in 1×PCR buffer. Amplification was carried out in a thermal cycler (GeneAmp® PCR System 9700, PE Applied Biosystems) under the following conditions: Samples were first incubated at 95 C for 5 min, and then subjected to 40 cycles of 95 C for 30 sec, 50 C for 30 sec, 74 C for 60 sec, and a final extension at 74 C for 10 min. Amplified DNA was visualized on 1.5% agarose gels in TAE buffer (400 mM Tris, 10 mM EDTA, 200 mM acetic acid) after electrophoresis at 150 V for approximately 25 min, ethidium bromide staining, and illumination with UV light (33).

Results

Sequence Variation among the dnaJ, mip, *and 16S rDNA Sequences*

As shown in Figs. 1 and 2, *dnaJ* and *mip* nucleotide sequences from the *L. pneumophila* serogroups were analyzed by the neighbor-joining method. The *dnaJ* gene variation among the 15 serogroups ranged from 88.9 to 99.0%, while *mip* gene variation ranged from 95.1 to 100%. In particular, *mip* gene sequences for serogroups 10 and 12, and serogroups 11, 13 and 14 were identical, while the corresponding *dnaJ* sequences had 98.8%, 98.2%, 96.1%, and 96.5% similarity.

The *dnaJ* sequences from serogroups 4 and 5, which belong to *L. pneumophila* subspecies *fraseri* and *pascullei*, respectively, were apparently different from the other *L. pneumophila* subspecies *pneumophila* serogroups (Fig. 1). However, published *mip* gene sequences indicated that only serogroup 5, belonging to the subspecies *pascullei*, was different from the other *L. pneumophila* subspecies *pneumophila* serogroups. By *mip* gene sequence analysis, serogroup 4, belonging to subspecies *fraseri*, was within the group of remaining *L. pneumophila* subspecies *pneumophila* serogroups (Figs. 1 and 2). This indicated that the *dnaJ* gene was more useful in differentiating between *L. pneumophila* serogroups than the *mip* gene.

The *dnaJ* and 16S rDNA sequences from a number of *Legionella* species are compared in Table 3. The *dnaJ* sequences were more variable than the 16S rDNA sequences. Thus, our results suggest that the *dnaJ* gene is a useful tool for species identification of members of the genus *Legionella*.

Primer Specificity

To develop a rapid identification and detection method for *L. pneumophila* serogroups and members of the *Legionella* genus, we evaluated the W 19 (*dnaJ*) and W 3 (*mip*) primer sets for *L. pneumophila* and the W 8 (16S rDNA) primer set for genus *Legionella* members. As shown in Table 1, the DNA template from 15 *L. pneumophila* serogroups was subjected to PCR using the W 19 (*dnaJ*) and W 3 (*mip*) primer sets. The 285 bp W 19 (*dnaJ*) and 597 bp W 3 (*mip*) amplicons are shown in Fig. 3. Other *Legionella* species and other water-borne pathogens, such as *Acinetobacter calcoaceticus* GIFU 11962^T, *Burkholderia cepacia* GTC13^T, *Chryseobacterium meningosepticum* GTC8^T, *Escherichia coli* GTC 503^T, *Pseudomonas aeruginosa* GTC2^T, and *Sphingobacterium spiritivorum* GTC120^T, were not amplified by the species-specific W 19 (*dnaJ*) and W 3 (*mip*) primer sets (Table 1).

The W 8 (16S rDNA) primer set was used to detect *Legionella* genus members. In this study, we used 51 *Legionella* strains, including 38 species and 2 subspecies of *Legionella*, as well as 6 species from other genera as targeting species. As shown in Table 1, the W 8 (16S rDNA) primer set specifically amplified most members of the *Legionella* genus (Fig. 3). *Acinetobacter calcoaceticus* GIFU11962^T, *Burkholderia cepacia* GTC13^T, *Chryseobacterium meningosepticum* GTC8^T, *Escherichia coli* GTC503^T, *Pseudomonas aeruginosa* GTC2^T, and *Sphingobacterium spiritivorum* GTC120^T are commonly found in water environment, and produced no amplified PCR product using the W 8 (16S rDNA) primer set.

When *Legionella* species were amplified using the W 8 (16S rDNA) primer set, the species *L. brunensis* GTC515^T, *L. cherrii* GTC374^T, *L. israelensis* GIFU 11367^T, *L. jamestowniensis* GIFU10741^T, *L. londiniensis* GTC741^T, *L. longbeachae* GIFU9245^T, *L. oakridgensis* GIFU10061^T, and *L. waltersii* GTC1737^T, could not be amplified. Analysis of the corresponding 16S rDNA sequences deposited in the GenBank showed that the eight species differed at the primer binding sites by 1–2 bases. Therefore, new 16S rDNA-specific primer sets were designed for these species: W 8 forward 1, 5′-AGCACGGACTAGTTTACTAG-3′ and W 8 reverse 1, 5′-TCCTCCCCAACAAAAGTG-3′ for *L. oakridgensis*; W 8 forward 2, 5′-AGCATGACCTAGCTTGCTAG-3′, for *L. longbeachae*; W 8 forward 3, 5′-AGCATGGTCCAGCTTGCTGA-3′, for *L. israelensis*; W 8 forward 5, 5′-AGCATGGTCTAGCTTGCTAG-3′, for *L. cherrii*; W 8 forward 6, 5′-AGCATTTTCTAGCTTGCTAG-3′, for *L. jamestowniensis* and *L. brunensis*; W 8 forward 7, 5′-CGGCGCATTCTGCTTGCTAG-3′, for *L. londiniensis*; and W 8 reverse 2, 5′-TTCGGGAACACTGATACAGGTG-3′, for *L. waltersii*. Use of these primers allowed the amplification of 16s rDNA from all species tested.

Primer Sensitivity

While the minimal DNA concentration detected by the W 3 (*mip*) primer set was 2.5 ng, the minimal concentration detected by the W 19 (*dnaJ*) primer set was 0.25

