JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1993, p. 824–830
0095-1137/93/040824-07$02.00/0

Vol. 31, No. 4

Polymerase Chain Reaction Using 16S rRNA Gene Sequences Distinguishes the Two Biovars of *Ureaplasma urealyticum*

J. A. ROBERTSON,[1]* A. VEKRIS,[2] C. BEBEAR,[2] AND G. W. STEMKE[3]

Department of Medical Microbiology and Infectious Diseases[1] and Department of Microbiology,[3] University of Alberta, Edmonton, Alberta T6G 2H7, Canada, and Departement de Bacteriologie, Universite de Bordeaux II, Bordeaux 33076, France[2]

Received 2 November 1992/Accepted 4 January 1993

Several fundamental phenotypic and genotypic differences have separated strains of the genital mycoplasma *Ureaplasma urealyticum* into two clusters or biovars. However, the lack of an easily performed and unambiguous test to discriminate between them has hampered investigation of the relationship between these biovars and disease. We determined the 16S rRNA nucleotide sequence of *U. urealyticum* 27, the serovar 3 standard and representative of the parvo biovar (serovars 1, 3, 6, and 14). This sequence was compared with the published sequence of *U. urealyticum* T960, which is the type strain and the serovar 8 standard and is representative of the T960 biovar which is composed of the 10 intervening serovars. Homology between the two sequences was 98.8%; differences were exploited to provide primers for biovar-specific polymerase chain reactions (PCRs). The results of these reactions placed all 14 serovar standard strains into the correct biovar. The PCRs were also applied to 10 cloned and 8 noncloned isolates that had been serotyped earlier. For 16 of them, we deduced their biovars from the serotyping data and then confirmed them by PCR. One unpredictable isolate and one nonserotypeable isolate were also classified as to biovar. Thus, we have developed a method for biotyping *U. urealyticum* that is applicable to both laboratory-adapted strains and wild-type isolates and that is appropriate for testing large numbers of clinical isolates. The amplification by the T960 biovar PCR protocol of DNAs from ureaplasmas of animals and certain *Mycoplasma* species suggested that the parvo biovar has diverged from the mainstream of the evolution of this clade.

The genus *Ureaplasma*, a genus within the family *Mycoplasmataceae*, class *Mollicutes*, has been defined by its ability to hydrolyze urea (29, 36). Subdivisions within this genus have been based largely on the host species and antigenic heterogeneity (9, 11, 13, 29). *Ureaplasma urealyticum* is the designation for all ureaplasmas isolated from humans. A 14-member serotyping scheme (24) has been widely accepted. The nonabsorbed, polyclonal antisera used for serotyping have been raised against whole ureaplasmal cells but are believed to detect differences in surface antigenicity. On the basis of several criteria, the serovars can be divided into two distinct clusters or biovars (2, 4, 8, 12, 17, 19, 21, 23, 35). One biovar is composed of 10 serovars, which are numbered 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13. Because the serovar 8 standard, strain T960, is the type strain, we gave this cluster that designation. The other biovar is composed of the four other serovars: 1, 3, 6, and 14. On the basis of the markedly smaller genomes of these strains (23), we recently designated them the parvo biovar (12). DNA-DNA hybridization has been determined for the first eight serotypes of *U. urealyticum* (2). The degree of hybridization among the first three of the four parvo biovar strains was 91 to 92%, whereas that among the first five of the T960 biovar strains was 69 to 97%. Hybridization between strains of the two clusters ranged from 38 to 60% (2). No hybridization data for the more recently identified serovars 9 through 14 are available. The apparently fundamental genomic division between the two *U. urealyticum* biovars is reflected by differences in other properties: restriction fragment length polymorphism (8, 19), polypeptide patterns from one- and two-dimensional gel electrophoresis (17, 35), sensitivity to Mn^{2+} (21), and enzyme profiles (4).

Woese and coworkers have employed comparisons of 16S rRNA (small subunit) gene sequences to describe the phylogenetic organization of prokaryotes (39), including members of the class *Mollicutes* (38). Since the 16S rRNA nucleotide sequence of *U. urealyticum* T960 had been determined previously (38), we needed only to determine the sequence for a representative of the parvo biovar to gain a better understanding of the relationship between the two groups of strains. We chose the serovar 3 standard to represent the parvo biovar because this is the most commonly identified determinant of this cluster (3, 18, 22, 28). Differences between the two sequences allowed us to develop biovar-specific polymerase chain reactions (PCRs).

