Phylogenetic analysis of *Ureaplasma urealyticum* – support for the establishment of a new species, *Ureaplasma parvum*

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In this study, the phylogenetic relationships between the two biovars and 14 serovars of *Ureaplasma urealyticum* were studied using the sequences of four different genes or genetic regions, namely: 16S rRNA genes; 16S–23S rRNA gene spacer regions; urease gene subunits *ureA*, *ureB*, partial *ureC* and adjoining regions upstream of *ureA*, *ureA–ureB* spacer and *ureB–ureC* spacer; the 5′-ends of the multiple-banded antigen (MBA) genes. *U. urealyticum* genotypes, based on all four genomic sequences, could be clearly separated into two clusters corresponding with currently recognized biovars 1 and 2. Sequences were generally conserved within each biovar. However, there was heterogeneity within the 5′-end regions of the MBA genes of the four serovars of biovar 1; the sequence of serovar 3 was identical with the previously published sequence and differed by only three bases from that of serovar 14; but there were significant differences between the sequences of serovars 3 and 14 and those of serovars 1 and 6. Based on the phylogenetic analysis, support is given to previous recommendations that the two biovars of *U. urealyticum* be classified as distinct species, namely *U. parvum* and *U. urealyticum* for biovars 1 and 2, respectively. In the future, the relationship between the new species and clinical manifestations of ureaplasma infections should be studied.

Keywords: *Ureaplasma urealyticum*, *Ureaplasma parvum*, phylogeny

INTRODUCTION

Ureaplasma urealyticum is a recognized cause of urethritis (Kong *et al.*, 1996; Taylor-Robinson & Furr, 1997; Taylor-Robinson *et al.*, 1985) and has been implicated in complications of pregnancy and prematurity (Abele-Horn *et al.*, 1997a, b; Cassell *et al.*, 1988; Kundsin *et al.*, 1996; Nelson *et al.*, 1998). As a common genital tract commensal (Tully, 1993), its pathogenic role in individual cases is difficult to confirm. Currently, *U. urealyticum* includes two biovars and 14 serovars (Lin & Kass, 1980; Naessens *et al.*, 1988; Razin & Yogev, 1986; Robertson & Stemke, 1982; Robertson *et al.*, 1993). Some serovars have been implicated in disease syndromes more commonly than others (Abele-Horn *et al.*, 1997b; Grattard *et al.*, 1995b; Naessens *et al.*, 1988; Zheng *et al.*, 1992), but any differences in pathogenicity among serovars is unproven. Investigations have been limited by technical difficulties and cross-reactions associated with conventional serotyping methods even when monoclonal antibodies are used (Cheng *et al.*, 1994; Naessens *et al.*, 1988; Quinn *et al.*, 1981; Robertson & Stemke, 1982; Watson *et al.*, 1990; Wiley & Quinn, 1984).

Phylogenetic analysis of the relationships between the biovars and serovars of *U. urealyticum* would provide the basis for a molecular typing system and allow further investigation of the pathogenic potential of individual types (Razin & Yogev, 1986; Robertson & Stemke, 1982; Robertson *et al.*, 1994; Weisburg *et al.*, 1989). The target sequences chosen for such an analysis should be relatively conserved and have biovar-specific and serovar-specific differences. In this study, we sequenced four gene regions of all 14 serovars of *U. urealyticum* to study the phylogenetic relationships

between them. They were: 16S rRNA genes; 16S–23S rRNA gene spacer regions; the urease gene subunits *ureA*, *ureB*, partial *ureC* and adjoining regions upstream of *ureA*, *ureA–ureB* spacer, and *ureB–ureC* spacer; and the 5′-end region of the MBA (multiple-banded antigen) genes. All have been used, individually, in previous phylogenetic studies of other bacteria and/or mycoplasmas, including ureaplasmas (Blanchard, 1990; Harasawa & Cassell, 1996; Harasawa *et al.* 1996; Robertson *et al.*, 1994; Zheng *et al.*, 1995). However, they have not previously been studied together to compare the sequences of all 14 serovars of *U. urealyticum*. We believed that combined data from analysis of several important genetic regions from all serovars would provide a better understanding of the phylogeny. The sequences obtained could also be used to develop methods for biotyping and serotyping of *U. urealyticum* isolates and for detection and subtyping of *U. urealyticum* directly from clinical specimens.

METHODS

Bacterial strains. Reference strains of each *U. urealyticum* serovar were obtained directly from the American Type Culture Collection (ATCC reference set) as follows: serovars 1, ATCC 27813; 2, ATCC 27814; 3, ATCC 27815; 4, ATCC 27816; 5, ATCC 27817; 6, ATCC 27818; 7, ATCC 27819; 8, ATCC 27618; 9, ATCC 33175; 10, ATCC 33699; 11, ATCC 33695; 12, ATCC 33696; 13, ATCC 33698; 14, ATCC 33697. In addition, a set of reference strains of serovars 1–14 were kindly provided by Dr H. L. Watson, Department of Microbiology, University of Alabama at Birmingham, AL, USA (UAB reference set). These had been obtained originally from E. A. Freundt, Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark (serovars 1–8) and J. A. Robertson, Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, AB, Canada (serovars 9–14).

Oligonucleotide primers. The oligonucleotide primers used in the paper are shown in Table 1. Previously published primers were used as follows: P1, P6, U3, U8 (Robertson *et al.*, 1993), GPO-1, Mseq-3, GPO-3 and MGSO (van Kuppeveld *et al.*, 1992) were used to amplify and sequence the 16S rRNA genes; MCGpF11, R23-1R, R16-2 and MCG-pR21 (Harasawa *et al.*, 1993) were used to amplify and sequence the 16S–23S rRNA gene spacer regions; UUS1, UUA1, U1A, U1B (Blanchard, 1990), U2B, U2C (Ruifu *et al.*, 1997) – as well as additional primers designed by us, UUSP, UCA1 and UCA2 – were used to amplify and

Table 1. Sequences of oligonucleotide primers used in the paper

Target gene/region	Reference	Primer name	Primer sequence
16S rRNA genes	Robertson *et al.* (1993)	P1	AGA GTT TGA TCC TGG CTC AGG A
	Robertson *et al.* (1993)	U3	TAG AAG TCG CTC TTT GTG G
	Robertson *et al.* (1993)	U8	GAA GAT GTA GAA AGT CGC GTT TGC
	Robertson *et al.* (1993)	P6	GGT AGG GAT ACC TTG TTA CGA CT
	van Kuppeveld *et al.* (1992)	GPO-1	ACT CCT ACG GGA GGC AGC AGT A
	van Kuppeveld *et al.* (1992)	Mseq-3	TGT ATT ACC GCG GCT GCT G
	van Kuppeveld *et al.* (1992)	GPO-3	GGG GAG CAA ATA GGA TTA GAT ACC CT
	van Kuppeveld *et al.* (1992)	MGSO	TGC ACC ATC TGT CAC TCT GTT AAC CTC
16S–23S rRNA gene spacer regions	Harasawa *et al.* (1993)	MCGpF11	AAA CTA TGG GAG CTG GTA AT
	Harasawa *et al.* (1993)	R16-2	GTG GGG ATG GAT CAC CTC CT
	Harasawa *et al.* (1993)	MCGpR2	GCA TTC ACC ATA AAC TCT T
	Harasawa *et al.* (1993)	R23-1R	CTC CTA GTG CCA AGG CAT C/TC
Urease gene subunits and adjacent regions	–	UUSP	AAT TCT (C/T)(C/T)A (A/T)TA AGA ATA (A/G)CA CAT
	Blanchard (1990)	UUS1	CAC AGA TGT CCT TGA TGT AC
	Blanchard (1990)	UUA1	TAC TTC ACG AGC AGA TTG CA
	Blanchard (1990)	U1A	GAT GGT AAG TTA GTT GCT GAC
	Blanchard (1990)	U1B	ACG ACG TCC ATA AGC AAC T
	Ruifu *et al.* (1997)	U2B	CGA AAT TGT GAT GAA CGA AGG
	Ruifu *et al.* (1997)	U2C	CTC CTA ATC TAA CGC TAT CAC C
	–	UCA1	TTC AT(C/T) CCC ATA CCT TCA CG
	–	UCA2	GTG AAC GTG AGT ATC TAA AC
The 5′ end of MBA genes	Teng *et al.* (1994, 1995)	UMS-125	GTG AAC GTG AGT ATC TAA AC
	Teng *et al.* (1994, 1995)	UMA226	CAG CTG ATG TAA GTG CAG CAT TAA ATT C
	Teng *et al.* (1994, 1995)	UMS51	TTC TGG GCT ATG ACA TTA GGT GTT ACC
	Teng *et al.* (1994, 1995)	UMA427	ACC TGG TTG TGT AGT TTC AAA GTT CAC
	Teng *et al.* (1994, 1995)	UMS-170	GTA TTT GCA ATC TTT ATA TGT TTT CG
	Teng *et al.* (1994, 1995)	UMA263	TTT GTT GTT GCG TTT TCT G