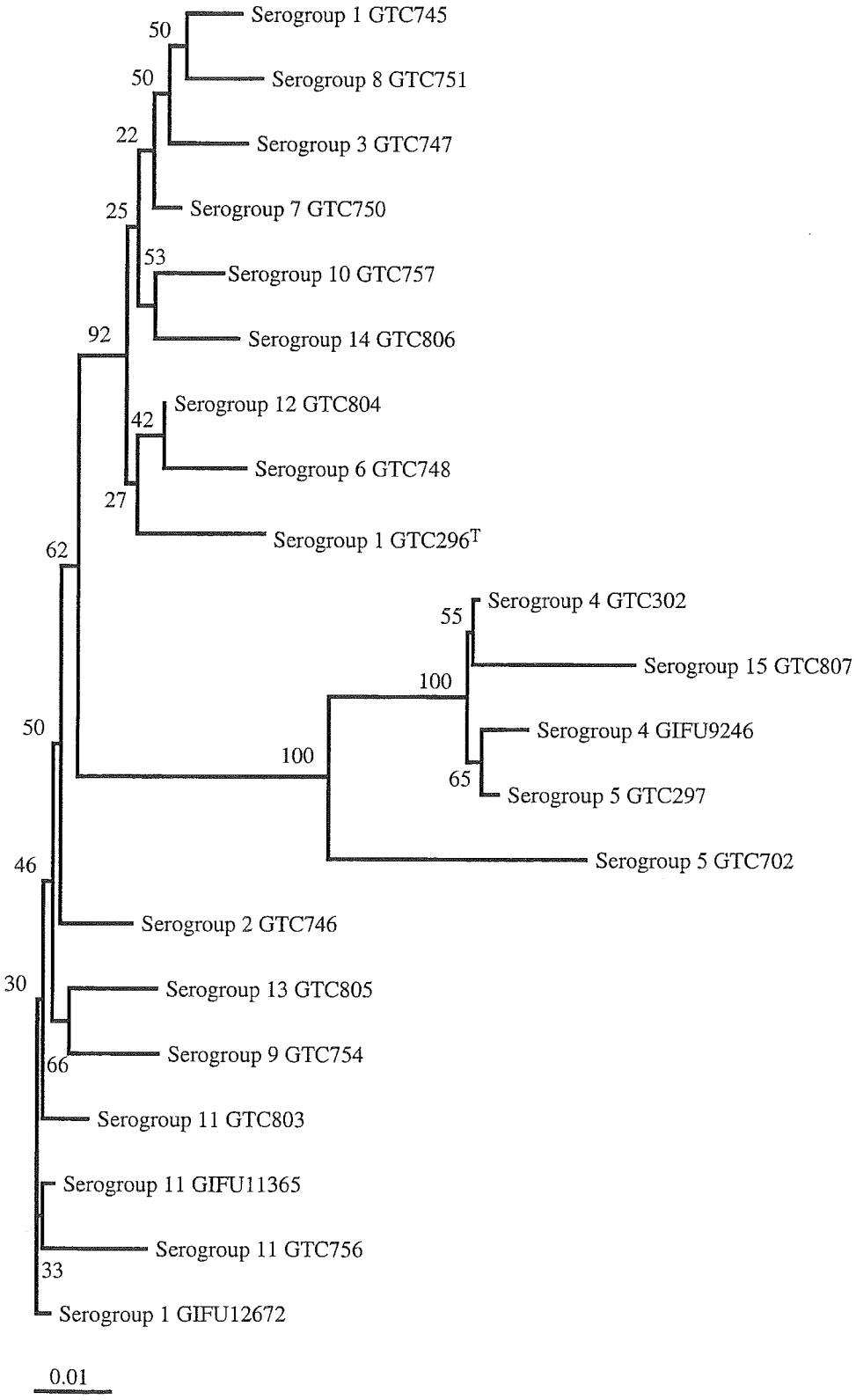

Fig. 1. Phylogenetic tree of *L. pneumophila* serogroups by *dnaJ* gene nucleotide sequencing. The sources of these strains are list in Table 1, except for the following strains: Serogroup 1 GIFU12672, Serogroup 11 GTC803, and Serogroup 11 GIFU11365 are *L. pneumophila* subsp. *pneumophila*. The numbers in this figure are bootstrap values.

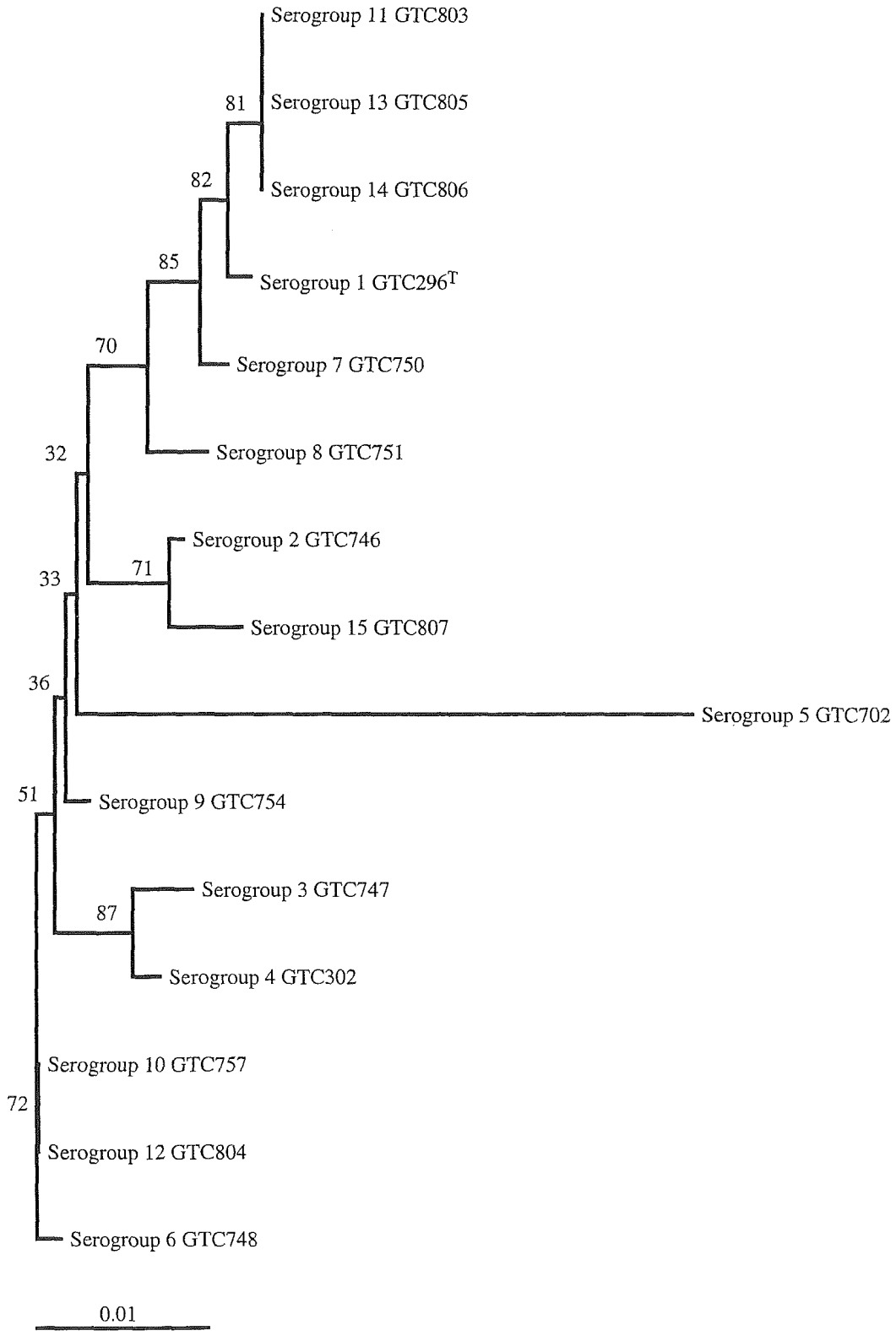

Fig. 2. Phylogenetic tree of *L. pneumophila* serogroups by *mip* gene nucleotide sequencing. The numbers in this figure are bootstrap values.

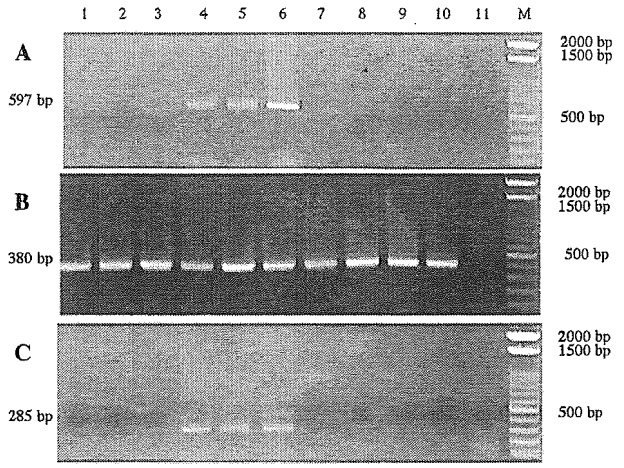

Fig. 3. Primer specificity for *Legionella* spp. A: W 3 (*mip*) primers; B: W 8 (16S rDNA) primers; C: W 19 (*dnaJ*) primers. Lane 1, *Legionella anisa* GTC464^T; Lane 2, *Legionella feeleii* GTC322^T; Lane 3, *Legionella parisiensis* GIFU10061^T; Lane 4, *Legionella pneumophila* subsp. *fraseri* GTC302^T; Lane 5, *Legionella pneumophila* subsp. *pneumophila* GTC296^T; Lane 6, *Legionella pneumophila* subsp. *pascullei* GTC702^T; Lane 7, *Legionella bozemanii* GIFU9141^T; Lane 8, *Legionella dumoffii* GIFU9244; Lane 9, *Legionella gomonii* GTC300^T; Lane 10, *Legionella micdadei* GTC299^T; Lane 11, *Pseudomonas aeruginosa* GTC2^T; Lane M, 100 bp ladder.