MATERIALS AND METHODS

Sources of microorganisms: *Ureaplasma* species. Colonies of all ureaplasmas gave a characteristic appearance on agar and a positive urease spot test (27). The *U. urealyticum* serovar 3 standard strain (U3) had been isolated as strain 27 from a patient with nongonococcal urethritis by D. K. Ford, who had demonstrated its antigenic distinctiveness (6); F. T. Black then cloned it three times and used it as the serovar 3 standard in his eight-member serotyping scheme (1) before giving it to our source, D. Taylor-Robinson, Harrow, England. Thus, this strain is a progenitor of strain ATCC 27815. The sources of the other serovar standard strains have been described elsewhere (23). Strain T960 (U8) was the progenitor of strains NCTC 10177 and ATCC 27618. The identities and sources of the other laboratory-adapted *Ureaplasma* species and members of the class *Mollicutes* that we examined are indicated in Table 2. The serovars of laboratory-

* Corresponding author.

TABLE 1. The serotypes and deduced biovars of serovar standard strains and wild-type isolates of *U. urealyticum* examined by the biovar-specific PCRs

Strain[a]	Serotype(s)[b]	Deduced biovar[c]	PCR result for biovar	
			parvo	T960
Serovar standard strains				
7	1	parvo	+	−
23	2	T960	−	+
27	3	parvo	+	−
58	4	T960	−	+
354 (NIH)	5	T960	−	+
Pi	6	parvo	+	−
Co	7	T960	−	+
T960 (CX8)	8	T960	−	+
Vancouver	9	T960	−	+
Western	10	T960	−	+
K2	11	T960	−	+
U24	12	T960	−	+
U38	13	T960	−	+
U26	14	parvo	+	−
Wild-type isolates				
T444/77	4, 7, 10, 11, 12, 13	T960	−	+
T154/78	10, 11, 13	T960	−	+
T223/78	4, 9, 10, 11, 13	T960	−	+
T388/78	2, 9	T960	−	+
T325/80	3	parvo	+	−
T522/80	No type obtained	NA[d]	−	+
T2477/80	3, 10, 11, 13	parvo	+	−
TJR65	4, 11	T960	−	+
TSH	2, 9, 13	T960	−	+
TSF	10	T960	−	+
RH507	13	T960	−	+
RH638	11, 14	parvo	+	−
RH774	1, 2, 3, 4, 8, 11, 12, 13, 14	NA	+	−
RH1261	2, 4, 6, 11, 12, 13	T960	−	+
RH1299	1, 2, 9, 12, 13	T960	−	+
RH1336	4, 11, 12, 13	T960	−	+
RH1396	2, 3, 6, 11, 12, 13, 14	parvo	+	−
RH2057	1, 11	parvo	+	−

[a] Strains prefixed with T were tetracycline-resistant strains that had been cloned three times by a broth dilution method (26); strains prefixed by RH, from tissues of abortion (21), had been passaged twice but not further purified.
[b] Serotype(s) was determined by a colony epifluorescence test (31).
[c] Biovar was deduced as follows. Serovars 1, 3, 6, and 14 were of the parvo biovar, and the other serovars were of the T960 biovar. For strains which had reacted with antisera to serovars of both biovars, we used the following criteria: the majority representation of serovars numbered 1 to 10 determined the biovar. If none of these serovars had been detected, serovar 14 was considered more dominant than any one of 11, 12, or 13.
[d] NA, not applicable.

TABLE 2. The identities and sources of members of the class examined by *U. urealyticum* biovar-specific PCRs

Species	Strain designation	Source	Reaction with primer	
			PCR-U3	PCR-U8
Ureaplasma diversum	2065-B	Cow[a]	−	+
Ureaplasma gallorale	D6-1	Chicken[b]	−	+
Ureaplasma felinum	FT2B	Cat[b]	−	+
Ureaplasma sp.	D6P-C	Dog[b]	−	+
Ureaplasma sp.	1655	Sheep[c]	−	+
Ureaplasma sp.	CH33	Monkey[b]	−	+
Mycoplasma pneumoniae	15531	Human[d]	−	+
Mycoplasma fermentans	Incognitus	Cell culture[e]	−	+
Mycoplasma hominis	14027	Human[d]	−	−
Mycoplasma arginini	23838	Mouse[d]	−	−
Mycoplasma hyopneumoniae	25934	Pig[d]	−	−
Mycoplasma flocculare	27716	Pig[d]	−	−
Mycoplasma hyorhinis	17981	Pig[d]	−	−
Acholeplasma laidlawii	B	Sewage[f]	−	−
Acholeplasma axanthum	25176	Murine cell culture[d]	−	−
Acholeplasma modicum	NCTC 10134	Bovine cell culture[a]	−	−
Spiroplasma citri	Maroc	Citrus plant[g]	−	−

[a] E. V. Langford and B. Kingscote, Animal Diseases Research Laboratory, Lethbridge, Alberta, Canada.
[b] K. Koshimizu, University of Tokyo, Tokyo, Japan.
[c] L. Ruhnke, Ontario Ministry of Agriculture and Food, Guelph, Ontario, Canada.
[d] American Type Culture Collection, Rockville, Md.
[e] S.-C. Lo, Armed Forces Institute of Pathology, Washington, D.C.
[f] R. McElhaney, University of Alberta, Edmonton, Alberta, Canada.
[g] R. Whitcomb, U.S. Department of Agriculture, Beltsville, Md.

adapted strains and wild-type isolates of *U. urealyticum* (Tables 1 and 2) had been determined previously by using polyclonal antisera (24) with a colony epifluorescence test (32). Certain wild-type isolates had been purified three times by a broth dilution method (25).

