNE

Departments of Epizootiology, [1)]Experimental Animals, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069, and [2)]Institute for Animal Experimentation, Hokkaido University School of Medicine, Sapporo 060, Japan

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ABSTRACT. Comparative 16S rRNA gene sequencing was used to assign four isolates of spontaneous urinary calculus in young laboratory rats. The phylogenetic relationships among the rat isolates and selected species of corynebacteria were also inferred. Based on the homology and evolutionary distance analysis, the 16S rRNA genes of the rat isolates were almost identical with that of *Corynebacterium renale* ATCC 19412^T. Also the results of the phylogenetic analysis showed a close relationship among the isolates and *C. renale,* but they were clearly different from *C. pilosum, C. cystitidis, C. kutscheri* and *Rhodococcus equi*. The results of the present study and previously published biochemical data demonstrate that the organism involving urinary infections in young rats is identified to be *C. renale*.—KEY WORDS: *Corynebacterium renale*, rat, 16S ribosomal RNA.

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Young inbred LEW/Hkm rats up to 10 weeks old suffered naturally from urinary calculus with hematuria and severe marasmus. More than a quarter (54/200) of the females of this line of rats had similar symptoms. A chart adapted from a scheme used to identify bacteria in routine laboratories [1] indicated that the biochemical profile of these organisms was similar to that of *Corynebacterium renale* [6]. Although the isolates and a type strain of *C. renale* (ATCC 19412^T) were similar in biological properties, they were not apparently identical in immunodiffusion patterns with the hyper-immune sera against the type strain or an isolate [6].

To know whether the immunological difference was based on variation within the species of *C. renale* or on the inter-species gap, genetical characterization of the isolates and selected species of corynebacteria was done in this study. Chromosomal DNA-DNA hybridization and calculation of the G+C composition ratio were commonly employed in the classification of bacteria [4], but these approaches have limitations in more precisely analyzing the order of the bacteriological taxonomy. Recent developments and improvements in genetical techniques like a polymerase chain reaction (PCR) gene amplification method made it easier to figure out the DNA sequences of bacterial genes. The sequence information was integrated as several data bases, in which we retrieved the sequence of the 16S rRNA gene of *C. renale* ATCC 19412^T [9].

Out of numerous genes of bacteria, sequencing of small-subunit (16S) rRNA genes has become a powerful tool for determining genetical assignments. The sequence of the 16S rRNA gene varies in an orderly fashion across phylogenetic boundaries so that organisms which are phylogenetically related have similar sequences. This property of 16S rRNA is utilized in the study of molecular evolution and systematics [10]. By means of 16S rRNA sequence analysis, we have studied the phylogenetic relationship between the isolates associating urinary calculus in rats and *C. renale*.

Four isolates, HR-56 to -59, were obtained from cultures of the urinary tract of rats with severe symptoms. The isolation was performed in the early stage of the outbreak of urinary calculus in the rats. The codes of the isolates and the source specimen of the isolation are summarized in Table 1. The pure culture of each organism was further incubated in 50 m*l* of brain heart infusion broth (BBL, Becton Dickinson, Cockeysville, U.S.A.) supplemented with 0.1% glycine at 37°C for 24 hr and packed by centrifugation at 1,200 × g for 10 min. The bacterial sediment was washed once with phosphate buffered saline, pH 7.0 by centrifugation and suspended in 2 m*l* of 10 mM Tris (pH 8.0) containing 1 mM EDTA (TE). The suspension was dounce homogenated 30 strokes and then chromosomal DNA was extracted with water saturated phenol/chloroform. The ethanol precipitate of the extract was used for PCR as template DNA.

The PCR procedure was followed at basic protocols [7] with a pair of generic primers for Gram-positive bacteria, primer A (+); 5' AGAGTTTGATCCTGGCTC 3', and primer B; 5' GGTTACCTTGTTACGACTT 3'. By this method, the 16S rRNA gene was amplified for cloning into plasmid. The amplified PCR product was tailed with oligo-dC at its 3' termini and then ligated with oligo-dG-tailed pBR322 (BRL, Ithaka, MD, U.S.A.).

The positive clone of recombinant plasmid was used for DNA sequencing performed by the dideoxy method [13] with a panel of generic primers for Gram-positive bacteria. The primers were designed on the basis of multialignment data reported by Neefs *et al.* [5]. The 5' termini of the primers were labeled with fluorescein isothiocyanate and the procedures for primer extension and determining DNA sequences were done according to the instructions with the AutoRead Sequencing kit and A. L. F. DNA Sequencer II (Pharmacia Biotech, Upsala, Sweden).