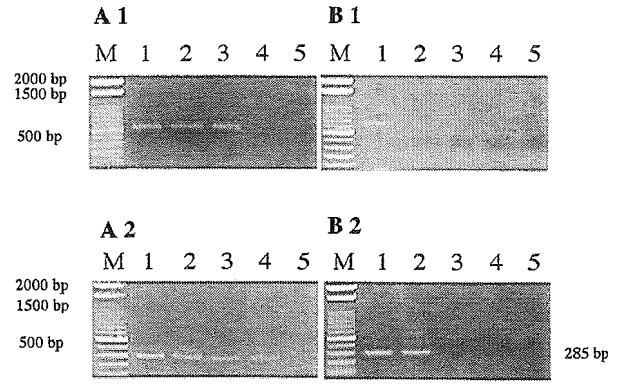

Fig. 4. Primer sensitivity for *Legionella pneumophila* GTC296^T. A: Purified DNA of *L. pneumophila* GTC296^T used as the template DNA. Lane M, 100 bp ladder; Lane 1, 5.0 ng DNA/PCR; Lane 2, 1.25 ng DNA/PCR; Lane 3, 2.5 ng DNA/PCR; Lane 4, 0.25 ng DNA/PCR; Lane 5, 0.025 ng DNA/PCR. A1: W 3 (*mip*) primer set; A 2: W 19 (*dnaJ*) primer set. B: Denatured *L. pneumophila* GTC296^T cells used as the template DNA. Lane M, 100 bp ladder; Lane 1, 10,000 cells/PCR; Lane 2, 1,000 cells/PCR; Lane 3, 100 cells/PCR; Lane 4, 10 cells/PCR; Lane 5, 1 cell/PCR. B 1: W 3 (*mip*) primer set; B 2: W 19 (*dnaJ*) primer set.

ng (Fig. 4). When live *L. pneumophila* GTC296^T cells were used as the template for PCR after 5-min boiling, the minimal colony-forming unit (CFU) in the PCR solution detected by the W 3 (*mip*) primer set was 1,000 compared to 100 CFU for the W 19 (*dnaJ*) primer set.

Discussion

Although 16S rDNA sequencing is one of the most common methods of bacterial identification, it cannot be used to differentiate between *L. pneumophila* serogroups 1 to 15 due to the very high degree of similarity between their 16s rDNA sequences.

The *dnaJ* gene is conserved in Eubacteria and Eucarya (2, 22). DnaJ, an Hsp 40 homolog, triggers the hydrolysis of DnaK-bound ATP, converting DnaK from its ATP-bound low-affinity state to its ADP-bound high-affinity state (16, 18). The *dnaJ* gene has been used as a highly effective tool to discriminate between species or subspecies. Bhattacharya (3) used part of the *dnaJ* gene to identify *Mycobacterium* species. In the present study, we first focused on the *dnaJ* gene analysis of *L. pneumophila* serogroups 1 to 15. We also assessed the use of the *mip* gene that has been used to discriminate between *L. longbeachae* and some other *Legionella* species (10, 19, 30) and between *L. pneumophila* serogroups (27).

The analysis of DNA sequences from the 15 *L. pneumophila* serogroups revealed that the variation between the *dnaJ* genes was higher than between the *mip* gene sequences (Table 2). The average gene variation between genus *Legionella* species was 94.9%±4.9% (from 88.9% to 99.2%) for the *dnaJ* gene, but 98.6%±3.5% (from 95.1% to 100.0%) for the *mip* gene. Thus, it appeared that *dnaJ* gene sequences would more easily discriminate between the *L. pneumophila* serogroups 1 to 15 than the *mip* gene, and we hypothesized that the *dnaJ* gene was a suitable target for PCR-based identification of legionellae. Therefore, we designed species-specific and genus-specific primer sets to allow the identification of *Legionella* species, and demonstrated that the *dnaJ* gene was indeed a better target compared to the *mip* gene.

We designed the specific primer sets W 3 to amplify a 597 bp fragment of the *L. pneumophila mip* gene, W 8 to amplify a 380 bp fragment of *Legionella* genus 16S rRNA, and W 19 to amplify a 285 bp fragment of the *L. pneumophila dnaJ* gene. The W 3 (*mip*) and W 19 (*dnaJ*) primer sets specifically amplified *L. pneumophila* sequences. Neither the *L. pneumophila*-specific primer set W 19 (*dnaJ*) nor W 3 (*mip*) (Fig. 3) amplified *Legionella* species other than *L. pneumophila*. The *Legionella* genus-specific primer set W 8 (16S rDNA) also worked for most pathogenic *Legionella* (Table 1).

The annealing temperatures between the two *Legionella* species-specific primer sets were different, being 50 C for the W 3 (*mip*) primer set compared to 57 C for the W 19 (*dnaJ*) primer set. When the annealing temperature

was decreased to 50 C for amplification using the W 19 (*dnaJ*) primer set, *L. pneumophila* serogroups 4 (GIFU 9246), 5 (GTC297), 14 (GTC806), and 15 (GTC807) were amplified as two amplicons.

Both the W 19 (*dnaJ*) and W 3 (*mip*) primer sets were designed to be *L. pneumophila*-specific. As shown in Fig. 4, the W 19 (*dnaJ*) primer set was more sensitive than the W 3 (*mip*) primer set, such that the W 3 (*mip*) primer set could detect 2.5 ng purified DNA and 1,000 CFU *L. pneumophila* GTC296^T in a PCR reaction, whereas the W 19 (*dnaJ*) primer set could detect 0.25 ng purified DNA and 100 CFU *L. pneumophila* GTC296^T.

In conclusion, the species-specific W 19 (*dnaJ*) primer is recommended for the detection of serogroups of *L. pneumophila*. For the detection of the members of the genus *Legionella*, nine different primer mixtures to amplify 16S rDNA gene is recommended.

Because of their poor biological activities, biochemical identification of the genus *Legionella* members is not possible. Molecular identification, such as, chromosomal DNA/DNA hybridization and 16S rDNA sequencing are only available methods. Chromosomal DNA/DNA hybridization is a demanding method and only used by taxonomists for classification. Identification by small ribosomal DNA sequencing is becoming a relatively easy method for microbiologists at research laboratories. However, sequencing is still difficult to introduce as a routine method at clinical laboratories. Furthermore, the variation of 16S rDNA among members of the genus *Legionella* is not variable enough for their species identification. Variation found in the *dnaJ* gene gives enough information for species identification of the genus *Legionella* members. In the near future, the OligoDNA microarray designed from *dnaJ* sequences would be a powerful candidate to identify species of the genus *Legionella* without sequencing.

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Editor-Communicated Paper

Quantitative Microarray-Based DNA-DNA Hybridization Assay for Measuring Genetic Distances among Bacterial Species and Its Application to the Identification of Family *Enterobacteriaceae*

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Abstract: **Quantitative DNA-DNA hybridization to measure the genetic distances among bacterial species is indispensable for taxonomical determination. In the current studies, we developed a method to determine bacterial DNA relatedness on a glass microarray. Reference DNAs representing a total 93 species of *Enterobacteriaceae* were arrayed on a glass microplate, and signal intensities were measured after 2 hr of hybridization with Cy3-labeled bacterial DNAs. All immobilized DNAs from members of the family *Enterobacteriaceae* were identified by this method except for DNAs from *Yersinia pseudotuberculosis* and *Y. pestis*. These results suggest that quantitative microarray hybridization could be an alternative to conventional DNA-DNA hybridization for measuring chromosome relatedness among bacterial species.**

Key words: **Microarrray, Species identification, *Enterobacteriaceae***

The sequences of nearly 6,000 taxonomically described species and bacteria have been reclassified according to their 16S ribosomal DNA (rDNA) sequences. As a result, ribosomal RNA sequencing has become a powerful method for determining the phylogenetic position of unidentified strains. All together, the sequences of more than 60,000 16S rDNAs covering more than 97% of the sequences of officially described species has been accumulated. This data has revealed that many established species have nearly identical rRNA sequences. Thus, given sequencing errors and variations among the five to eight copies of the 16S rDNA operon on a chromosome from a single species, it is practically impossible to identify an isolated strain by sequencing if the strain less than 2% different than a closely related established species. The Ad Hoc Committee of the International Systematic Bacteriology recommended that, if the 16S rDNA sequence variation between a new strain and an established species was less than 3%, chromosomal DNA-DNA hybridization data is needed to describe the new species (18). Since the last century, members of the family *Enterobacteriaceae* have been classified according to their biochemical traits (3). For this reason, genetically identical organisms, such as four species of genus *Shigella* and *Escherichia coli*, had been misclassified as different species (26, 27). Also, early in the 20th century, more than 2,000 *Salmonella* serovars had been classified as independent species, but they are now known to be a single species (33).

Chromosomal DNA-DNA hybridization can solve the problems of phenotypic identification and 16S rDNA sequencing. A bacterial species is genetically defined as a group of strains that shares more than 70%

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Abbreviations: GTC, Gifu Type Culture Collection; PCR, polymerase chain reaction; rDNA, ribosomal RNA gene; SSC, saline-trisodium-citrate buffer.