Cultivation of members of the class *Mollicutes*. Ureaplasmas were grown in bromothymol blue broth (20); for the more-fastidious bovine and ovine strains, we doubled the serum and yeast extract supplementation (26). *Mycoplasma* and *Acholeplasma* species were cultivated in a standard Hayflick medium (10), except for *Mycoplasma pneumoniae* and the porcine mycoplasmas, which were grown in SP4 (37) and Friis (7) media, respectively. *Spiroplasma citri* was cultivated in C-3G medium (16). All cultures were incubated in air at 37°C, except for *S. citri*, which was cultivated at 30°C.

Preparation of DNA. Cells from broth cultures were harvested from late logarithmic growth by centrifugation at 20,000 × *g* for 25 min at 5°C in a type 19 rotor in a Beckman L5-50 ultracentrifuge. Cells were lysed by sodium dodecyl sulfate. DNA was isolated by treatment with DNase-free RNase and proteinase K and then by phenol, phenol-chloroform, and chloroform extraction. DNA was precipitated with sodium acetate and ethanol. The washed and dried pellets were hydrated in small volumes of Tris-EDTA buffer, and the concentration of DNA was estimated on ethidium bromide-containing agarose.

Vector, host, and cloning procedures. Vector, host, and cloning procedures were obtained from Stratagene, La Jolla, Calif. (30). DNA from *U. urealyticum* 12738 was partially hydrolyzed with *Eco*RI according to the manufacturer's directions. The resulting digest was used with the lambda ZAP II-*Eco*RI cloning kit to produce a recombinant library. Recombinant plaques in the *Escherichia coli* XL1-Blue host were transferred to nitrocellulose membranes, and the membranes were exposed to alkaline lysis and then probed with plasmid pR136 containing a *S. citri* 16S rRNA gene (14) to detect homologous sequences in these recombinants. Selected recombinant phage were purified three times. Recombinant 28a gave a consistently strong hybridization signal and was selected for further study. The pBluescript plasmid was excised from lambda ZAP II according to the Stratagene

protocol and was used for large-batch-plasmid preparation, and its DNA was prepared by standard methods for sequencing.

Sequencing. Although pBluescript is a phagemid that allows rescue of single-stranded target DNA, the double-stranded plasmid form allowed us to sequence from both strands without subcloning. We used the double-stranded DNA sequencing protocol of a dideoxy T7 polymerase kit (Pharmacia, Uppsala, Sweden) with ^{35}S-dATP. The resulting reactions were resolved in 6% acrylamide–6 M urea gels as described previously (31). The initial sequencing primers were the universal 16S primers described by Lane et al. (15); the sequences so obtained were extended by being primed with oligonucleotides based on the extremes of these sequences. Some of these had been employed earlier for the 16S rRNA sequencing of *Mycoplasma flocculare* (31). Radioautographs were read with a light box and the DNA Parrot DP100-PC digital reading device (T&t Research, Etobicoke, Ontario, Canada) to yield computer-readable files.

Data analysis. Files of strain 27 sequences were ordered relative to the 16S rRNA sequence of *U. urealyticum* T960 (38) by using the National Institutes of Health-DCRT analysis program of M. Kanehisa (IBM PC version). The PC GENE (IntelliGenetics, Inc., Mountain View, Calif.) program Clustal was used to align multiple sequences, including those obtained from the GenBank data base. The sequence of the *U. urealyticum* serovar 3 standard 16S rRNA gene was based on both strands of DNA.

PCRs. PCRs were with primers based on 16S rRNA gene sequences. The first PCR (5) was with a modified buffer (see below) with well-conserved 5′ and 3′ end primers to confirm that the samples contained DNA from the class *Mollicutes* or other organisms of gram-positive lineage. The primers were P1 (AGAGTTTGATCCTGGCTCAGGA) and P6 (GGTAGG GATACCTTGTTACGACT), the annealing temperature was 54°C, and the product was ca. 1,500 bp. The primers for the biovar-specific PCRs were developed during the present study. The specific oligonucleotide sequences for the top primers were as follows: U3, TAGAAGTCGCTCTTTGT GG; U8, GAAGATGTAGAAAGTCGCGTTTGC. Primer 6 (P6 above), the common negative-strand oligonucleotide primer for the mollicutes probe (5), also served for both biovar-specific reactions. The primer pair U3 and P6 is designated PCR-U3, and the primer pair U8 and P6 is designated PCR-U8. The amplified 16S fragments of both biovar-specific PCRs were ~1,300 bp. The 100-μl PCR mixture was composed of 2.5 μl of target DNA, 2 U of *Taq* DNA polymerase (Boehringer Mannheim), 0.07 M Tris (pH 8.8), 4 mM $MgCl_2$, and 0.1% Triton X-100. Each deoxyribonucleotide triphosphate was at a final concentration of 100 μM. Thermocycling was as follows: a single cycle of 95°C for 5 min followed by 30 cycles each of 95°C for 30 s, 58°C for 30 s, and 72°C for 2 min and 30 s. DNA was precipitated in 95% ethanol, rehydrated in Tris-EDTA buffer, resolved by electrophoresis in a 1% agarose gel at 100 V, and visualized by staining with ethidium bromide. The gels were photographed with appropriate filtering. Fragment sizes were determined relative to GelMarker II (Research Genetics, Huntsville, Ala.) molecular size markers. The products of the PCR amplification of strain 27 and T960 DNA were further identified by hybridization of Southern transfers with an internal, well-conserved, 16S rRNA probe; the products of other DNAs were identified by product size.