Table 2. Summary of PCR results showing sizes of bands (amplicons) produced by all 14 serovars of *U. urealyticum* using 11 primer pairs to amplify four different target genes/regions

Target gene/region	Primer pair*	Amplicon size (bp)		See Fig.:	Reference
		Biovar 1†	Biovar 2†		
16S rRNA genes	P1, P6	1488	1484	–	Robertson *et al.* (1993)
	P6, U3	1299	–	–	Robertson *et al.* (1993)
	P6, U8	–	1301	1	Robertson *et al.* (1993)
16S–23S rRNA gene spacer regions	MCGpF11, R23-1R	471	472	–	Harasawa *et al.* (1993)
	R16-2, MCGpR2	344	345	–	Harasawa *et al.* (1993)
Urease gene subunits and adjacent regions	U2B, U2C	425	418	–	Ruifu *et al.* (1997)
	UUS1, UUA1	–	313	–	Blanchard (1990)
	UUSP, UCA2	1354	1350	–	–
5′ end of MBA genes	UMS-125, UMA226	403/404‡	448	2	Teng *et al.* (1994, 1995)
	UMS51, UMA427	447	–	–	Teng *et al.* (1994, 1995)
	UMS-170, UMA263	–	476	3	Teng *et al.* (1994, 1995)

* See text for primer sequences.

† Biovar 1 includes serovars 1, 3, 6 and 14; biovar 2 includes serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, 13.

‡ Serovars 1, 3 and 14 produce bands of 403 bp and serovar 6, a band of 404 bp.
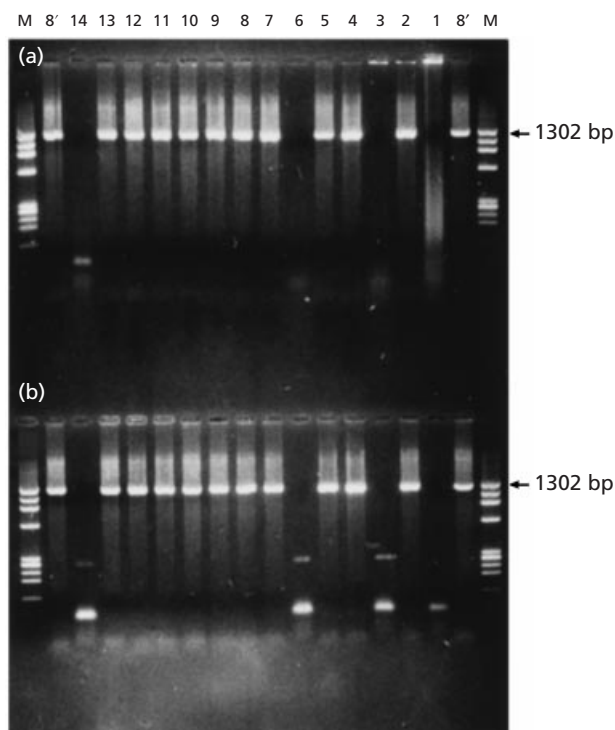
Fig. 1. Results of PCR amplification of the 16S rRNA genes of all 14 serovars of *U. urealyticum* using primers P6 and U8. (a) ATCC strains and (b) UAB reference strains. Biovar 1 consists of serovars 1, 3, 6 and 14, the other ten serovars belong to biovar 2. Lane M, molecular mass markers ϕX174 DNA/*Hae*III; lane numbers in each panel correspond with *U. urealyticum* serovar numbers.
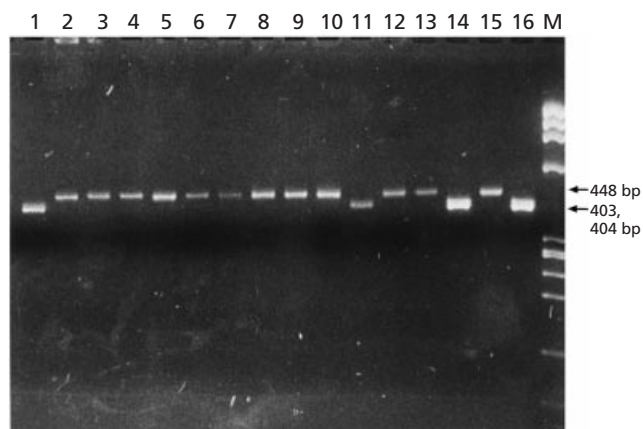
Fig. 2. Results of PCR amplification of the 5′-end of MBA genes of all 14 serovars of *U. urealyticum* using primers UMS-125 and UMA226. Biovar 1 consists of serovars 1, 3, 6 and 14, the other ten serovars belong to biovar 2. Lanes: M, molecular mass markers ϕX174 DNA/*Hae*III; 1–14, correspond with *U. urealyticum* serovars, ATCC strains; 8′, UAB reference strain of *U. urealyticum* serovar 8.

sequence *U. urealyticum* urease gene subunits *ureA*, *ureB*, partial *ureC* and adjoining upstream region of *ureA*, *ureA–ureB* spacer and *ureB–ureC* spacer. Three previously published oligonucleotide primer pairs were used to amplify the 5′-end region of the MBA genes of *U. urealyticum* serovars 1–14 namely: UMS-125, UMA226 for all 14 serovars; UMS51 and UMA427 for the four serovars of biovar 1 (Teng *et al.*, 1994); UMS-170, UMA 263 for the ten serovars of biovar 2 (Teng *et al.*, 1995).

DNA preparations. Cells from 0·5 ml of ureaplasma broth (10B) cultures of each *U. urealyticum* serovar were harvested from late exponential growth by centrifugation at 14000 *g* for 20 min. DNA was isolated from cultures by treatment with 500 µl digestion buffer (10 mM Tris/HCl, pH 8·0, 0·45% Triton X-100 and 0·45% Tween 20) and proteinase K, 20 g l⁻¹, at 55 °C for 1 h, 95 °C for 20 min and then extraction with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated with 0·1 vol. of 3 M sodium acetate (pH 5·2) and 2 vols of ethanol. The washed and dried pellets were hydrated in 200 µl ultrapure and sterile water.

PCR. The 25 µl amplification reaction mixtures contained 2·5 µl 10 × PCR buffer (1 × is 10 mM Tris/HCl, pH 8·8 at 25 °C, 1·5 mM MgCl₂, 50 mM KCl and 0·1% Triton X-100), 0·5 U *Taq* polymerase (Finnzymes), 200 mM of each dNTP (dATP, dCTP, dGTP, dTTP; Boehringer Mannheim), 10 pmol each primer, 5 µl sample DNA, and added ultrapure sterile water to 25 µl.

The denaturation, annealing and elongation temperatures and times used were 95 °C for 30 s, 55–62 °C (according to the T_m values of different primers) for 30 s and 72 °C for 1 min, respectively, for 40 cycles using a Perkin Elmer thermocycler (Blanchard, 1990; Harasawa *et al.*, 1993; Robertson *et al.*, 1993; Ruifu *et al.*, 1997; Teng *et al.*, 1994, 1995; van Kuppeveld *et al.*, 1992).