Table 1. List of 93 type strains immobilized on a microarray and three strongly cross-hybridized strains to reference DNAs

Array position		Type strain	Labeled strains strongly cross-hybridized to reference DNAs	
			GTC 109	GTC 118
1	A-1	*Arsenophonus nasoniae* GTC 1349	–	–
2	A-2	*Budvicia aquatica* GTC 1689	–	–
3	A-3	*Buttiauxella agrestis* GTC 1505	–	–
4	A-4	*Cedecea davisae* GTC 345	–	–
5	A-5	*Cedecea lapagei* GTC 346	–	–
6	A-6	*Cedecea neteri* GTC 1717	–	–
7	A-7	*Citrobacter freundii* GTC 108	–	–
8	A-8	*Citrobacter amalonaticus* GTC 1718	–	–
9	A-9	*Citrobacter braakii* GTC 1320	–	–
10	A-10	*Citrobacter farmeri* GTC 1319	–	–
11	A-11	*Citrobacter koseri* GTC 113	–	–
12	A-12	*Citrobacter rodentium* GTC 1323	–	–
13	B-1	*Citrobacter sedlakii* GTC 1322	–	–
14	B-2	*Citrobacter werkmanii* GTC 1321	–	–
15	B-3	*Edwardsiella tarda* GTC 112	–	–
16	B-4	*Edwardsiella hoshinae* GTC 337	–	–
17	B-5	*Edwardsiella ictaluri* GTC 1340	–	–
18	B-6	*Enterobacter cloacae* GTC 109	100	–
19	B-7	*Enterobacter aerogenes* GTC 121	–	–
20	B-8	*Enterobacter amnigenus* GTC 340	–	–
21	B-9	*Enterobacter asburiae* GTC 1719	–	–
22	B-10	*Enterobacter cancerogenus* GTC 1704	–	–
23	B-11	*Enterobacter dissolvens* GTC 1514	53 (16)[b]	–
24	B-12	*Enterobacter gergoviae* GTC 350	–	–
25	C-1	*Enterobacter hormaechei* GTC 1318	–	–
26	C-2	*Enterobacter intermedius* GTC 1678	–	–
27	C-3	*Enterobacter kobei* GTC 1531	–	–
28	C-4	*Enterobacter nimipressuralis* GTC 1513	–	–
29	C-5	*Enterobacter pyrinus* GTC 1516	–	–
30	C-6	*Enterobacter sakazakii* GTC 336	–	–
31	C-7	*Erwinia aphidicola* GTC 1688	–	–
32	C-8	*Escherichia coli* GTC 503	–	–
33	C-9	*Escherichia blattae* GTC 1342	–	–
34	C-10	*Escherichia fergusonii* GTC 1720	–	–
35	C-11	*Escherichia hermannii* GTC 347	–	–
36	C-12	*Hafnia alvei GTC* 104	–	–
37	D-1	*Klebsiella pneumoniae* GTC 107	–	–
38	D-2	*Raoultella ornithinolytica* GTC 490	–	–
39	D-3	*Klebsiella oxytoca* GTC 132	–	–
40	D-4	*Raoultella planticola* GTC 1343	–	–
41	D-5	*Raoultella terrigena* GTC 489	–	–
42	D-6	*Kluyvera ascorbata* GTC 338	–	–
43	D-7	*Kluyvera cryocrescens* GTC 339	–	–
44	D-8	*Kluyvera georgiana* GTC 1508	–	–
45	D-9	*Kluyvera cochleae* GTC 1506	–	–
46	D-10	*Leclercia adecarboxylata* GTC 1267	–	–
47	D-11	*Leminorella grimontii* GTC 1369	–	–
48	D-12	*Moellerella wisconsensis* GTC 1370	–	–
49	E-1	*Morganella morganii* GTC 1500	–	–
50	E-2	*Obesumbacterium proteus* GTC 1507	–	–
51	E-3	*Pantoea agglomerans* GTC 1261	–	–
52	E-4	*Pantoea ananatis* GTC 1705	–	–
53	E-5	*Pantoea citrea* GTC 1680	–	–

(Table 1. Continued)

Array position		Type strain	Labeled strains strongly cross-hybridized to reference DNAs	
			GTC 109	GTC 118
54	E-6	*Pantoea dispersa* GTC 1472	–	–
55	E-7	*Pantoea punctata* GTC 1511	–	–
56	E-8	*Pantoea stewartii* GTC 1666	–	–
57	E-9	*Pantoea terrea* GTC 1512	–	–
58	E-10	*Pragia fontium* GTC 1557	–	–
59	E-11	*Photorhabdus luminescens* GTC 1341	–	–
60	E-12	*Proteus vulgaris* GTC 134	–	–
61	F-1	*Proteus inconstans* GTC 1502	–	–
62	F-2	*Proteus mirabilis* GTC 137	–	–
63	F-3	*Proteus penneri* GTC 349	–	–
64	F-4	*Providencia alcalifaciens* GTC 111	–	–
65	F-5	*Providencia heimbachae* GTC 1501	–	–
66	F-6	*Providencia rettgeri* GTC 1263	–	–
67	F-7	*Providencia rustigianii* GTC 1504	–	–
68	F-8	*Providencia stuartii* GTC 1722	–	–
69	F-9	*Rahnella aquatilis* GTC 1499	–	–
70	F-10	*Salmonella enterica* serovar *Choleraesuis* GTC 103[a]	–	–
71	F-11	*Serratia marcescens* GTC 135	–	–
72	F-12	*Serratia entomophila* GTC 1316	–	–
73	G-1	*Serratia ficaria* GTC 343	–	–
74	G-2	*Serratia grimesii* GTC 1268	–	–
75	G-3	*Serratia odorifera* GTC 1344	–	–
76	G-4	*Serratia plymuthica* GTC 351	–	–
77	G-5	*Serratia proteamaculans* GTC 1278	–	–
78	G-6	*Serratia rubidaea* GTC 136	–	–
79	G-7	*Serratia liquefaciens* GTC 323	–	–
80	G-8	*Tatumella ptyseos* GTC 344	–	–
81	G-9	*Trabulsiella guamensis* GTC 1379	–	–
82	G-10	*Xenorhabdus nematophilus* GTC 1515	–	–
83	G-11	*Yersinia bercovieri* GTC 1317	–	–
84	G-12	*Yersinia enterocolitica* GTC 127	–	–
85	H-1	*Yersinia frederiksenii* GTC 1264	–	–
86	H-2	*Yersinia intermedia* GTC 1723	–	–
87	H-3	*Yersinia kristensenii* GTC 1724	–	–
88	H-4	*Yersinia mollaretii* GTC 1690	–	–
89	H-5	*Yersinia pseudotuberculosis* GTC 118	–	100
90	H-6	*Yersinia rohdei* GTC 1315	–	–
91	H-7	*Yersinia ruckeri* GTC 1266	–	–
92	H-8	*Yokenella regensburgei* GTC 1377	–	–
93	H-9	*Yersinia pestis* GTC 3P417	–	87 (15)[b]
94	H-12	*Herring sperm* DNA	–	–

[a] Other *Salmonella* serovars and *Shigella* species were not spotted on a microarray.

[b] Values in parenthesis are range of variation.

No. 1 to 17, 31 to 46, 48 to 83, 85 to 88, and 91 to 93 did not cross-hybridize to reference DNAs.

similarity in chromosomal DNA under optimal experimental conditions (15, 31). Many chromosomal DNA-DNA hybridization methods to measure genetic relatedness among bacterial strains have been published and have been indispensable for describing new species. To measure chromosomal DNA similarity among bacterial strains, two methods have been used: free solution hybridization membrane filter hybridization and a hydroxyl apatite method (5, 13). However, these methods require a large amount of purified chromosomal DNA.

In previous studies, we introduced a 96-well microplate-based protocol to reduce the amount of chromosomal DNA needed for DNA-DNA hybridiza-

tion (7, 8, 17). In this technique, DNA is fixed to the surface of the microtiter wells by physical adsorption. The method does not require radioisotopes and requires only microgram level of DNA. However, the DNA must be highly purified because of the limited DNA binding capacity of the microplate. Also, binding of DNA to the microtiter well can be competed by contaminating polysaccharides. Therefore microgram quantities of highly purified DNA have been needed for the method (1, 2, 22).

DNA microarray techniques using synthesized DNA (11, 14, 24), PCR amplicons, or covalently attached presynthesized oligoDNAs or complimentary DNA (25, 26, 30) have become popular. These microarray technologies require only nanogram levels of DNA (23), an advantage that has been applied to species identification (4, 21, 32).

