The phylogeny of *Ureaplasma urealyticum* based on the *mba* gene fragment

Christine L. Knox, Philip Giffard and Peter Timms

Author for correspondence: Peter Timms. Tel: +61 7 3864 2120. Fax: +61 7 3864 1534.
e-mail: p.timms@qut.edu.au

Centre for Molecular
Biotechnology, School of
Life Sciences, Queensland
University of Technology,
Brisbane, Queensland,
Australia 4001

Sequencing of *mba* gene fragments of reference strains of *Ureaplasma urealyticum* serovars 1, 3, 6, 14 and 8, in addition to 33 clinical *U. urealyticum* isolates is reported. A phylogenetic tree deduced from alignment of these sequences clearly demonstrates two major clusters (confidence limit 100%), which equate to the parvo and T960 biovars, and five types, which have been designated *mba* 1, 3, 6, 8 and X. These relationships are supported by bootstrap analysis. Polymorphisms within the *mba* fragment of types *mba* 1, 3 and 6 were used to define nine subtypes (*mba* 1a, 1b, 3a, 3b, 3c, 3d, 3e, 6a and 6b), thus facilitating high resolution typing of *U. urealyticum*. Inclusion of reference strains for serovars 1, 3, 6 and 8 in the *mba* typing scheme showed that the results of this analysis are broadly consistent with currently accepted serotyping. In addition, a *ure* gene fragment from nine of the clinical isolates was amplified and sequenced. Comparisons of the sequences clearly distinguished the two biovars of *U. urealyticum*; however, this fragment was invariant within the parvo biovar. This study has shown that the *mba* sequence can reveal the fine details of the relationships between *U. urealyticum* isolates and also supports the significant evolutionary gap between the two biovars.

Keywords: *Ureaplasma urealyticum*, molecular subtyping, phylogeny, *mba* gene, *ure* gene

INTRODUCTION

Members of the class *Mollicutes* are small, free-living, wall-less prokaryotes that are characterized by small genomes with a low G+C content and unusual nutritional requirements (Razin & Freundt, 1984). The largest family within the *Mollicutes* is the *Mycoplasmataceae* of which both the mycoplasmas and the ureaplasmas are members. The ureaplasmas are distinguished phenotypically by their ability to hydrolyse urea (Shepard & Luncford, 1976).

The phylogeny of the *Mollicutes*, the genus *Ureaplasma* and the species *urealyticum* has been defined by comparison of 16S rRNA gene sequences (Weisburg *et al.*, 1989; Maniloff, 1992; Robertson *et al.*, 1994; Harasawa & Cassell, 1996; Stemke & Robertson, 1996). There are five phylogenetic groups within the *Mollicutes* and the ureaplasmas belong to the *Mollicutes pneumoniae*–*Ureaplasma* clade (Weisburg *et al.*, 1989; Maniloff, 1992). Six phylogenetically distinct ureaplasma species, each isolated from a different host (humans and five different animals) have been defined (Harasawa & Cassell, 1996; Stemke & Robertson,

1996). Ureaplasmas isolated from humans are currently classified as a single species, *Ureaplasma urealyticum*. However, strains within this species can be clearly separated into two biovars by a variety of techniques including 16S rRNA sequencing (Robertson *et al.*, 1994). Other methods which have been used to distinguish the biovars of *U. urealyticum* include: DNA–DNA hybridization (Christiansen *et al.*, 1981), restriction endonuclease cleavage patterns (Razin *et al.*, 1983), RFLP analysis (Harasawa *et al.*, 1991), one- and two-dimensional gel electrophoresis of cell proteins (Mouches *et al.*, 1981; Swenson *et al.*, 1983), genome size as determined by PFGE (Robertson *et al.*, 1990), sensitivity to manganese salts (Robertson & Chen, 1984), and enzyme profiles (Davis & Villaneuva, 1990). More recently the biovars have also been distinguished by PCR of the 16S rRNA gene (Robertson *et al.*, 1993), PCR of the Multiple Banded (MB) antigen gene (*mba*) (Teng *et al.*, 1994), and by arbitrarily primed PCR (Grattard *et al.*, 1995a, b; Kong *et al.*, 1996).

Whilst the biovar grouping is easily determined, the subgroups within these biovars are less well-defined.

U. ureaplasma strains are most commonly identified by serotyping. The parvo biovar (biovar 1) contains serovars 1, 3, 6 and 14 and the T960 biovar (biovar 2) contains serovars 2, 4, 5 and 7–13. There are a number of different serotyping techniques (Lin *et al.*, 1972; Shepard & Luncford, 1978; Robertson & Stemke, 1979, 1982; Quinn *et al.*, 1981; Naessens & Lauwers, 1987). However, these are difficult to perform since there are no commercially available antisera.

Apart from 16S rRNA comparisons, the two biovars have rarely been distinguished by direct gene sequencing since very few *U. urealyticum* genes have been characterized. Polymorphisms which differentiate the two biovars have, however, been detected in the urease gene (*ure*) (Blanchard, 1990; Neyrolles *et al.*, 1996), and recently the *mba* gene of serovars 3 and 14 have been compared (Zheng *et al.*, 1996). Whilst the phylogeny of the ureaplasmas has been broadly defined using 16S rRNA sequencing (Maniloff, 1992; Robertson *et al.*, 1994; Harasawa & Cassell, 1996), it has not been supported by detailed analysis of other gene loci. Therefore, the main objective of the present study was to determine the relationships between *U. urealyticum* reference serovars and clinical isolates by sequence analysis of gene fragments which are more likely to be polymorphic than the 16S rRNA. Two fragments were used in this study: (i) the 5' end and the upstream control region of the *mba* gene, and (ii) an internal fragment of the *ure* gene.