Nucleotide sequence accession numbers. The nucleotide sequence described below has been deposited in the GenBank data base and is available under accession number L08642; the GenBank accession number for *U. urealyticum* T960 is M23935.

RESULTS

The 16s rRNA gene sequences of U3 and U8 are shown in Fig. 1. The *U. urealyticum* 27 (U3) sequence was 1,529 nucleotides in length (Fig. 1); only the first 1,455 nucleotides of the *U. urealyticum* T960 (U8) sequence had been determined previously (38). Over the length of these two sequences where alignment was possible, U3 was 2 nucleotides longer than U8. Excluding the 4 undetermined nucleotides in the available sequence of U8, all but 17 nucleotides of the U3 and U8 sequences were identical. Thus, the degree of homology between the two representative strains of *U. urealyticum* was about 98.8%. Most differences occurred early in the sequences, especially between positions 185 and 285; some of these differences were exploited to develop biovar-specific PCRs.

The DNA from each sample was subjected to three PCRs. The first PCR, with primers P1 and P6, generated a nearly complete 16S sequence and established that the test sample contained an adequate amount of appropriate DNA. The two other PCRs, with primer pairs PCR-U3 and PCR-U8, were based on biovar-specific differences that we had identified in the two representative strains (Fig. 1). The specific oligonucleotides used as PCR primers are described in Materials and Methods; their locations in the gene sequences are indicated in Fig. 1. The test based on PCR-U3 amplified DNA from the serovar 3 standard strain and also from the standard strains for serovars 1, 6, and 14, the other members of the parvo biovar. PCR-U3 did not amplify DNA from any of the 10 members of the T960 biovar; PCR-U8 gave the appropriate, opposite pattern of results (Table 1). For both tests, the size of the amplified, biovar-specific products, ca. 1,300 bp, was as anticipated from the determined sequences. Thus, all serovar standard strains fit the biovar cluster that had been deduced from the serovar determinations and would be predicted from the other biovar-specific criteria (see Introduction).

On this firm basis, we turned our attention to wild-type isolates. Such isolates tend to show more-complex serotyping patterns than do the serovar standard strains (33). From a bank of previously serotyped isolates, we selected a few strains that had reacted typically, i.e., with one or two antisera. However, most of the isolates that we selected had shown atypical serotyping patterns and thus had given us problems in data interpretation. One isolate (T522/80) had not reacted with any antisera, whereas others had reacted only with reagents for serotypes 11 to 14, determinants which we consider less definitive than the others. The remaining isolates that we chose for examination had reacted with multiple antisera; seven of them had reacted with antisera to strains of both biovars (e.g., RH774). Before biotyping these isolates by PCR, we used the serotyping data to predict their biovars. The predictions were based on our experience with serotyping; the guidelines which we used are described in footnote *c* to Table 1.

As with DNA from the serotype standards, each wild-type isolate was amplified by only one of the two biovar-specific PCRs. Of the 10 cloned isolates, 9 gave the predicted response. Because the 10th isolate from this study, T555/80, had not reacted with any antisera, no biovar prediction could be made; the PCRs placed this isolate in the parvo biovar. Of the eight noncloned isolates, seven had the predicted PCR