The nucleotide sequences of the 16S rRNA genes of *C. pilosum, C. cystitidis, C. kutscheri* and *Rhodococcus equi*, were also determined in the same manner, because we suspected that the isolates could be *C. kutscheri* at an early stage of the chemotaxonomical examination [6]. *C. pilosum* and *C. cystitidis* have been known as species related to *C. renale* because of their pathogenicities in cattle [12]. The sequence of *R. equi* was also compared as an outgroup. The sequence data determined in this study

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   1 CGAACGCTGG CGGCGTGCTT AACACATGCA AGTCGAACGG AAAGGCCACT GCTTGCAGTG GTGCTCGAGT GGCGAACGGG TGAGTAACAC GTGGGTGATC
 101 TGCCCTGTAC TTTGGGATAA GCCTGGGAAA CTGGGTCTAA TACCGGATAG GACCATCGTT TAGTGTCGGT GGTGGAAAGC TTTTGCGGTA CGGGATGAGC
 201 TCGCGGCCTA TCAGCTTGTT GGTGGGGTAA TGGCCTACCA AGGCGTCGAC GGGTAGCCGG CCTGAGAGGG TGGACGGCCA CATTGGGACT GAGATACGGC
 301 CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGCAA GCCTGATGCA GCGACGCCGC GTGAGGGATG ACGGCCTTCG GGTTGTAAAC
 401 CTCTTTCGCT AGGGACGAAG CTTTTGTGAC GGTACCTAGA GAAGAAGCAC CGGCTAACTA CGTGCCAGCA GCCGCGGTAA TACGTAGGGT GCGAGCGTTG
 501 TCCGGATTTA CTGGGCGTAA AGAGCTCGTA GGTGGTTTGT CGCGTCGTCT GTGAAATTCC GGGGCTTAAC TCCGGGCGTG CAGGCGATAC GGGCATAACT
 601 TGAGTACTGT AGGGGAGACT GGAATTCCTG GTGTAGCGGT GGAATGCGCA GATATCAGGA GGAACACCGA TGGCGAAGGC AGGTCTCTGG GCAGTTACTG
 701 ACACTGAGGA GCGAAACGAT GGGTAGCGAA CAGGATTAGA TACCCTGGTA GTCCATGCCG TAAACGGTGG GCGCTAGGTG TGAGACCCTT CCACGGGTTT
 801 TGTGCCGTAG CTAACGCATT AAGCGCCCCG CCTGGGGAGT ACGGCCGCAC ATGGAAAACT CAAAGGAATT GACGGGGGCC CGCACAAGCG GCGGAGCATG
 901 TGGATTAATT CGATGCAACG CGAAGAACCT TACCTGGGTT TGACATACAC CAGATCGGGC CAGAGATGGT CTTTCCCTTT GTGGTTGGTG TACAGGTGGT
1001 GCATGGTTGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA AGTCCCGTAA CGAGCGCAAC CCTTGTCTTA TGTTGCCAGC ACGTTGTGGT GGGGACTCAT
1101 GAGAGACTGC CGGGGTTAAC TCGGAGGAAG GTGGGGATGA CGTCAAATCA TCATGCCCCT TATGTCCAGG GCTTCACACA TGCTACAATG GTCGGTACAA
1201 CGCGTGTGCT ACTTCGTGAG GAGATCGTAA TCGCTGAAAG CCGGCCTTAG TTCGGATTGG GGTCTGCAAC TCGACCCCAT GAAGTCGGA
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Fig. 1. Nucleotide sequence of 16S rRNA gene from rat isolate HR56. The underlined sequences were found uniquely in rat isolates and *C. renale* ATCC 19412^T.

Table 1. Rat isolates and the other bacteria used in this study

Bacteria	Specimen for isolation	Accession no. of 16S rRNA gene sequence
HR56	Bladder swab	D37803
HR57	Urine	D37804
HR58	Bladder swab	D37805
HR59	Kidney	D37806
Corynebacterium renele (ATCC 19412^T)		M29553
C. pilosum (ATCC 29592^T)		D37914
C. cystitidis (ATCC 29593^T)		D37915
C. kutscheri (ATCC 15677^T)		D37802
Rhodococcus equi (ATCC 33701)		D37876

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence data bases with the accession numbers.

are not shown excepting for that of a rat isolate, HR-56, in Fig. 1, but all the data have been submitted to the DNA Databank of Japan (DDBJ), and their accession numbers are shown in Table 1. with the sequence data for *C. renale* ATCC 19412^T [9], which were obtained from DDBJ, the similarities and phylogenetic relationships among their genes were analyzed with computer software packages for the PC Gene (Intelligenetics, Mountain View, CA, U.S.A.) and Clustal V [3].

The sequences were aligned manually and the results were used for further analyses of homology and evolutionary distance values. As shown in Table 2, the sequence similarities were presented as sequence homology (%) and evolutionary distance by using the method of Fitch and Margoliash [2]. No major differences among the rat isolates and *C. renale* type strain were demonstrated since the homologies were 99.6% or more and the evolutionary distances were 0.4% or less in any combinations in the group. Similarities of the group of *C. renale* to the other corynebacteria and *R. equi* ranged 93.7 to 96.6% and 91.5 to 92.5%, respectively. These results agreed with the biological characteristics of the rat isolates [6], which were nearly identical with the type strain of *C. renale*.