Eight microlitres of PCR products were analysed by electrophoresis on 2·0% (w/v) agarose gels which were stained with 0·5 µg ethidium bromide ml⁻¹. A visible band with appropriate size on UV translumination was considered a positive result.

Sequencing and analysis. The PCR products were sequenced with Applied Biosystems (ABI) *Taq* DyeDexoy terminator cycle-sequencing kits according to standard protocols. The multiple sequence alignments were performed with PILEUP and PRETTY programs from the Multiple Sequence Analysis program group, provided in WebANGIS, ANGIS (Australian National Genomic Information Service) version 3. Phylogenetic relationships were studied using CLUSTAL and trees were bootstrapped with 100 replications.

RESULTS

PCR

The results of PCR for all 14 *U. urealyticum* serovars, using 11 primer pairs to amplify the four different gene regions are summarized in Table 2 and representative examples are shown in Figs 1–3. Five primer pairs were specific for either biovar 1 or biovar 2 and the others produced different sized bands for each biovar. With one exception, band sizes produced by individual primer pairs were consistent for all serovars within

Table 3. Comparative study of the sequences of 16S rRNA genes of 14 serovars of *U. urealyticum* (ATCC strains)

Numbers in parentheses are serovars affected by changes.

Site	Biovar 1	Biovar 2	Biovar 1/2
87	T	C	
176	T	G	
177	C	T	
180–183	TGTG	–	
200	A	G	
350		A (5)	C
430	A (1, 14)		G
807	C	T	
808	T (1)		C
812	T	C	
814	G	A	
974	T	C	
1090	A	G	
1270	T	C	
1407		T (5, 7*)	C

* For serovar 7 UAB reference strain, the base position 1407 was C not T.

Fig. 3. Results of PCR amplification of the 5′-end of MBA genes of all 14 serovars of *U. urealyticum* using primers UMS-170 and UMA263. Biovar 1 consists of serovars 1, 3, 6 and 14, the other ten serovars belong to biovar 2. Lanes: M, molecular mass markers φX174 DNA/*Hin*fI; 1–14 correspond with *U. urealyticum* serovars, ATCC strains; 8′, UAB reference strain of *U. urealyticum* serovar 8.

Fig. 4. Multiple sequence alignment of the sequences of the 16S–23S rRNA gene spacer region of 14 serovars of *U. urealyticum* (ATCC strains).

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             1upstream of ureA                                        50
UUU.msf{biovar 1}  ---------- ---tct-t-- a-tt-ac--t ----t----- -aac-ct---
UUU.msf{biovar 2}  ---------- ---cac-g-- g-cc-tg--g ----c----- -gca--.---
        Consensus  CACATTTTTT TAT---A-AT -T--T--AT- TACC-AAAAA A---A--TTT

             51                                                     100
UUU.msf{biovar 1}  --t-a----- -g--t-tt-- ----t----- ---------- ----------
UUU.msf{biovar 2}  --c-t----- -a----a--- ----c----- ---------- ----------
        Consensus  TT-T-ATTTT A-TG-A--TT TTTG-TTAA AAGCGTTAAA ATAAAATTGC

             101                       upstream of ureA|ureA     150
UUU.msf{biovar 1}  ---------- --------ag ----*----- ---------- ----------
UUU.msf{biovar 2}  ---------- --------ga ---------- ---------- ----------
        Consensus  ATTATTACTT AATATACA-- ATATATTAGA GGTAAATAAA TGAATCTATC

             151                                                    200
UUU.msf{biovar 1}  ---------- g-------a- -----a---- ---t------ ----------
UUU.msf{biovar 2}  ---------- a------g-- -----g---- ---a------ ----------
        Consensus  ATTAAGAGAA -TCCAAAA-T TATTG-TAAC AGT-GCTGCT GACGTTGCAA

             201                                                    250
UUU.msf{biovar 1}  ---------- ---------- ---------- ----t----- ------#---
UUU.msf{biovar 2}  ---------- ---------- ---------- ----c----- ------c---
        Consensus  GAAGACGTTT AGCTAGAGGT TTAAAATTAA ACTA-TCAGA AGCTGT-GCT

             251                                                    300
UUU.msf{biovar 1}  ---------- -t--t--a-- ---------- ---------- --c-------
UUU.msf{biovar 2}  ---------- --c--c---g- ---------- ---------- --t-------
        Consensus  TTAATTACTG A-CA-GTA-T GGAAGGGGCA AGAGATGGTA AG-TAGTTGC

             301                                                    350
UUU.msf{biovar 1}  ----c----- ---------- --------c- ---------- ----------
UUU.msf{biovar 2}  ----t----- ---------- --------t- ---------- ----------
        Consensus  TGAC-TAATG CAATCTGCTC GTGAAGTA-T ACGTGTTGAT CAAGTTATGG

             351                                                    400
UUU.msf{biovar 1}  ---------- ---------- --------t- ---------- ---------t
UUU.msf{biovar 2}  ---------- ---------- g-------c- ---------- ---------a
        Consensus  AAGGTGTAGA TACAATGGTT -GTATAAT-C AAGTTGAAGT TACTTTCCC-

             401                                        ureA|ureA-ureB
UUU.msf{biovar 1}  ---------- ---------- -------gat ------c--- -----ca---
UUU.msf{biovar 2}  ---------- ---------- -------agc ------t--- -----tt---
        Consensus  GATGGTACTA AACTAGTTTC TGTACAC--- CCAATT-ACA AATAA--TTT

             spacer                                                 500
UUU.msf{biovar 1}  ---------- ---.....-- -tt--a--a- ---------- ----------
UUU.msf{biovar 2}  ---------- ---tcaat-- -aa--t--t- ---------- ----------
        Consensus  ACAATTCGTA AAA-----TT T--TT-TA-A AGGAGATAAT GATTATATGT

             501                           ureA-ureB  spacer|ureB
UUU.msf{biovar 1}  ---------- --g------t -g-------- ---------- ---g------
UUU.msf{biovar 2}  ---------- --a------c -c-------- ---------- ---a------
        Consensus  CAGGATCATC AA-TCAATT- A-TCCAGGTA AATTAGTACC AGG-GCAATT

             551                                                    600
UUU.msf{biovar 1}  --t------a gt--t----- ---------t ---------- -g--------
UUU.msf{biovar 2}  --c------g aa--c----- ---------c ---------- -a--------
        Consensus  AA-TTCGCT- --GG-GAAAT TGTGATGAA- GAAGGTAGAG A-GCAAAAGT

             601                                                    650
UUU.msf{biovar 1}  ---t--t--- ---------- -g-------- ---a------ ----------
UUU.msf{biovar 2}  ---c--c--- ---------- -t-------- ---c------ ----------
        Consensus  AAT-AG-ATT AAAAATACTG G-GACCGTCC TAT-CAAGTT GGATCACATT

             651                                                    700
UUU.msf{biovar 1}  -t-----g-- ----gtg--- ---------- ----t----- ----------
UUU.msf{biovar 2}  -c-----a-- ----aca--- ---------- ----c----- ----------
        Consensus  T-CACTT-TT TGAA---AAT AGTGCATTAG TATT-TTTGA TGAAAAAGGA

             701                                                    750
UUU.msf{biovar 1}  --t-----t- -------c-- ---------- -----a---- ----------
UUU.msf{biovar 2}  --c-----c- -------t-- ---------- -----t---- ----------
        Consensus  AA-GAAGA-A AAGAACG-AA AGTTGCTTAT GGACG-CGTT TCGATATTCC

             751                                                    800
UUU.msf{biovar 1}  ---------- ---------- ---------- ---t------ ---------a
UUU.msf{biovar 2}  ---------- ---------- ---------- ---c------ ---------g
        Consensus  ATCAGGTACT GCTATTCGTT TTGAACCAGG AGA-AAAAA GAAGTTTCA-