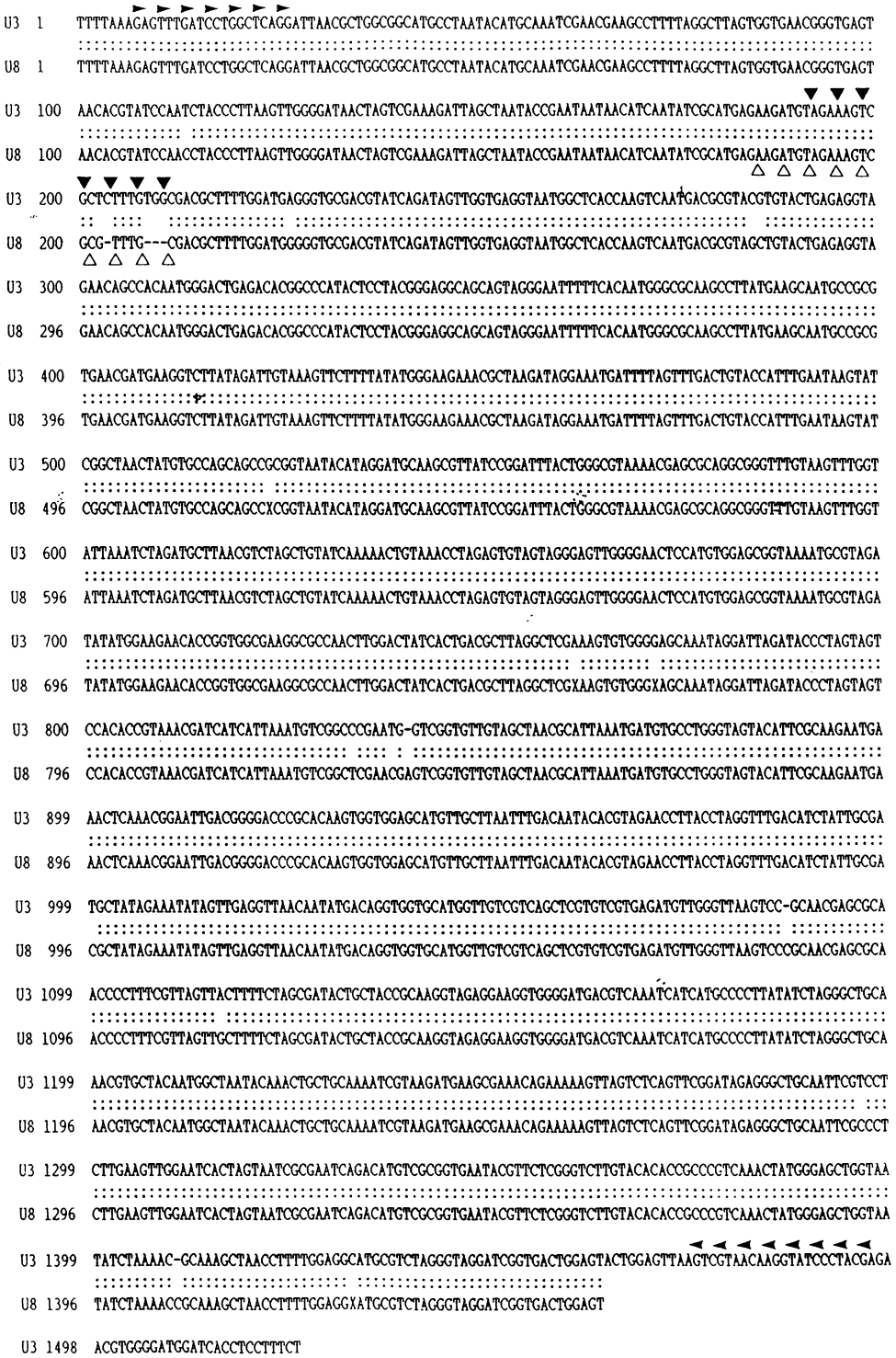

FIG. 1. The 16S rRNA sequence of *U. urealyticum* 27, the serovar 3 standard and representative of the parvo biovar, was aligned with the published sequence of *U. urealyticum* T960 (38), the type strain and serovar 8 standard (GenBank accession no. M23935 [38]) representing the T960 biovar. Identical (::), missing (---), and undetermined (X) nucleotides are indicated. The positions of the top primers for the mollicutes PCR, P1 (►), the parvo biovar PCR-U3 (▼), and the T960 biovar PCR-U8 (△) are indicated. Primer P6, the common bottom primer for all three PCRs (◄), is before position 1500.

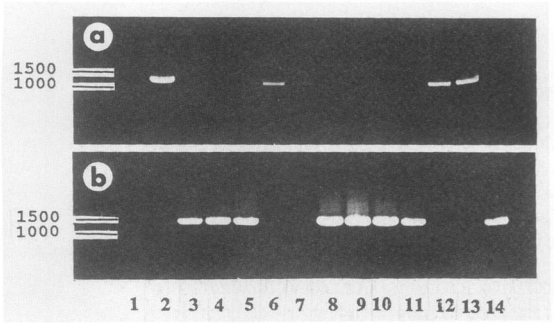

FIG. 2. Biovar determinations of selected serovar standard strains and cloned and noncloned clinical isolates of *U. urealyticum* by PCRs. (a) DNA amplification with PCR-U3; (b) DNA amplification with PCR-U8. Consecutive lanes contain barely distinguishable molecular size markers (in base pairs; lane 1) and the products of amplification of the following ureaplasmas (lanes 2 through 14): RH1396 (lane 2), RH1336 (lane 3), RH1299 (lane 4), RH1261 (lane 5), RH774 (lane 6), RH638 (lane 7), RH507 (lane 8), the serovar standards 11, 12, and 13 (lanes 9 through 11, respectively), and the isolates RH2057 (lane 12), T325/80 (lane 13), and T522/80 (lane 14). All products except RH638 can be readily distinguished.