The phylogeny of 16S rRNA genes of the rat isolates are shown in Fig. 2 as an unrooted tree that was drawn by the Neighbor-joining method [8]. As a result, the genes of the rat isolates were located in the same cluster as *C. renale*, although there were some minor differences in the cluster. The difference between one cluster containing HR56, HR57, and a *C. renale* type strain and another cluster containing HR58 and HR59 was due to only four or five changes out of 1,289 bases, whereas the difference between *C. renale* and other corynebacteria was based on at least 45 base changes. These results suggest that the rat isolates represent *C. renale*. This means that the immunological diversity of the rat isolates from the *C. renale* type strain might be explained by the fact that the species have several serotypes.

As shown in Table 2 and Fig. 2, the 16S rRNA gene of *C. kutscheri* is located somewhat closer to *C. renale* than to *C. pilosum* or *C. cystitidis*. These results suggest that the three corynebacterial species which had been the same species [11, 12] were substantially different from each other, although this might depend on the method of phylogenetic analysis employed in this study. The most important point, however, is that the approach by means of 16S rRNA gene sequencing makes brings us to intuitive understanding of the bacterial phylogeny even among closely related species.

Table 2. Evolutionary distance (upper right hand triangle) and homology (lower left) values for 16S rRNA gene sequences of rat isolates and other bacteria

	Bacteria	Evolutionary Distance (per 100 bases) HR56	HR57	HR58	HR59	Cr	Cp	Cc	Ck	Re
Homology (%)	HR56		0.0	0.3	0.3	0.4	4.3	5.5	3.5	8.2
	HR57	100.0		0.3	0.3	0.4	4.3	5.5	3.5	8.2
	HR58	99.7	99.7		0.0	0.0	4.3	5.2	3.5	7.9
	HR59	99.7	99.7	100.0		0.0	4.3	5.2	3.5	7.9
	C. renale ATCC 19412^T	99.6	99.6	99.9	99.9		4.3	5.3	3.6	7.9
	C. pilosum ATCC 29592^T	96.2	96.2	96.2	96.2	96.1		3.9	6.4	9.1
	C. cystitidis ATCC 29593^T	94.8	94.8	95.0	95.0	94.9	96.4		5.0	9.6
	C. kutscheri ATCC 15677^T	96.6	96.6	96.6	96.6	96.5	93.7	95.1		8.5
	R. equi ATCC 33701	92.2	92.2	92.5	92.5	92.5	91.5	91.0	91.8	

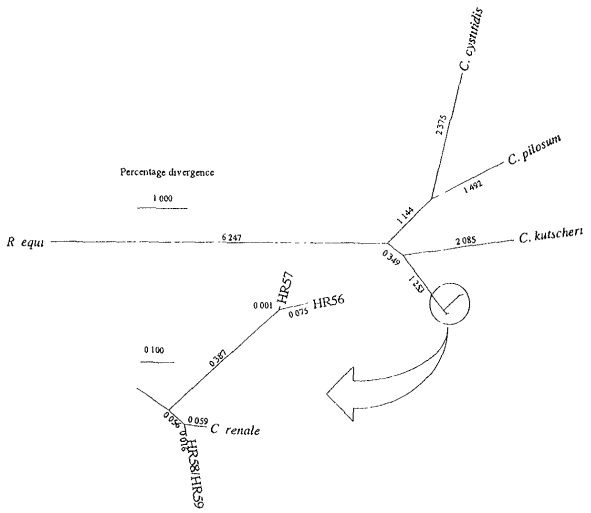

Fig. 2. Unrooted phylogenetic tree of 16S rRNA sequences showing the relationships among rat isolates (HR56, HR57, HR58 and HR59) and the type strains *Corynebacterium renale* (ATCC 19412^T), *C. pilosum* (ATCC 29592^T), *C. cystitidis* (ATCC 29593^T) and *C. kutscheri* (ATCC 15677^T). The sequences were also compared with that of *Rhodococcus equi* (ATCC 33701) as an outgroup. The tree was drawn by the Neighbor-joining method based on a comparison of ca. 1,289 nucleotides. Bar=1.000 (or 0.100 as a magnified scale) percentage divergence.

In this report, we did not specify any species specific sequence because only a few species of genus Corynebacterium were analyzed. However, as shown in Fig. 1, several regions were found uniquely in all *C. renale* strains. They might be group or species specific sequences in the 16S rRNA gene. Confirmation and further search for such sequences conserved in species are in progress. Detecting specific sequences to identify pathogenic agents is more useful in the case of slow growing organisms such as corynebacteria. Generally, the 16S rRNA sequence of a pathogenic organism provides much new information for research and treatment of the disease. For example, the urinary calculus in the laboratory rat infected with *C. renale* had never been known before, but in our studies these isolates were identified as *C. renale* [6]. Knowledge of the causative agent could help in determining appropriate treatment of the disease. Furthermore, the information provided in this report could be used for rapidly finding similar diseases in other animals, because it is possible that the organism could settle in the urinary systems of a wider range of mammals.

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