             801                                                    850
UUU.msf{biovar 1}  ---------- --c------- --c------- ---------- t---------
UUU.msf{biovar 2}  ---------- --t------- --t------- ---------- c---------
        Consensus  TTATTGATTT AG-CGGAACA CG-GAAGTTT GAGGTGTAAA -GGCTTAGTT

             851             ureB|ureB-ureC spacer              900
UUU.msf{biovar 1}  --t------ --------- -----aac----t- ---------- cta-----g-
UUU.msf{biovar 2}  --c------ --------- -...,----.- ---------- ...-----t-
        Consensus  AA-GGAAAC TTAAAAAATA A---TCTA-T TTACAAGTTT ---CTATA-A

             ureB-ureC spacer|ureC                           950
UUU.msf{biovar 1}  t-aa------ ---------- ---------- ---------- --t------t
UUU.msf{biovar 2}  g-cg------ ---------- ---------- ---------- --c------c
        Consensus  -A--AAGGGG AACATTATGT TTAAAATTTC AAGAAAAAAT TA-TCAGAT-

             951                                                    1000
UUU.msf{biovar 1}  ----c----- t--------- ---------- ---------- t---------
UUU.msf{biovar 2}  ----t----- c--------- ---------- ---------- c---------
        Consensus  TATA-GGTAT -ACAACTGGT GATAGCGTTA GATTAGGAGA -ACAAATCTT

             1001                                                   1050
UUU.msf{biovar 1}  ---------- ---------- ---------- ---------- -a-----t--
UUU.msf{biovar 2}  ---------- ---------- ---------- ---------- -c-----c--
        Consensus  TGAGTTAAAG TTGAAAAAGA CTTAACTACT TATGGTGAAG A-TCTGT-TT

             1051                                                   1100
UUU.msf{biovar 1}  t--------a ------c---- ---------- ---a-----t ----------
UUU.msf{biovar 2}  c--------t ------t---- ---------- ---g-----c ----------
        Consensus  -GGTGGTGG- AAAAC-CTAC GTGAAGGTAT GGG-ATGAA- TCTACTATGA

             1101                                                   1150
UUU.msf{biovar 1}  ------c-- t--a-----t ---------- -------t-- ----------
UUU.msf{biovar 2}  ------c-- c--g----g ---------- -------c-- ----------
        Consensus  AGTTAGA-GA -AA-TTAGG- AATGCTGAAG TAATGGA-TT AGTTATTACA

             1151                                                   1200
UUU.msf{biovar 1}  --t---c--- --g----t-- t--------t --t-----a- ----t-----
UUU.msf{biovar 2}  --c---t--- --c----c-- c--------c --c----t- ----c-----
        Consensus  AA-GCA-TAA TT-TTGA-TA -ACAGGTAT- TA-AAAGC-G ATAT-GGTAT

             1201                                                   1250
UUU.msf{biovar 1}  ------t--- --------tg -g-------- ---t--a--t -a--------
UUU.msf{biovar 2}  ------c--- --------at -t-------- ---a--t--c -t--------
        Consensus  TAAAAA-GGA AAAATTGC-- C-ATTGGTAA ATC-GG-AA- CC-CATTTAA

             1251                                                   1300
UUU.msf{biovar 1}  -a---aat-- t--t---a-- --g-----c- --------a- ----------
UUU.msf{biovar 2}  -t---gga-- a--c---g-- --t-----t- --------g- ----------
        Consensus  C-GAT---GT -GA-ATG-TT GT-GGTAT-T CAACTGAA-T TTCAGCTGGT

             1301          1320ureC
UUU.msf{biovar 1}  --g------- ----------
UUU.msf{biovar 2}  --a------- ----------
        Consensus  GA-GGTAAAA TTTATACAGC
```
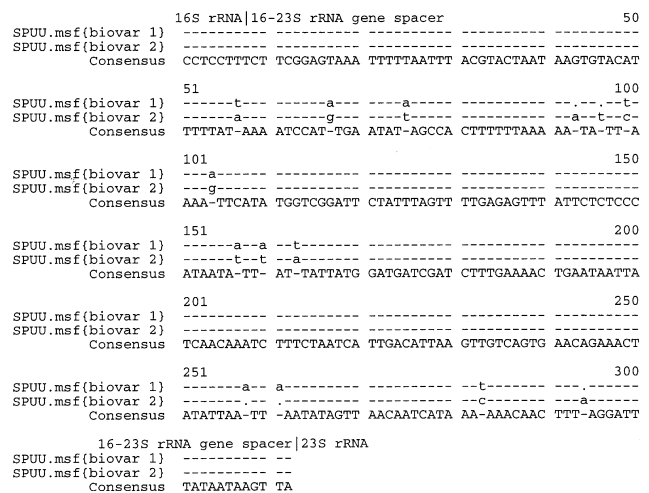
Fig. 5. Multiple sequence alignment of the sequences of urease gene subunits of 14 serovars of *U. urealyticum* (ATCC strains). *, Base for serovar 2 is G instead of A; #, base for serovar 2 is T, for the other serovars of biovar 2 it is C.

each biovar. The exception was the primer pair UMS-125 and UMA226, which gave fragments of 403 bp for serovars 1, 3 and 14 and of 404 bp for serovar 6 (due to a single base insertion in serovar 6 at position −46) (Fig. 7).

Comparative study of the sequences of four genetic regions

16S rRNA genes. There were 14 ($14/1439 = 0.97\%$) base differences in the sequences of 16S rRNA genes between the two biovars. Heterogeneity was found at two sites among four serovars of biovar 1 and at two sites among ten serovars of biovar 2 (Table 3).

UAB reference isolates of *U. urealyticum* serovars 1, 2, 3, 5, 6, 7, 8 and 14 were sequenced and the results were identical to those of the corresponding ATCC serovar strains, with the exception of serovar 7. In the UAB reference strain of serovar 7, the base at position 1407 was C, as it is for the other eight serovars of biovar 2, rather than T as it was for ATCC serovars 5 and 7.

16S–23S rRNA gene spacer regions. The DNA sequence alignment for the sequences of 16S–23S rRNA gene spacer regions showed 14 ($14/312 = 4.5\%$) base differences between the two biovars (Fig. 4), but sequences were similar among serovars within each biovar.

Urease subunits *ureA*, *ureB*, partial *ureC* genes and adjoining upstream of *ureA*, *ureA*–*ureB* spacer and *ureB*–*ureC* spacer. The sequences of the four serovars of biovar 1 were identical. Sequences of nine of the ten serovars of biovar 2 were identical, but serovar 2 differed by two bases, at positions 126 (G instead of A) and 248 (T instead of C). There were 141 base differences ($141/1320 = 10.7\%$) between the sequences of biovars 1 and 2. There were 25 base differences ($25/149 = 16.8\%$) in the region upstream of *ureA*; 19 ($19/306 = 6.2\%$) in *ureA*; 11 ($11/51 = 20.4\%$) in the *ureA*–*ureB*

```
                      -200                                              -151        101                                              150
MBA.msf{MBAUU-11}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-7}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-10}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-12}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-13}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-4}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-2}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-5}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-8}  ---------- ---------- ---------- ------....  .-------      ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-9}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-14}  ---------- ---------- ---------- ---------- -----a--g-     c--------- ---g------ -ac---t--- c--------c -----gt--a
 MBA.msf{MBAUU-3}  ---------- ---------- ---------- ---------- -----a--g-     c--------- ---g------ -ac---t--- c--------c -----gt--a
 MBA.msf{MBAUU-1}  ---------- ---------- ---------- ---------- -----a--g-     cc-------- ---------- -ac---t--- c--------c ------t--a
 MBA.msf{MBAUU-6}  ---------- ---------- ---------- ---------- -----a----     c--------- ---g------ --c---t--- ---------c ---t--t--a
       Consensus   GTATTTGCAA TCTTTATATG TTTTCGTTAA AATTAAAAAT TAATTCCTAT     ATGTTAAATC TAAATTAAGT AGTCAACTTG TTAAATCAAA AGACGAAAAG