In the present study, we developed a new method to determine genetic relatedness among bacterial species that utilizes a chromosomal DNA microarray with several thousand immobilized chromosomal DNA fragments. We measured the similarity in chromosomal DNA between the members of the *Enterobacteriaceae* family. With this method, we correctly identified 93 species covering almost all of the genera in the members of this family.

Materials and Methods

Bacterial strains. Bacterial strains were obtained from Gifu Type Culture Collection (GTC). The reference strains from which DNA was extracted and immobilized on glass slides are listed in Table 1. A total of 92 strains were used for these studies.

Preparation of reference DNAs. Strains were cultured on heart infusion agar for 2 days. Sedimented cells (1 g wet cells) were resuspended in 10 ml saline-EDTA buffer (0.15 M NaCl/0.1 M EDTA · 2Na, pH 8.0), and then lysed with lysozyme (1 mg/ml) followed by sodium dodecyl sulfate (2%) for 10 min at 60 C. The lysate was treated with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol according as described previously (6). Ethanol precipitated DNA was resuspended in saline-EDTA buffer and treated with RNase A (50 μg/ml) at 37 C for 30 min and followed by proteinase K (50 μg/ml) treatment for 60 min at 55 C. The DNA was then purified using a standard phenol-chloroform-isoamyl alcohol method (8) followed by ethanol precipitation.

Immobilization of DNA. Four different DNA concentrations (100, 50, 25, and 12.5 ng/μl) were prepared for spotting on the glass slides. These solutions were heat-denatured with 3× Standard Saline-Citrate buffer, pH 7.2 and were spotted on poly-L-lysine-coated glass slides (Matsunami Glass Industries, Ltd.) using a GTMASS stamping system (Nippon Laser and Electronics Laboratories), and the glass slide was stored in a dry chamber at room temperature. Next, 20 μl of prehybridization solution (2× SSC, 5× Denhardt's solution (8), 0.1 mg/ml of denatured salmon sperm DNA, and 50% formamide) was added and the slide was covered with a cover slip. Slides were kept in a humid chamber and incubated for 30 min at 37 C. After the prehybridization, the slide was placed in 1× SSC, washed for 2 min, and then dried.

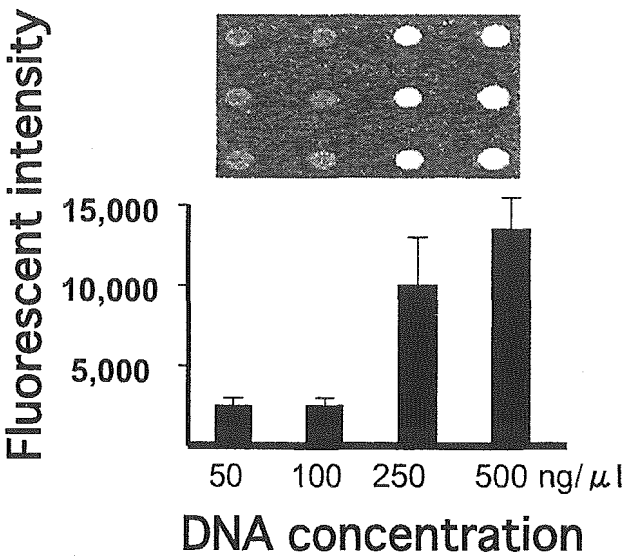

Fig. 1. Selection of an optimal DNA concentration for the microarray using a 2-hr hybridization. DNA from *E. coli* GTC 503 (500, 250, 100, and 50 ng/μl) was hybridized to spots of its own DNA for 2 hr at 45 C.

Fluorescence labeling of extracted DNA. Extracted genomic DNA (1 μg) was labeled and denatured using a Label IT Cy3 labeling kit (Mirrus) according to the manufacturer's recommendations.

DNA-DNA hybridization. Labeled DNA (10 μg/μl) solution was mixed with 20 μl of hybridization solution (2× SSC, 5× Denhardt's solution, 50% formamide, and 2.5% dextran sulfate). Twenty microliters of the solution was applied to DNA-immobilized glass and covered with a cover slip. Slides were kept in a humid chamber and hybridized for 30, 120, or 240 min at 35, 40, 45, 50, or 55 C. After hybridization, the slides were washed twice with 1× SSC buffer for 2 min and then centrifuged to remove excess buffer.

Data analysis. Hybridization slides were scanned with a ScanArray 4000 confocal laser scanner (GSI Lumonics). The average signal intensity of hybridized spots and local background signals were analyzed by QuantArray software (GSI Lumonics).

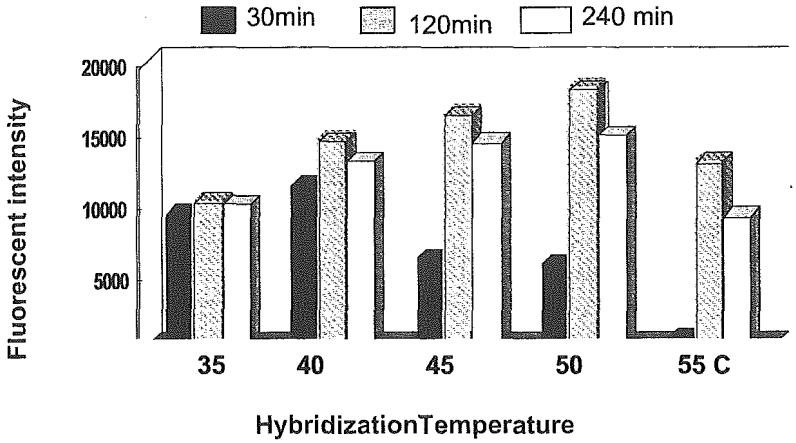

Fig. 2. Optimal hybridization temperature and time for the microarray. Labeled DNA from *E. coli* GTC 503 was hybridized to spots of its own DNA at different temperatures (35, 40, 45, 50, and 55 C) and hybridized for 30,120, or 240 min.

Results

Selection of the Optimal DNA Concentration for the Microarray

To find optimal DNA concentration for spotting, three different DNA concentrations were prepared. These spotted DNAs were hybridized with heat-denatured Cy3-labeled DNA from *E. coli* GTC 503 (Fig. 1). Two concentrations, 250 and 500 ng/μl at 50 C gave strong signal after 2 hr of hybridization. We selected 500 ng/μl as an optimal concentration for rapid identification.

Determination of the Optimal Time and Temperature for Hybridization

Five different hybridization temperatures (35, 40, 45, 50, and 55 C) were examined (Fig. 2). When 500 ng/μl of DNA was used for spotting, the hybridization signal at 30 min was too weak at 45, 50, and 60 C, but strong signals were detected after 120 min at approximately 45–50 C. Based on these results, we selected 2 hr hybridization at 50 C for the identification of *Enterobacteriaceae* on the microarray.

Fluorescent Signal Intensities of Hybridized Spots on a Glass Slide

DNA from the 92 species in the *Enterobacteriaceae* family were arrayed and hybridized with Cy3-labeled reference DNAs (Fig. 3). Although all species were labeled and hybridized to the reference DNA spots on the glass array, only two species cross-hybridized with other type species (Table 1). Specifically, DNA from *Enterobacter cloacae* GTC 109 cross-hybridized with DNA from *E. dissolvens* GTC 1514. The variance in their 16S rDNA sequences was 2.4%. Also, DNA from *Yersinia pseudotuberculosis* GTC118 cross-hybridized with DNA from *Y. pestis* GTC 3P 417. This is not surprising because, based on a DNA-DNA similarity higher than 80% and identical 16S rDNA sequences, the two species are considered genetically identical (3).

Genetic Relatedness among Species of the Genus Escherichia *and* Shigella

We next determined the interspecies similarity of members of the genus *Escherichia* and the genus *Shigella* (Table 2). Among members of the genus *Escherichia*, 16S rRNA similarity between *E. coli* and *E. fergusonii* was the most similar. The relatedness was also reflected in the similarities of their chromosomes as determined by microarray analysis. All four *Shigella* species were genetically identical to *E. coli* GTC 503 because their similarities with *E. coli* GTC 503 were higher than 70%.