results. The eighth isolate, RH774, was the most reactive that we have yet encountered; with repeated testing, it had given positive reactions with 9 of the set of 14 antisera. These included 3 of the 4 parvo biovar determinants and 6 of the 10 T960 biovar determinants. By our criteria, this isolate would be placed in the T960 cluster. However, we knew that the three other *U. urealyticum* isolates from tissues of the same patient had reacted with antiserum to serovar 6 and thus carried every determinant associated with the parvo biovar. On this basis, we excluded isolate RH774 from our predictions. Figure 2 shows the gels of the products of amplification for some ureaplasmas examined in this study. The multiply reactive isolate RH774 (lane 6) was amplified by PCR-U3 but not by PCR-U8 and thus belonged to the parvo biovar.

We tested DNA from representative members of the class *Mollicutes* with these PCRs. DNAs from the six ureaplasmas from animal hosts were amplified by PCR-U8 but not PCR-U3 (Table 2). DNAs from three other genera were also examined. *M. pneumoniae* and *Mycoplasma fermentans* DNAs gave the typical 1,300-bp product, but the other five strains of *Mycoplasma* species, the three *Acholeplasma* species, and *S. citri* were not amplified.

The sensitivity of amplification (i.e., the minimal amount of DNA giving a visible product) was determined to be 10 pg for PCR-U3 with U3.

DISCUSSION

Amplification of DNAs from the serotype standards of *U. urealyticum* has indicated that the differences in the variable regions of 16S rRNA genes that had been exploited for the PCR tests were consistent within each biovar (Table 1). Amplification of the 16 wild-type isolates according to the prediction based on the serovar(s) (Table 1) showed that the biovar-specific differences in sequences were not artifacts of laboratory adaptation and that this test is appropriate for biotyping clinical isolates. The ability to predict the biovar from serovar patterns suggests that serovars have some phylogenetic importance, albeit at a hierarchical level lower than that of biovar. None of the ureaplasmas that we examined was positive or negative for both biovar-specific tests (e.g., Fig. 2 and Table 1). Since the wild-type isolates examined in this study included the most problematic of hundreds that we have serotyped (e.g., see references 21 and 33), we do not expect to find ureaplasma strains of an intermediate biovar or of another distinct biovar in humans of the Western world.

In serotyping *U. urealyticum*, our greatest difficulties have been associated with the serovar 13 determinant. The initial problems occurred when we tried to assign isolates to biovar on the basis of manganese susceptibility. In all of many trials, the serovar 13 standard strain took an intermediate position between the strains of the two biovars (21, 34). Other parameters, namely, enzyme profiles (4) and restriction fragment length polymorphism (8), and now our biovar-specific PCR tests, placed the serovar 13 standard clearly into the T960 cluster. We have commonly found the serovar 13 determinant as a cross-reactant but rarely by itself, as in isolate RH507 (Table 1). Because of our problems with this determinant, we overrepresented it in our selection of test isolates (Table 1). Of the 11 wild-type isolates expressing that determinant, 8 were biotyped to the T960 cluster and 3 were biotyped to the parvo cluster. One explanation for our continuing difficulty with this determinant is that despite a titer of 2,560 in a metabolic inhibition test (24), the homologous antiserum has low specificity. At present, we can state only that in our hands, the serovar 13 determinant remains equivocal.

The obligatory nature of protein synthesis and the attendant requirement for functioning ribosomes have clearly constrained variation in 16S rRNA genes (38, 39). The 98.8% DNA sequence homology between the two *U. urealyticum* biovars (Fig. 1) attests to their close phylogenetic relationship. However, despite this similarity, their many common peptides (17, 35), and their enzymatic activities (4), differences of 17 nucleotides within the highly conserved 16S sequence are significant. Furthermore, as indicated in the Introduction, the degree of DNA hybridization among strains of the two biovars (2) is low. We are currently determining the 16S rRNA sequences of the serovar 1, 6, and 14 standard strains to establish the overall homogeneity-heterogeneity within the parvo biovar. Confirmation that these sequences are similarly distinct from those of the T960 cluster would support separation of the two biovars of *U. urealyticum* into separate species.

This study provides further evidence of the strength of 16S rRNA homology as a phylogenetic tool. Two observations are of particular interest in this regard. One is that PCR-U8 but not PCR-U3 amplified all ureaplasmas isolated from nonhuman sources (Table 2). These strains included four of the five named species of the genus; the fifth species, *Ureaplasma cati*, was not available for testing. The second observation is that PCR-U8 also amplified DNA from certain *Mycoplasma* species that we examined (Table 2), namely, *M. pneumoniae* and *M. fermentans*. On the basis of the work of Weisburg et al. (38), of all *Mycoplasma* species that we tested, these two species have the closest relationship to *U. urealyticum*. Thus, our data are consistent with evolutionary divergence as it has been postulated for members of the class *Mollicutes*. Taken together, these data suggest that the parvo biovar has diverged further from the mainstream of evolution than strains of the T960 biovar. Sequencing of the 16S rRNA genes of selected ureaplasmas would confirm or deny this hypothesis.