                      -150                                              -101        151                                              200
MBA.msf{MBAUU-11}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-7}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-10}  ---------- ---------- ---------- --------g- ----------     ---------- ---------- ---------- ---------- ---a------
MBA.msf{MBAUU-12}  ---------- ---------- ---------- --------g- ----------     ---------- ---------- ---------- ---------- ---a------
MBA.msf{MBAUU-13}  ---------- ---------- ---------- --------g- ----------     ---------- ---------- ---------- ---------- ---a------
 MBA.msf{MBAUU-4}  ---------- ---------- ---------- --------g- ----------     ---------- ---------- ---------- ---------- ---a------
 MBA.msf{MBAUU-2}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-5}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-8}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-9}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-14}  -g---tt-tg -a-------. .t------- g----ca--- -t-t--c-t-     --t-----t- -g-------- a--------c --ta-a---c ---g-a-tg-
 MBA.msf{MBAUU-3}  -g---tt-tg -a-------. .t------- g----ca--- -t-t--c-t-     --t-----t- -g-------- a--------c --ta-a---c ---g-a-tg-
 MBA.msf{MBAUU-1}  -g---tt-tg -a-----gc. .t------- g----ca--- -t-t--c---     --t-----t- -g-------- a--------c --ta-a---c ---g---t--
 MBA.msf{MBAUU-6}  -----tt-tg -a-------. .t------- g----ca--- -t-t--ctag     --t-----t- -a------- a--------c --ta-a---c ---g---t--
       Consensus   AAAAACAACA TGAGATTAAA CAAAATCTTA ATGTTGTTAT TATCTATACA     AGCTTTTACG CTGTTTACGA CATTGAAAAT TTCGATGATT TAACTGAAAA

                      -100                                              -51         201                                              250
MBA.msf{MBAUU-11}  ---------- ---------- ---------- ---------- ----------     ---------- --------t- --a------- ---------- ----------
 MBA.msf{MBAUU-7}  ---------- ---------- ---------- ---------- ----------     ---------- --------t- --a------- ---------- ----------
MBA.msf{MBAUU-10}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-12}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-13}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-4}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-2}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-5}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-8}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-9}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-14}  -at----.t- ---...... .......... ---------- ...a-c----     ---------- t-----gta -cat------ ta---c---- c---------
 MBA.msf{MBAUU-3}  -at----.t- ---...... .......... ---------- ...a-c----     ---------- t-----gta -cat------ ta---c---- c---------
 MBA.msf{MBAUU-1}  -at----.t- --g....... .......... ---------- ...a-c----     ---------- t------t- -cat------ ta---c---- c---------
 MBA.msf{MBAUU-6}  -at----tt- ---....... .......... ---------- ...acc----     ---------- t- -cat------ ta---c---- c---------
       Consensus   TTCTAAAGAA AAATATATTT GCAAAACTAT AAATAGACAC AAAAAACAAT     TGATAAAAAA GCATTAAACG AAGCTGAATT CCATGTTGCA ATTACATCAG

                      -50                                               -1          251                                    281
MBA.msf{MBAUU-11}  ---------- ---------- -g-------- ---------- ----------     t--------- ---------- ---------- -
 MBA.msf{MBAUU-7}  ---------- ---------- -g-------- ---------- ----------     t--------- ---------- ---------- -
MBA.msf{MBAUU-10}  ---------- ---------- -g-------- ---------- ----------     ---------- ---------- ---------- -
MBA.msf{MBAUU-12}  ---------- ---------- -g-------- ---------- ----------     ---------- ---------- ---------- -
MBA.msf{MBAUU-13}  ---------- ---------- -g-------- ---------- ----------     ---------- ---------- ---------- -
 MBA.msf{MBAUU-4}  ---------- ---------- -g-------- ---------- ----------     ---------- ---------- ---------- -
 MBA.msf{MBAUU-2}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- -
 MBA.msf{MBAUU-5}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- -
 MBA.msf{MBAUU-8}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- -
 MBA.msf{MBAUU-9}  ---------- ---------- ---------- ---------- ----------     ---------- ---------- ---------- -
MBA.msf{MBAUU-14}  ........-c --ta----ga -------tt- a--------- --.a-c----     -------c-- --------gt a--cttga-- -
 MBA.msf{MBAUU-3}  ........-c --ta----ga -------tt- a--------- --.a-c----     -------c-- --------gt a--cttga-- -
 MBA.msf{MBAUU-1}  ........-c --ta----ga -------tt- a--------- --.a-c----     t-----c-- --------t ct-gtt---- -
 MBA.msf{MBAUU-6}  ........-c --ta----ga -------tt- a--------- --.acc----     t-----c-- --------t ct-gtt---- -
       Consensus   AGAATAATAA AACTAAATTT CATATTTAGT TTATTAGGAG ATCGTTATAA     CTGAAAATAA AACAGAAAAC GCAACAACAA A

                   1start codon                                        50
MBA.msf{MBAUU-11}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-7}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-10}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-12}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-13}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-4}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-2}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-5}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-8}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-9}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-14}  ---------- ---------- a-----c-t- --t--g---t ---t--t--
 MBA.msf{MBAUU-3}  ---------- ---------- a-----c-t- --t--g---t ---t--t--
 MBA.msf{MBAUU-1}  ---------- ---------- a----c--- --t--g---t ----a-t--
 MBA.msf{MBAUU-6}  ---------- ---------- a-----c--- --t--g---t ----a-t--
       Consensus   ATGAAATTAT TAAAAAATAA GAAATTTTGA GCAATTACAC TAGGGGTAAC