DNAs of serovars of *Escherichia coli*, *Salmonella enterica*, and clinical strains of *Serratia marcescens* and *Klebsiella pneumoniae* were spotted on a glass slide, and their similarities with each species in the genus are shown in Fig. 4. Twelve strains of *Salmonella enterica* serovars made an independent cluster from 4 subspecies of *Salmonella enterica*. *Escherichia coli* and the 8 serovars and 4 species of genus *Shigella* made a single cluster and apparently different from 3 independent species of the genus *Escherichia*. *S. flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei* were confirmed to belong to *E. coli* even in this method. Four species of the genus *Escherichia* had different level of 16S rDNA sequence similarity to the type species. Especially *E. blattae* had only 96% 16S rDNA similarity to the type species, *E. coli* GTC 503 (as in Table 2 and Fig. 4).

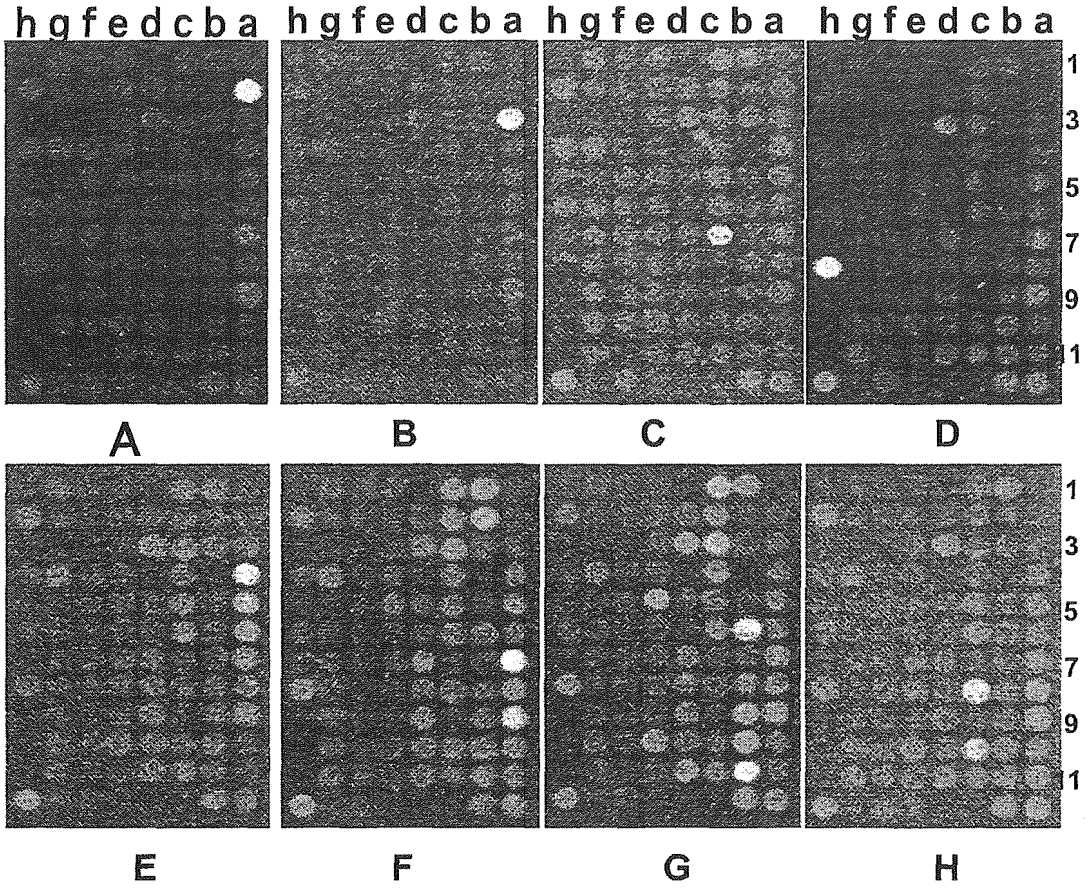

Fig. 3. Hybridization results for selected strains. The DNA spots on the microarray are described in Table 1. Labeled strain A, *Budvicia aquatica* GTC 1689 (position A2); labeled strain B, *Buttiauxella agrestis* GTC 1689 (position A3); labeled strain C, *Erwinia aphidicola* GTC 1688 (position C7); labeled strain D, *Yokenella regensburgi* GTC 1377 (position H8); labeled strain E, *Cedecea davisae* GTC 345 (position A4); labeled strain F, *Citrobacter freundii* GTC 108 (position A7); labeled strain G, *Enterobacter cloacae* TC 109 (position B6); labeled strain H, *Escherichia coli* GTC 503 (position C8).

Table 2. Similarities among species of genus *Escherichia* and *Shigella*

Type strain	16S rDNA homology to	Relative similarity values agaist labeled strain (calculated from spots on microarray)			
	E. coli GTC 503	GTC 503	GTC 1342	GTC 1720	GTC 347
E. coli GTC 503	100–98.6[a)]	100	–	55.2	–
E. blattae GTC 1342	96.2	–[b)]	100	–	–
E. fergusonii GTC 1720	99.6	37.0	–	100	–
E. hermannii GTC 347	97.4	–	31.6	–	100
S. dysenteriae GTC 786	99.8	95.0	–	45.3	–
S. flexneri GTC 780	99.3	98.0	–	46.2	–
S. boydii GTC 779	98.8	95.0	–	38.7	–
S. sonnei GTC 781	98.8	92.6	–	40.1	–

[a)] Variation among 7 copies of rDNA operon of *E. coli* GTC 503 was 1.4% (18 base difference).
[b)] –, signal lower than 30%.

Six clinical strains of *Serratia marcescens* made a close cluster to the type strains and their similarity values to the type species was higher than 80% as in Fig. 4. Established 8 species of the genus *Serratia* had DNA-DNA similarities to the type strain less than 45% with 96–98% 16S rDNA sequence similarities. Seven clinical strains of *Klebsiella pneumoniae* made identical cluster with the type strain with more than 99% 16S rDNA similarities. While 2 species of the genus *Klebsiella* and 3 species of the genus *Raoultella* were proved to be independent species with less than 45% DNA-DNA similarities by this microarray method but