These 16S rRNA-based PCRs have met the long-standing need for a means of differentiating the two *U. urealyticum*

biovars. We now have the means to readily biotype not only the laboratory-adapted serovar standard strains but also the wild-type isolates of ureaplasmas isolated from humans, including those which have given equivocal serotyping results. We will apply these PCRs to isolates of *U. urealyticum* that have been obtained from well-characterized patients and appropriate control subjects in order to determine the relationship between biovar and disease.

ACKNOWLEDGMENTS

The serotyping was performed by J. Kakulphimp, and the sequencing was performed by L. Howard. R. Phan and M. Fillion performed the PCRs.

This study was supported by an operating grant from the Medical Research Council of Canada (to J.A.R.) and the National Science and Engineering Research Council (to G.W.S.), both of Ottawa, Ontario, Canada.

REFERENCES

1. **Black, F. T.** 1970. Serological methods for classification of human T-mycoplasmas, p. 407–411. *In* Fifth International Congress on Infectious Diseases, vol. 1. Wiener Medizinische Akademie, Vienna.
2. **Christiansen, C., F. T. Black, and E. A. Freundt.** 1981. Hybridization experiments with deoxyribonucleic acid from *Ureaplasma urealyticum* serovars I to VIII. Int. J. Syst. Bacteriol. **31**:259–262.
3. **Cracea, E., D. Botez, S. Constantinescu, and M. Georgescu-Braila.** 1982. Ureaplasma urealyticum serotypes isolated from cases of female sterility. Zentralbl. Bakteriol. Hyg. Abt. 1 Orig. A **252**:535–539.
4. **Davis, J. W., Jr., and I. Villaneuva.** 1990. Enzyme differences in serovar clusters of *Ureaplasma urealyticum*, *U. diversum*, and ovine ureaplasmas. Zentralbl. Bakteriol. Suppl. 20:661–665.
5. **Deng, S., C. Hiruki, J. A. Robertson, and G. W. Stemke.** 1991. Detection by PCR and differentiation by restriction fragment length polymorphism of *Acholeplasma*, *Spiroplasma*, *Mycoplasma*, and *Ureaplasma*, based upon 16S rRNA genes. PCR Methods Appl. **1**:202–204.
6. **Ford, D. K.** 1967. Relationships between mycoplasma and the etiology of nongonococcal urethritis and Reiter's syndrome. Ann. N. Y. Acad. Sci. **143**:501–504.
7. **Friis, N. F.** 1975. Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*. A survey. Nord. Veterinaermed. **27**:337–339.
8. **Harasawa, R., K. Dybvig, H. L. Watson, and G. H. Cassell.** 1991. Two genomic clusters among 14 serovars of *Ureaplasma urealyticum*. Syst. Appl. Microbiol. **14**:393–396.
9. **Harasawa, R., Y. Imada, M. Ito, K. Koshimizu, G. H. Cassell, and M. F. Barile.** 1990. *Ureaplasma felinum* sp. nov. and *Ureaplasma cati* sp. nov. isolated from the oral cavities of cats. Int. J. Syst. Bacteriol. **60**:45–51.
10. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl.):285–303.
11. **Howard, C. J., and R. N. Gourlay.** 1982. Proposal for a second species within the genus *Ureaplasma*, *Ureaplasma diversum* sp. nov. Int. J. Syst. Bacteriol. **32**:446–452.
12. **Kakulphimp, J., L. R. Finch, and J. A. Robertson.** 1991. Genome sizes of mammalian and avian ureaplasmas. Int. J. Syst. Bacteriol. **41**:326–327.
13. **Koshimizu, K., R. Harasawa, I.-J. Pan, H. Kotani, M. Ogata, E. B. Stephens, and M. F. Barile.** 1987. *Ureaplasma gallorale* sp. nov. from the oropharynx of chickens. Int. J. Syst. Bacteriol. **37**:333–338.
14. **Laigret, F., O. Grau, and J. M. Bove.** 1990. Comparison of 16S rDNA sequences of various mollicutes, p. 435–440. *In* G. Stanek, G. H. Cassell, J. G. Tully, and R. F. Whitcomb (ed.), Recent advances in mycoplasmology. Gustav Fischer Verlag, Stuttgart, Germany.
15. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic studies. Proc. Natl. Acad. Sci. USA **82**:6955–6985.
16. **Liao, C. H., and T. A. Chen.** 1977. Culture of corn stunt spiroplasma in simple medium. Phytopathology **67**:802–808.
17. **Mouches, C., D. Taylor-Robinson, L. Stipkovits, and J. M. Bove.** 1981. Comparison of human and animal ureaplasmas by one- and two-dimensional protein analysis on polyacrylamide slab gel. Ann. Microbiol. (Paris) **132B**:171–196.