                   51                                                 100
MBA.msf{MBAUU-11}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-7}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-10}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-12}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-13}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-4}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-2}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-5}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-8}  ---------- ---------- ---------- ---------- ----------
 MBA.msf{MBAUU-9}  ---------- ---------- ---------- ---------- ----------
MBA.msf{MBAUU-14}  c-----t--- --t-aa--- ----a-a-- ---------- ----at----
 MBA.msf{MBAUU-3}  c-----t--- --t-aa--- ----a-a-- ---------- ----at----
 MBA.msf{MBAUU-1}  c-----t--- --t-aa--- ----a-a-- ---------- ----at----
 MBA.msf{MBAUU-6}  c-----t--- --t-aa--- ----a-a-- g--------- ----at----
       Consensus   TTTAGTGGGA GCAGGGGTAG TTGCTGTGGC AGCTTCATGT TCTAGCTCAA
```
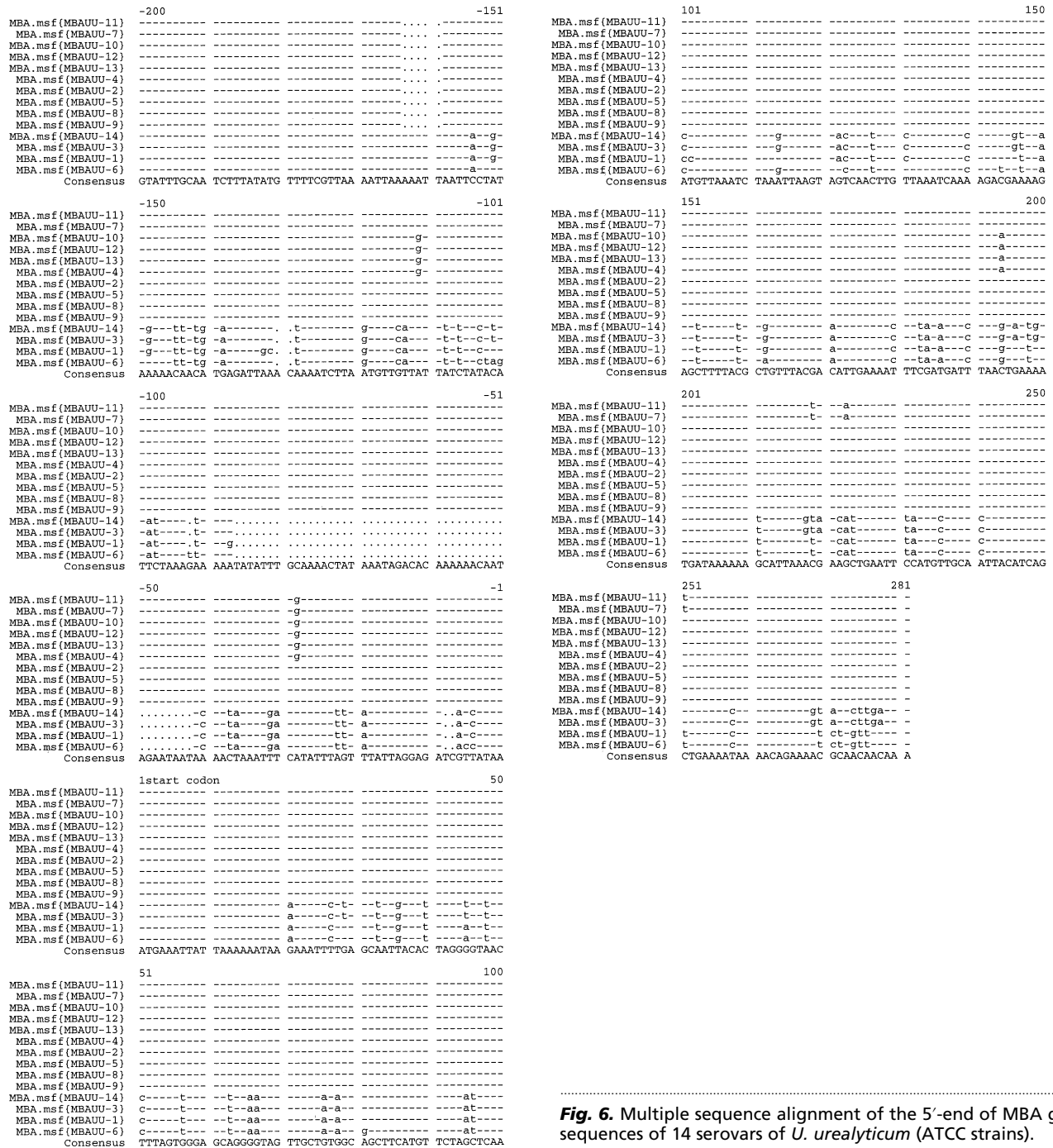
Fig. 6. Multiple sequence alignment of the 5′-end of MBA gene sequences of 14 serovars of *U. urealyticum* (ATCC strains).

spacer; 30 (30/375 = 8·0%) in *ureB*; 11 (11/45 = 24·4%) in the *ureB–ureC* spacer and 45 (45/404 = 11·1%) in partial *ureC* (Fig. 5). UAB reference isolates of serovars 2, 6, 7, 10, 11, 12 and 13 were sequenced and the results were identical to those of the corresponding ATCC serovar strains.

The 5′-end region of the MBA genes. The amplified fragments of the 5′-end of the MBA gene of serovars belonging to biovar 1 were shorter than those of biovar 2, mainly because of the deletion of a 45 bp segment of the biovar 1 sequence upstream of the start codon of

the MBA genes (−87 to −43). There were 155 (155/481 = 32·2%) base differences between the sequences for the two biovars – 73 (73/281 = 26·0%) in the region downstream from the start codon and 82 (82/200 = 41·0%) in the upstream region. These differences included deletions at 49 sites in biovar 1 and at five sites in biovar 2 (Fig. 6). All 14 serovars of UAB reference strains were sequenced, and the results were identical to the corresponding ATCC serovar strains.

There were base differences at 37 (37/603 = 6·1%)