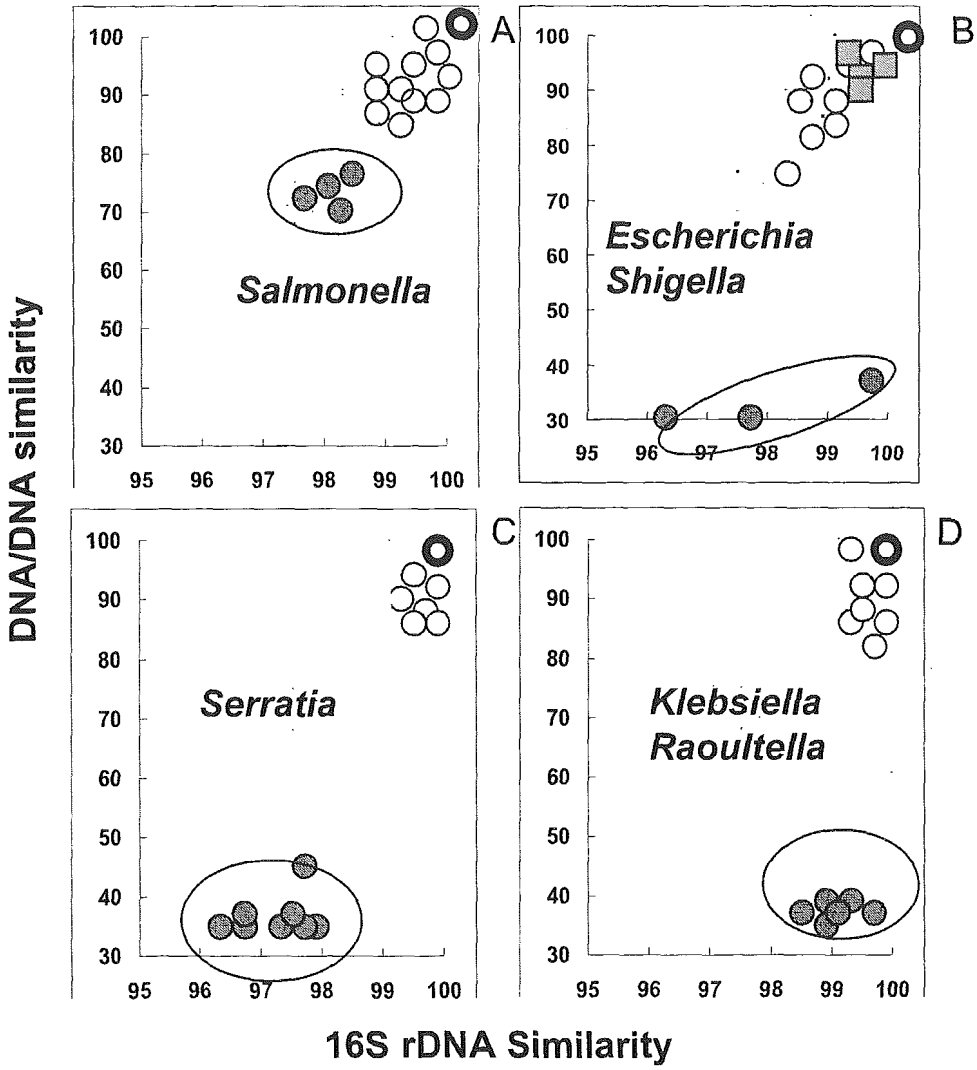

Fig. 4. DNA-DNA similarity of members of the genus *Shigella*, *Escherichia*, *Salmonella*, *Klebsiella*, and *Serratia* and species from each genera as determined by the microarray method and 16S rDNA sequence homology. Open bold circles, *S. enterica* subspecies *enterica* ATCC 13311 (A), *E. coli* GTC 503 (B), *S. marcescens* GTC135 (C), and *K. pneumoniae* GTC 107 (D). (A) Closed circle, four subspecies of *Salmonella enterica* (*S. enterica* subsp. *arizonae* NCTC 8297, *S. enterica* subsp. *indica* DSM 14848, *S. enterica* subsp. *bongori* CIP 8233); open circle, 11 members of *Salmonella enterica* subsp. *enterica* (including serovars Typhi, Choleraesuis, Patatyphi A, and Dublin). (B) Closed circle, three species of genus *Escherichia* (see Table 2); open circle, eight serovars of *Escherichia coli* (including O157 and O121); closed square, four species of the genus *Shigella* (see Table 2). (C) Closed circle, eight species of genus *Serratia* (see Table 1); open circle, six clinical strains of *S. marcescens*. (D) Closed circle, two species of the genus *Klebsiella* and three species and one clinical strain of the genus *Raoultella* (see Table 1); open circle, seven clinical strains of *K. pneumoniae*.

their 16S rDNA sequence were close to the type species because their 16S rDNA sequence similarities were higher than 98% as in Fig. 4.

Discussion

The taxonomy of the *Enterobacteriaceae* family was thought to have been well established by conventional biochemical methods, but genetic information has since shown that many species that were previously thought to be distinct are, in fact, genetically identical. For example, *Escherichia coli* and four species of the genus *Shigella* are taxonomically identical species (26), and four DNAs of the genus *Shigella* strongly hybridize to the reference DNA of *E. coli* even by this microarray method. Also, many earlier attempts at bacterial taxonomy classified many *Salmonella* serovars as independent species (10, 11), but they have now been proposed to be part of a single species (33). The potent human pathogen *Salmonella typhi* and the animal pathogen *Salmonella enteritidis* have been shown to be genetically identical and it has been proposed that they be classi-

fied as *Salmonella enterica* subspecies *enterica* (9, 10).

Ribosomal DNA sequence-based methods are currently widely accepted as rapid and accurate techniques for determining the taxonomic position of unknown bacteria. However, the interspecies distinction in a genus is almost impossible because of their high sequence similarities. For example, we found that all established species in the genus *Klebsiella* have more than 98% similarity in their 16S rDNAs.

We previously reported a highly quantitative fluorometric microtiter plate hybridization method that uses chromosomal DNA-DNA hybridization to differentiate species such as these that share almost identical 16S rDNA sequences (8). Our current microarray technique is an even more promising method because much less DNA is required. This new microarray technique utilizes thousands of individual DNAs printed in a high-density array on a glass slide. Using this microarray technique, we were able to hybridize the unknown labeled DNAs to DNAs from all 5,000 established bacterial species at once.

Generally, chromosomal hybridization has been used only in some taxonomy laboratories to determine bacterial species because highly purified DNA has been necessary (1, 2, 5, 6, 16, 28, 29). Modern taxonomic guidelines suggest that DNA-DNA similarity values among strains within an established species should be more than 70% under optimal conditions (12, 31). This indicates species are considered new when the strains share less than 70% similarity with established species.

Based on the G+C content of the DNAs from the *Enterobacteriaceae* family, the optimal temperature for DNA-DNA hybridization should be approximately 36 C in the 50% formamide-containing solution. However, we used 45 to 50 C as the hybridization temperature to increase the stringency of the experiment and to minimize cross-hybridization. By employing this stringent condition, most established species among *Enterobacteriaceae* were found to share less than 30% similarity on the microarray. Our final conditions for hybridizing to DNA from members of *Enterobacteriaceae* on the microarray included 0.5 mg/ml DNA concentration for spotting and hybridization for 120 min at 45 C in 2× SSC containing 50% formamide.

Values of chromosomal DNA similarity reported by multiple laboratories have differed even when the same strains have been used because each laboratory has used their own method for determining chromosome similarity. Thus, the Ad Hoc Committee of International Systematic Bacteriology had released recommendation that the DNA sequence for at least five housekeeping genes be used for taxonomy as alternative to using chromosomal DNA-DNA similarity (28). *rpoB*, *gyrB*, ITS, and *groEL* are candidate because they have larger sequence variation than the 16S rDNA sequence (17). The *rpoB* sequence of members of the *Enterobacteriaceae* family has been determined in detail and has proven to be promising for differentiating closely related species within this family *Enterobacteriaceae* (20). We have recently reported the variation in the *dnaJ* sequence among the genus *Legionella* is another candidate for classification (19). Our ongoing sequencing project for the members of family *Enterobacteriaceae* has, in fact, shown that the *dnaJ* sequence is much more variable than *rpoB* (our unpublished observations). However, we still do not have enough data to definitively establish a species using this gene. Until enough sequence data for these multilocus genes is accumulated and is used for setting new criteria for species, the chromosomal DNA-DNA similarity value will remain important for the identification and classification of bacteria.

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Note

Comparison of Loop-Mediated Isothermal Amplification (LAMP) and Conventional Culture for the Detection of *Legionella* Species in Hot Spring Water Samples in Japan

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We detected *Legionella* species in 125 samples of hot spring bath water from various places in Japan using the culture and LAMP methods, and compared the results of the 2 methods. *Legionella* spp. was detected in 40 samples in the culture test, and 38 of these (95.0%) were also positive in the LAMP test, showing a high rate of consistency. Of the 85 negative samples in the culture test, 38 samples (44.7%) were positive in the LAMP test. The positive rate in the LAMP test was higher than that in the current culture test ; the test procedure was simple, and judgments could be made in a few hours, showing that the LAMP method is useful for the rapid detection of *Legionella* spp.

Key words : *Legionella* species/Hot spring water/Loop-mediated isothermal amplification (LAMP).

Mass infection through bath water as the source of infection has recently occurred in Japan (National Institute of Infectious Diseases, 2003), and improved hygienic management of bath water is needed. For monitoring the bacterial counts of *Legionella* spp. in bath water, a culture test is necessary. However, 7-10 days are needed to obtain the test results because bacterial growth on selective media is very slow, which is a limitation of the culture method. Such time-consuming procedures cause delays in applying effective coutermeasures and increases the risk of infection. Furthermore, in the case of the public bath business, delays in inspection after bactericidal measures results in great economic losses due to the long-term suspension of business licenses. Therefore, the establishment of a rapid method to detect *Legionella* spp. in bath water that replaces the culture method is urgently required.

Detection of pathogenic microorganisms by gene amplification takes only a few hours (Coleman et al., 1996). Particularly, a new gene amplification method, loop-mediated isothermal amplification (LAMP), is superior in sensitivity and the ease of implementation to the current polymerase chain reaction (PCR) method (Horisaka et al., 2004 ; Inoue et al., 2004b), and basic investigations of the detection of *Legionella* spp. have progressed (Annaka et al., 2004).

In this study, we detected *Legionella* spp. in hot spring bath water from various places in Japan using both the culture and LAMP methods, and compared the results of the 2 methods.

Between March and November 2004, 125 samples (500 ml) of hot spring water from 20 prefectures were subjected to testing. Samples from local regions were transported in a refrigerator. As a rule, the

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samples were bath water, but some samples were collected from water sources and hot-water taps.