18. **Naessens, A., W. Foulon, J. Breynaert, and S. Lauwers.** 1988. Serotypes of *Ureaplasma urealyticum* isolated from normal pregnant women and patients with pregnancy complications. J. Clin. Microbiol. **26**:319–322.
19. **Razin, S., R. Harasawa, and M. F. Barile.** 1983. Cleavage patterns of the mycoplasma chromosome, obtained by using restriction endonucleases, as indicators of genetic relatedness among strains. Int. J. Syst. Bacteriol. **33**:201–206.
20. **Robertson, J. A.** 1978. Bromothymol blue broth: improved medium for detection of *Ureaplasma urealyticum* (T-strain mycoplasma). J. Clin. Microbiol. **7**:127–132.
21. **Robertson, J. A., and M. C. Chen.** 1984. Effects of manganese on the growth and morphology of *Ureaplasma urealyticum*. J. Clin. Microbiol. **19**:857–864.
22. **Robertson, J. A., L. H. Honore, and G. W. Stemke.** 1986. Serotypes of *Ureaplasma urealyticum* in spontaneous abortion. Pediatr. Infect. Dis. J. **5**(Suppl.):S270–S272.
23. **Robertson, J. A., L. E. Pyle, G. W. Stemke, and L. R. Finch.** 1990. Human ureaplasmas show diverse genome size by pulsed-field electrophoresis. Nucleic Acids Res. **18**:1451–1455.
24. **Robertson, J. A., and G. W. Stemke.** 1982. Expanded serotyping scheme for *Ureaplasma urealyticum* strains isolated from humans. J. Clin. Microbiol. **9**:673–678.
25. **Robertson, J. A., G. W. Stemke, S. G. Maclellan, and D. E. Taylor.** 1988. Characterization of tetracycline-resistant strains of *Ureaplasma urealyticum*. J. Antimicrob. Chemother. **21**:319–332.
26. **Robertson, J. A., M. E. Stemler, and G. W. Stemke.** 1984. Growth characteristics of ureaplasmas from animals and human sources. Isr. J. Med. Sci. **20**:935–937.
27. **Shepard, M. C., and D. R. Howard.** 1970. Identification of "T" mycoplasmas in primary agar culture by means of a direct test for urease. Ann. N. Y. Acad. Sci. **174**:809–819.
28. **Shepard, M. C., and C. D. Lunceford.** 1978. Serological typing of *Ureaplasma urealyticum* isolates from urethritis patients by an agar growth inhibition method. J. Clin. Microbiol. **8**:566–574.
29. **Shepard, M. C., C. D. Lunceford, D. K. Ford, R. H. Purcell, D. Taylor-Robinson, S. Razin, and F. T. Black.** 1974. *Ureaplasma urealyticum* gen. nov., sp. nov.: proposed nomenclature for the human T (T-strain) mycoplasmas. Int. J. Syst. Bacteriol. **24**: 160–171.
30. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. λ Zap: a bacteriophage expression vector with *in vivo* excision properties. Nucleic Acids Res. **16**:7583–7600.
31. **Stemke, G. W., F. Laigret, O. Grau, and J. M. Bove.** 1992. Phylogenetic relationships of three porcine mycoplasmas, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis*, and complete 16S rRNA sequence of *M. flocculare*. Int. J. Syst. Bacteriol. **42**:220–225.
32. **Stemke, G. W., and J. A. Robertson.** 1981. Modified colony indirect epifluorescence test for serotyping *Ureaplasma urealyticum* and an adaptation to detect common antigenic specificity. J. Clin. Microbiol. **14**:582–584.
33. **Stemke, G. W., and J. A. Robertson.** 1985. Problems associated with serotyping strains of *Ureaplasma urealyticum*. Diagn. Microbiol. Infect. Dis. **31**:311–320.
34. **Stemler, M. E., G. W. Stemke, and J. A. Robertson.** 1987. ATP measurements obtained by luminometry provide a rapid estimation of *Ureaplasma urealyticum* growth. J. Clin. Microbiol. **25**:427–429.
35. **Swenson, C. E., J. VanHamont, and B. S. Dunbar.** 1983. Specific protein differences among strains of *Ureaplasma urealyticum* as determined by two-dimensional gel electrophoresis and a sensitive silver stain. Int. J. Syst. Bacteriol. **33**:417–421.

36. **Taylor-Robinson, D., and N. R. Gourlay.** 1984. Genus II. *Ureaplasma*, p. 770–775. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
37. **Tully, J., R. F. Whitcomb, H. F. Clark, and D. L. Williamson.** 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. Science **195:**892–894.
38. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171:**6455–6476.
39. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.