```
                 -151                                                      -102
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-3}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-1}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-6}   ---------- ---------- ---------- ---------- -------a-
       Consensus  GTATTTGCAA TCTTTATATG TTTTCGTTAA AATTAAAAAT TAATTACTGT
                 -101                                                       -52
MBA1.msf{MBAUU-14}  ---------- --------a- ---------- ---------- ----------
MBA1.msf{MBAUU-3}   ---------- --------cc ---------- ---------- ----------
MBA1.msf{MBAUU-1}   ---------- --------gc- ---------- ---------- -----c---
MBA1.msf{MBAUU-6}   -a-------- --------a- ---------- ---------- -----tag--
       Consensus  AGAAATTATG TAAGATTA-T AAATCTTAGT GTTCATATTT TTTACATATA
                 -51                                                        -2
MBA1.msf{MBAUU-14}  -----.---- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-3}   -----.---- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-1}   -----.---- g--------- ---------- ---------- ----------
MBA1.msf{MBAUU-6}   -----t---- ---------- ---------- ---------- -----c---
       Consensus  TTAAA-TAAA AACAATAAAA TGACATATTT TTTATATTAG GAGAATCATA
                  1start codon                                             49
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- -----t----
MBA1.msf{MBAUU-3}   ---------- ---------- --------t ---------- -----t----
MBA1.msf{MBAUU-1}   ---------- ---------- ---------- ---------- -----a----
MBA1.msf{MBAUU-6}   ---------- ---------- ---------- ---------- -----a----
       Consensus  AATGAAATTA TTAAAAAATA AAAAATTCTG AGCTATGACA TTAGG-GTTA
                  50                                                       99
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-3}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-1}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-6}   ---------- ---------- ---------- --g------- ----------
       Consensus  CCTTAGTTGG AGCTGGAATA GTTGCTATAG CAGCTTCATG TTCTAATTCA
                  100                                                      149
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- -----g---
MBA1.msf{MBAUU-3}   ---------- ---------- ---------- ---------- -----g---
MBA1.msf{MBAUU-1}   --c------- ---------- a----- ---------- -----a---
MBA1.msf{MBAUU-6}   ---------- ---------- --g------- -t-------- ----t-a--
       Consensus  ACTGTTAAAT CTAAGTTAAG TAACCAATTT GCTAAATCAA CAGACG-TAA
                  150                                                      199
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- -----a--g
MBA1.msf{MBAUU-3}   ---------- ---------- ---------- ---------- -----a--g
MBA1.msf{MBAUU-1}   ---------- ---------- ---------- ---------- -----g--a
MBA1.msf{MBAUU-6}   ---------- --a------- ---------- ---------- -----g--a
       Consensus  AAGTTTTTAT GCGGTTTACG AAATTGAAAA CTTTAAAGAT CTAAGT-AT-
                  200                                                      249
MBA1.msf{MBAUU-14}  ---------- --------g- a--------- ---------- ----------
MBA1.msf{MBAUU-3}   ---------- --------g- a--------- ---------- ----------
MBA1.msf{MBAUU-1}   ---------- --------a- g--------- ---------- ----------
MBA1.msf{MBAUU-6}   ---------- --------a- g--------- ---------- ----------
       Consensus  ATGATAAAAA ATCATTAA-T -ACATTGAAT TTAATGCTGC ACTTACATCA
                  250                                                      299
MBA1.msf{MBAUU-14}  -c-------- ---------g -ac-c--ga- ---------- -a--------
MBA1.msf{MBAUU-3}   -c-------- ---------g -ac-c--ga- ---------- -a--------
MBA1.msf{MBAUU-1}   -t-------- ---------a -ct-g--ac- ---------- -g--------
MBA1.msf{MBAUU-6}   -t-------- ---------a -ct-g--ac- ---------- -g--------
       Consensus  G-TGAAAACA AAACAGAAA- T--A-TT--A AAAGGTCATT T-GTTGGTGA
                  300                                                      349
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-3}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-1}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-6}   ---------- ---------- ---------- ---------- ----------
       Consensus  AAAAATTTAC GTTAAATTAC CTCGTGAACC AAAACCTAAT GAACAATTAA
                  350                                                      399
MBA1.msf{MBAUU-14}  --------g ---------- ---------- ---------- -------t--
MBA1.msf{MBAUU-3}   --------g ---------- ---------- ---------- -------t--
MBA1.msf{MBAUU-1}   --------a ---------- c--------- ---------- -------c--
MBA1.msf{MBAUU-6}   --------a ---------- ---------- ---------- -------c--
       Consensus  CTATTATTA- TAAAAGTGGA TTAATCAAGA CTTCAGGTTT GTTAATA-CT
                  400                                                      449
MBA1.msf{MBAUU-14}  ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-3}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-1}   ---------- ---------- ---------- ---------- ----------
MBA1.msf{MBAUU-6}   a--------- ---------- ---------- ---------- ----------
       Consensus  GATAATTTGA ATTATCAAAC AGAAAAGTG AACTTTGAAA CTACACAACC
                  453
MBA1.msf{MBAUU-14}  ----
MBA1.msf{MBAUU-3}   ----
MBA1.msf{MBAUU-1}   ----
MBA1.msf{MBAUU-6}   ----
       Consensus  AGGT
```

Fig. 7. Multiple sequence alignment of the 5′-end of MBA gene sequences of *U. urealyticum* serovars 1, 3, 6 and 14 (ATCC strains).

Phylogenetic tree of *U. urealyticum*

Because the sequences of the 16S rRNA genes, the 16S–23S rRNA gene spacer regions and the urease gene subunits were conserved within each biovar, they did not provide enough information to distinguish serovars. However, all three showed enough differences between biovars to separate them clearly into two clusters. We analysed the phylogenetic relationships further using sequence data for the MBA genes, based on the results of multiple sequence alignment (Fig. 9).

The phylogenetic tree, based on the sequences of the 5′-end of MBA genes, confirmed that the four serovars of *U. urealyticum* biovar 1 and 10 serovars of biovar 2 were clearly separated into two clusters. Within biovar 1, serovars 3 and 14 formed one cluster, and serovars 1 and 6 another cluster. The ten serovars of biovar 2 could be separated into three clusters: (i) serovars 2, 5, 8 and 9; (ii) serovars 4, 10, 12 and 13; (iii) serovars 7 and 11.

DISCUSSION

Various methods have been described previously to distinguish the two biovars of *U. urealyticum* including susceptibility to manganese (Robertson & Chen, 1984); enzyme profiles (Davis & Villanueva, 1990); protein or antigen epitope analysis (Horowitz *et al.*, 1986; MacKenzie *et al.*, 1996; Teng *et al.*, 1994); DNA–DNA hybridization (Christiansen *et al.*, 1981); RFLP (Harasawa *et al.*, 1991); one- and two-dimensional gel electrophoresis (Swenson *et al.*, 1983); genomic sizes (Robertson *et al.*, 1990); arbitrarily primed PCR fingerprinting (Grattard *et al.*, 1995a, b; Kong *et al.*, 1996); and PCR amplification of specific genes (Blanchard, 1990; Harasawa *et al.*, 1993; Robertson *et al.*, 1993; Teng *et al.*, 1994). However, to study the possible association of individual serovars with clinical disease, further identification of individual serovars is needed. The aim of this study was to analyse the phylogenetic relationships between the 14 serovars of *U. urealyticum* and define their genotypes, as the basis of a new molecular typing system, using sequence data from four genetic regions.

The 16S rRNA genes and the 16S–23S rRNA gene spacer regions have been used extensively in taxonomic studies of many different types of bacteria, including the *Mollicutes* and, specifically, ureaplasmas (Barry *et al.*, 1991; Everett & Andersen, 1997; Harasawa *et al.*, 1991, 1996; Harasawa & Cassell, 1996; Perez Luz *et al.*, 1998; Robertson *et al.*, 1994; van Kuppeveld *et al.*, 1992; Weisberg *et al.*, 1989). Both are relatively well-conserved but have sufficient heterogeneity to allow some differentiation within species. In the 16S rRNA genes, base differences occurred between biovars 1 and 2 at fewer than 1% of sites, several of which – at positions 176, 177 and 180–183 – have been previously described and used to design PCR primers to distinguish the two biovars (Robertson & Stemke, 1982). The 16S rRNA genes were highly conserved within

sites among four serovars of biovar 1 (Fig. 7) (Kong *et al.*, 1999). Sequences were more conserved between the ten serovars of biovar 2, with base changes at only six (6/476 = 1·3%) sites: base −112 of serovars 4, 10, 12, 13 changed from A to G; base 194 of serovars 4, 10, 12, 13 changed from C to A; base 219 of serovars 7, 11 changed from C to T; base 223 of serovars 7, 11 changed from G to A; base 251 of serovars 7, 11 changed from C to T; base −29 of serovars 4, 10, 12, 13 and 7, 11 changed from A to G (Fig. 8). Sequencing of UAB reference strains showed identical differences between serovars to those found with ATCC serovars.

```
                     -195                                          -146
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   GTATTTGCAA TCTTTATATG TTTTCGTTAA AATTAAAATT CCTATAAAAA

                     -145                                           -96
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---g----- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---g----- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---g----- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---g----- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   CAACATGAGA TTAAACAAAA TCTTAATGTT GTTATTATCT ATACATTCTA

                     -95                                            -46
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   AAGAAAAATA TATTTGCAAA ACTATAAATA GACACAAAAA ACAATAGAAT

                     -45                                   start 1 codon
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ------a--- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ------a--- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ------a--- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ------a--- ---------- ---------- ----------
        Consensus   AATAAAACTA AATTTCGTAT TTAGTTTATT AGGAGATCGT TATAAATGAA

                     6                                               55
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   ATTATTAAAA AATAAGAAAT TTTGAGCAAT TACACTAGGG GTAACTTTAG

                     56                                             105
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   TGGGAGCAGG GGTAGTTGCT GTGGCAGCTT CATGTTCTAG CTCAAATGTT

                     106                                            155
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   AAATCTAAAT TAAGTAGTCA ACTTGTTAAA TCAAAAGACG AAAAGAGCTT

                     156                                            205
MBA2.msf{MBAUU-11}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-7}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- --------a- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- --------a- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- --------a- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- --------a- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   TTACGCTGTT TACGACATTG AAAATTTCGA TGATTTAACT GAAAATGATA

                     206                                            255
MBA2.msf{MBAUU-11}   ---------- ---t---a-- ---------- ---------- -----t----
MBA2.msf{MBAUU-7}    ---------- ---t---a-- ---------- ---------- -----t----
MBA2.msf{MBAUU-10}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-12}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-13}   ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-4}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-2}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-5}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-8}    ---------- ---------- ---------- ---------- ----------
MBA2.msf{MBAUU-9}    ---------- ---------- ---------- ---------- ----------
        Consensus   AAAAAGCATT AAACGAAGCT GAATTCCATG TTGCAATTAC ATCAGCTGAA