Each sample was centrifuged at 6,000 rpm for 30 min, and re-suspended in 5 ml to prepare 100-times-concentrated samples.

Following the new version of the *Guidelines for the Control of Legionelosis* (Building Management Education Center, 1999), 1 ml of 0.2 M HCl-KCl solution (pH 2.2) was added to 1 ml of the 100-times-concentrated sample in a small sterilized test tube. After sufficient stirring, the mixture was kept at room temperature for 15 min. The samples, 0.1 ml each, were dropped on WYO α agar medium (Eiken Chemical Co., Ltd., Tokyo) and GVPC α agar medium (Nikken Biomedical Laboratory Co., Kyoto), and smeared by using a Conradi stick (Toyo Kizai Co., Saitama). The plates were incubated at 37℃ for 7 d, and colonies showing characteristics of *Legionella* spp. on the media were counted. A few colonies were selected and smeared on blood agar medium and BCYE α agar medium (Nikken Biomedical Laboratory Co., Kyoto). The plates were subjected to pure culture at 37℃, and a cysteine auxotrophy test was performed at the same time. After culturing for 3 d, strains that did not grow on the blood agar plate, but grew on the BCYE α agar plate, were assumed to be *Legionella* spp., and confirmed to have gram-negative rods. For identification of the bacterial species, the latex aggregation reaction (Kanto Chemical Co., Tokyo), immune serum aggregation reaction (Denka Seiken Co. Ltd., Tokyo) and DNA-DNA hybridization (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo) were used. The detection limit of this culture method was 10 colony forming units (CFU)/100 ml.

For LAMP, 'Loopamp® *Legionella* Screening kit *E*' (Eiken Chemical Co., Ltd., Tokyo) was used according to the package insert. A 100-times-concentrated sample of 2 ml was centrifuged at 13,000×g for 10 min at 4℃, and the supernatant was removed, leaving about 40 μl. 50 μl of 'Extraction Solution for *Legionella*' was then added to the precipitate. After being mixed by using a vortex mixer, the mixture was heated at 95℃ for 15 min, rapidly cooled, and neutralized with 8 μl of 1 M Tris-HCl buffer (pH 7.0). The mixture was centrifuged again under the same conditions, and the supernatant was used as the nucleic acid extract. The nucleic acid extract, 5 μl, was added to 20 μl of LAMP reaction reagent 'Master Mix', and the amplification reaction was performed at 65℃ for 60 min with a Loopamp® real-time turbidity measurement system. When a characteristic increase in turbidity with amplification was observed within 1 h, the sample was judged positive for *Legionella* spp. The enzyme was inactivated by heating at 80℃ for 2 min. The lower limit of detection of this test was 10 cells/100 ml.

TABLE 1. Distribution of *Legionella* spp. colony counts, and comparison of detection results using the LAMP method.

Legionella counts (CFU/100ml)	Culture	LAMP	
		Positive	Negative
Less than10	85(68.0)*	38(50.0)	47(95.9)
10-40	18(14.4)	16(21.1)	2(4.1)
50-90	6(4.8)	6(7.9)	0(0)
100-490	6(4.8)	6(7.9)	0(0)
500-990	3(2.4)	3(3.9)	0(0)
1,000-4,900	6(4.8)	6(7.9)	0(0)
5,000-9,900	1(0.8)	1(1.3)	0(0)
Subtotal	40(32.0)	38(50.0)	2(4.1)
Total	125(100.0)	76(100.0)	49(100.0)

* : Number of samples(%)

The results of detection of *Legionella* spp. in 125 samples using the culture and LAMP methods are shown in TABLE 1. *Legionella* spp. was detected in 38 samples (30.4%) with both methods, and not detected in 47 samples (37.6%) with either method. The results of the 2 methods were consistent in 85 samples (68.0%). Thirty-eight samples (30.4%) were negative in the culture test, but positive in the LAMP test. In contrast, 2 samples (1.6%) were positive in the culture test, but negative in the LAMP test. There was a significant difference in the results of detection between the 2 methods ($p<0.01$). When *Legionella* spp. was detected with the LAMP method, the risk magnification, relative risk level and attributable risk level were 0.303, 0.526 and 0.288, respectively, and the detection rate was higher than that of the culture method.

The distribution of bacterial counts in 40 *Legionella*-positive samples in the culture test is shown in TABLE 1. The count was 10-40 CFU/100 ml in 18 samples (45.0%), and was the most frequent count level. The count was 50-90, 100-400 and 1,000-4,000 CFU/100 ml in 6 samples (15.0%). The count was less than 10 CFU/100 ml (undetectable) in 85 samples (68.0%), and 7 of these (8.2%) were judged negative because bacteria other than *Legionella* spp. and/or fungi grew faster on the selective media, and the growth of *Legionella* spp. could not be confirmed. Forty-nine isolates were subjected to identification, and 42 isolates (85.7%) were identified as *L. pneumophila*. *L. micdadei* and *L. gormanii* were also identified. On serotyping *L. pneumophila*, 17 isolates (34.7%) were typed group 1, which was

the most frequent type, and next most frequent type was group 6, in 8 isolates (16.3%). A relatively high number of isolates were typed group 3, group 4 and group 10. These identification results were similar to the isolation situation of the *Legionella* spp. in the soil of Japan, which the authors investigated previously (Furuhata et al., 2002).

The results of the detection of *Legionella* spp. in 125 samples of hot spring bath water using the LAMP method are shown with the distribution of bacterial counts measured with the culture method in TABLE 1. Of the 85 undetectable samples (less than 10 CFU/100 ml) in the culture test, 38 samples (44.7%) were positive in the LAMP test. Of the 18 samples in which 10-40 CFU/100 ml *Legionella* spp. was detected in the culture test, 16 samples (88.9%) were also positive in the LAMP test, but 2 samples (11.1%) were negative. The *Legionella* counts in these 2 LAMP test-negative samples were 10 and 30 CFU/100 ml, respectively, and *L. pneumophila* was isolated from both samples, but the serotypes were not clear. All of the 22 samples in which 50 CFU/100 ml or higher *Legionella* spp. was detected were positive in the LAMP test.

The biggest disadvantage of the generally used culture method for the detection of *Legionella* spp. is the length of time required for testing. Detection of nucleic acid has been widely used as a rapid test method (Savan et al., 2004). As for differences between the 2 methods, the culture method detects only viable bacteria capable of forming colonies on medium, while the gene test is capable of detecting not only viable bacteria but also dead bacteria and viable but non-culturable bacteria (VNC). The gene test is also capable of detecting bacteria using nucleic acid alone. Thus, it is difficult to obtain a complete consistency between the results of the culture test and the gene test.

Legionella spp. in 125 samples of hot spring bath water was detected using the current culture method and the recently developed LAMP method, and the results were compared. Of the 40 samples in which *Legionella* spp. was detected in the culture test, 38 samples (95.0%) were positive in the LAMP test, showing a high rate of consistency. Two samples that were positive in the culture test but negative in the LAMP test contained abundant insoluble materials, suggesting that amplification-inhibiting substances present in the hot spring bath water affected the test. Of the 85 undetectable samples in the culture test, 38 samples (44.7%) were positive in the LAMP test. Ng et al. (1997) reported that the presence of dead bacteria and VNC bacteria were the factors causing the PCR test positivity of the culture test-negative samples in a *Legionella* spp. test of cooling tower water. There may be similar factors involved in the testing of hot spring bath water, and positive readings may be obtained with the gene test even when disinfection with chlorine is performed properly. A positive result in the gene test may indicate the existence of *Legionella* spp. contamination in the past, and it indicates that there is the potential for actual contamination in the future. Accordingly, positivity in the gene test does not directly mean infectivity, unlike the culture test, and gene test-positive hot spring bath water does not necessarily become a source of infection. Sufficient consideration is necessary at this point when the gene test is employed for rapid testing of *Legionella* spp.

When oxidizing bactericidal agents such as chlorine are added, the nucleic acid of dead bacteria is degraded, and become undetectable, depending on the concentration of the bactericidal agent and exposure time (Inoue et al., 2004a). NASBA and TRC methods developed as rapid RNA amplification techniques have recently been investigated in many fields (Templeton et al., 2003 ; Masuda et al., 2004). Introduction of these techniques may bring the results of the gene test and culture test into closer accordance.

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IV. 研究成果の刊行物・別刷

5．レジオネラ症の臨床疫学的研究