                     256                  281
MBA2.msf{MBAUU-11}   ---------- ---------- ------
MBA2.msf{MBAUU-7}    ---------- ---------- ------
MBA2.msf{MBAUU-10}   ---------- ---------- ------
MBA2.msf{MBAUU-12}   ---------- ---------- ------
MBA2.msf{MBAUU-13}   ---------- ---------- ------
MBA2.msf{MBAUU-4}    ---------- ---------- ------
MBA2.msf{MBAUU-2}    ---------- ---------- ------
MBA2.msf{MBAUU-5}    ---------- ---------- ------
MBA2.msf{MBAUU-8}    ---------- ---------- ------
MBA2.msf{MBAUU-9}    ---------- ---------- ------
        Consensus   AATAAAACAG AAAACGCAAC AACAAA
```
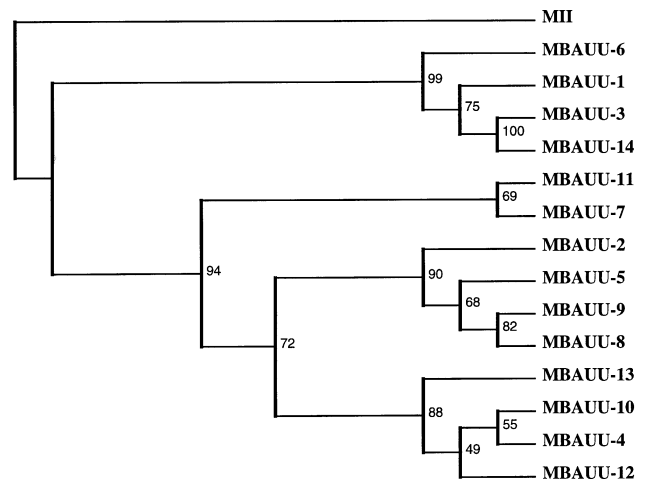

Fig. 9. Phylogenetic tree for the 14 serovars of *U. urealyticum* (based on the 5′-end of MBA gene nucleotide sequences of *U. urealyticum* ATCC strains). CLUSTAL was used for alignment, and PHYLIP was used for constructing the phylogenetic tree. The tree was formed using *Methanococcus jannaschii* as outgroup and was bootstrapped with 100 replications.

each biovar with few differences between serovars. The 16S–23S rRNA gene spacer region is shorter but more heterogeneous than the 16S rRNA gene. We found differences between the sequences of biovars 1 and 2 at 4·5% of sites, but none within the biovars.

Urease is a virulence factor in *U. urealyticum* and a number of other bacteria (Collins & D'Orazio, 1993; Ligon & Kenny, 1991; Smith *et al.*, 1993; Willoughby *et al.*, 1991). The urease subunit genes have been used to study the phylogenetic relationships of other urease-producing bacteria (Akashi *et al.*, 1996) as well as ureaplasmas (Blanchard, 1990; Ruifu *et al.*, 1997), in which it has been used to separate the two biovars. Using urease subunit genes *ureA*, *ureB*, partial *ureC* and adjoining upstream regions of *ureA*, *ureA–ureB* spacer and *ureB–ureC* spacer, we demonstrated base pair differences between the two biovars at 10·7% of sites. Again, sequences of these genes were well-conserved within biovars, although serovar 2 was distinguished from other serovars in biovar 2 by two separate single-base mutations, which were confirmed in a clinical isolate of the same serovar.

Urease subunit genes have been used previously to biotype isolates (Blanchard, 1990) and to demonstrate heterogeneity among different serovars (Ruifu *et al.*, 1997). In the former study (Blanchard, 1990), primers used for biotyping of *U. urealyticum* did not amplify urease genes of serovars 10 and 12 of biovar 2.

Fig. 8. Multiple sequence alignment of the 5′-end of MBA gene sequences of *U. urealyticum* serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, 13 (ATCC strains).

However, we were able to amplify all serovars of biovar 2 including serovars 10 and 12 (ATCC and UAB reference strains) but none of the serovars of biovar 1, using the same primers. Therefore these primers can be used to biotype *U. urealyticum*. Our results also showed that the homologies between the different serovars within each biovar were quite high and minor differences between sequences were inadequate to distinguish all serovars of *U. urealyticum* using this target (Ruifu *et al.*, 1997).

The MBAs of *U. urealyticum* are the major immunogens recognized during infection and are thought to be important virulence factors involved in interactions with host cells (Watson *et al.*, 1990; Zheng *et al.*, 1992, 1995). Their genes, which contain both biovar- and serovar-specific regions, were selected as appropriate targets for this phylogenetic study of *U. urealyticum*. We amplified and sequenced the 5′-end of MBA genes of all 14 serovars. The genes of biovar 2 were more highly conserved and longer than those of biovar 1 in the regions compared. The two biovars could be easily distinguished from each other. There were base changes in only six sites at the 5′-end of MBA gene of biovar 2, all of which were confirmed in UAB reference strains of the same serovars. They appear to be stable differences that could be used to subtype *U. urealyticum* biovar 2 by direct sequencing.

Base differences between sequences of the four serovars of biovar 1 were more numerous. However, serovars 3 and 14 were similar to each other, with only three base pair differences; serovars 1 and 6 differed by 16 bp. In common with others, we have shown that biovar 1 (serovars 1, 3, 6 and 14) is the predominant biovar isolated from the urogenital tract (Abele-Horn *et al.*, 1997a, b; Kong *et al.*, 1996). Therefore, we have developed a serovar-specific PCR/restriction endonuclease analysis procedure to differentiate these serovars (Kong *et al.*, 1999).

Our results showed that the heterogeneity of inter-gene spacer regions was higher than that of the genes themselves (Nakashima *et al.*, 1998). For example, the heterogeneity of the 16S–23S rRNA gene spacer region was 4·5%, compared with only 0·97% for the 16S rRNA gene; the heterogeneity in the sequence upstream of *ureA*, the *ureA–ureB* spacer and the *ureB–ureC* spacer genes varied from 17 to 24% compared with 6–11% for *ureA*, *ureB* and partial *ureC* genes; the heterogeneity in the sequence upstream of the 5′-end region of MBA genes was 41% compared with 26% in the 5′-end of MBA genes themselves. Therefore, primers based on the inter-gene spacer regions could be more discriminatory for biotyping than those based on the genes themselves. Biovar-specific primers based on the upstream region of the *ureA* subunit of the urease gene have been used previously (Blanchard, 1990).

Our study is the first phylogenetic analysis of *U. urealyticum* based on four important genes or DNA sequences from all 14 serovars. We confirmed and extended the findings of previous studies, that there were significant differences between the two biovars of *U. urealyticum*, which justify their being designated as different species (Robertson *et al.*, 1993; Teng *et al.*, 1995). This was first suggested in 1981, based on DNA–DNA hybridization, which showed only 40–60% homology between the biovars, enough to justify their separation into two species (Christiansen *et al.*, 1981). Subsequently other workers have supported this separation on the basis of differences in the sequences of 16S rRNA (Robertson *et al.*, 1993) and MBA genes (Teng *et al.*, 1994) between the two biovars. Although, in our study, the degree of heterogeneity in the 16S rRNA genes was relatively low (0·97%), it was higher in the other three gene regions sequenced, especially the MBA gene (32%). It has been shown previously that sequence identity of the 16S rRNA gene does not necessarily imply species identity. For example, DNA–DNA hybridization showed 99·5% sequence identity between the sequences of 16S rRNA genes of three different bacterial strains that were distinguishable as different *Bacillus* species on the basis of phenotypic and other genetic differences (Fox *et al.*, 1992).

Comparison of the sequences of the whole genomes of each biovar and serovar would be the most accurate way to study the phylogeny of *U. urealyticum* (Nakashima *et al.*, 1998). However, comparison of sequence data for several important genes, from each of the serovars, is more feasible and should provide almost as much information. Genes other than those that we sequenced, that are present in mycoplasmas (including ureaplasmas), and for which sequences have been reported by others, may be useful also for phylogenic analysis of *U. urealyticum*. They include the *tuf* gene which has been sequenced from *M. genitalium* (Loechel *et al.*, 1989), *M. pneumoniae* (Yogev *et al.*, 1990), *M. hominis* (S. A. Ladefoged & G. Christiansen, GenBank accession no. X57136), the 23S RNA gene and the 3′-end of the MBA gene of *U. urealyticum* (Zheng *et al.*, 1996). Like the urease and MBA genes, they may be more useful than the 16S rRNA gene, for phylogenetic analysis of *U. urealyticum* (Powers & Noller, 1993; Kamla *et al.*, 1996). However, we believe that the comparative sequence data provided in our study is adequate to justify our support for the division of *U. urealyticum* into two species.

The combination of DNA sequence data from several important genes with traditional methods of classification, may better reflect the phylogenetic relationships between different biovars and serovars better than either method alone. Our work provides further support for establishment of two different human *Ureaplasma* species as proposed by Robertson & Chen (1994) and Teng *et al.* (1994), namely *U. parvum* (currently *U. urealyticum* biovar 1) and *U. urealyticum* (currently biovar 2). In the future, clinical studies of human ureaplasma infection should distinguish these two new species to determine whether either is more likely to be associated with disease. Further work is

required to determine whether the current subdivision of these species into serovars requires modification, based on genetic data.

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