METHODS

Samples. *U. urealyticum* isolates used were: (i) the reference serovars (1, 3, 6, 14, and 8) which were provided by G. L. Gilbert (University of Sydney, Sydney, Australia), with the kind permission of H. L. Watson (University of Alabama, Birmingham, AL, USA) [serovars 1, 3, 6 and 8 were originally obtained from E. A. Freundt (Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark) and serovar 14 was from J. A. Robertson (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Canada)]; and (ii) 33 clinical samples from pregnant women, collected during a previous clinical study (Knox *et al.*, 1997). All ureaplasmas were cultured and stored as previously described (Knox *et al.*, 1997) using A8 agar medium (modified) (Becton Dickinson) and Shepard's U9B and 10B liquid broth (Shepard & Luncford, 1976, 1978).

DNA preparation. *Ureaplasma* DNA was prepared by one of three methods. (i) Initially stored clinical samples of *U. urealyticum* were thawed and subcultured in 1.8 ml 10B broth and incubated at 37 °C until an alkaline colour change was observed. Broths were boiled for 20 min, then centrifuged for 5 min at 15000 g (Beckman Microfuge E). The supernatant was collected and then used for *mba* gene PCR. Another previously described method (Blanchard *et al.*, 1991) was also used with greater success, i.e. (ii) stored clinical samples (U9B broth) of *U. urealyticum* were thawed and 250 µl sample was centrifuged at 15900 g (Beckman Microfuge E) for 20 min at 4 °C. The pellet was resuspended in 50 µl solution A (10 mM Tris/HCl pH 8.3, 100 mM KCl, 2.5 mM MgCl₂) and 50 µl solution B (10 mM Tris/HCl pH 8.3, 2.5 mM MgCl₂, 1%, v/v, Tween 20, 1%, v/v, Triton

X-100) with 120 µg proteinase K ml⁻¹. The suspension was incubated for 1 h at 60 °C, followed by 10 min at 94 °C. It was then cooled and 5 µl of each sample was used for *mba* gene PCR or stored at -20 °C until PCR analysis. (iii) Stored clinical samples and the reference strain serovars 1, 3, 6, 14 and 8, were inoculated into 25 ml 10B broth and incubated at 37 °C until an alkaline colour change was observed. The broth was then centrifuged at 24000 g (Beckman J2-21M/E) for 1 h. The pellet was washed in 2 ml cold TE buffer and re-centrifuged for 15 min at 15900 g (Beckman Microfuge E) at 4 °C. The pellet was resuspended in equal volumes (250 µl) of solution A and solution B with 120 µg proteinase K ml⁻¹ and then incubated for 1 h at 60 °C. Samples were cooled and the DNA was extracted (Van Kuppeveld *et al.*, 1992) with equal volumes of (a) phenol, (b) phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), and (c) chloroform/isoamyl alcohol (24:1, v/v) and then precipitated from the aqueous phase with 0.1 vol. 3 M sodium acetate (pH 5.2) and 2 vol. ethanol. The DNA pellet was resuspended in 25 µl water.

mba gene PCR and sequencing

Primers. The primers used in this study were derived from the published nucleotide sequence of the serovar 3 *mba* gene (Teng *et al.*, 1994). This gene encodes a surface exposed protein (MB) and contains a 5', 450 bp region plus a 3' region at position 451–1206 of identical 18 nucleotide repeats (Zheng *et al.*, 1995). Primers UMS-125 (5' GTATTGCAATCTTTATATGTTTTTCG 3') and UMA226 (5' CAGC-TGATGTAAGTGCAGCATTAAATTC 3') (Teng *et al.*, 1994) were selected to amplify the 5' region from position -150 to 253 (serovar 3) which produced a 403 or 404 bp product for parvo biovar isolates and a 448 bp product for biovar T960 isolates.

PCR conditions. The 50 µl reaction mixture contained 1 U *Taq* polymerase (Boehringer Mannheim), 10 × PCR buffer (100 mM Tris/HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3; Boehringer Mannheim), 200 µM each dNTP (Boehringer Mannheim), water, 0.25 µM each primer and DNA template (5 µl prepared clinical specimen or 1 µl extracted DNA). Amplification was performed in a thermal cycler (Perkin-Elmer) programmed for 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final cycle of 72 °C for 5 min.

DNA sequencing. The PCR products were separated by electrophoresis in a 1% (w/v) low-melting-point agarose gel and the *mba* gene product was excised and purified using a Wizard PCR Preps DNA Purification system (Promega). Nucleotide sequencing was performed by the dideoxy method (Sanger *et al.*, 1977) using an Applied Biosystems 373A DNA sequencer (Perkin Elmer Cetus). The products were sequenced in both directions using the forward (UMS-125) and reverse (UMA226) primers and repeat PCR and repeat sequencing were performed until all anomalies were resolved. All nucleotide sequences were first edited to a 310 or 311 bp fragment (parvo biovar) or a 315 bp fragment (T960 biovar) and then aligned with the *U. urealyticum mba* gene sequence of the serovar 3 reference strain, GenBank accession number L20329 (Zheng *et al.*, 1995), using CLUSTAL W (Thompson *et al.*, 1994).

Urease gene PCR and sequencing

Primers. For the amplification of the urease gene, the previously published primers (Willoughby *et al.*, 1991) 14b (5' CCAGGTAAATTAGTACCAGG 3'; sense) and 72c (5'

Fig. 1. Nucleotide sequence alignment of the *mba* fragments of the *mba* subtypes and the reference serovars 1, 3, 6, 8 and 14. A dash indicates that the position in the alignment is conserved. The dots indicate spaces between adjacent nucleotides introduced for maximum alignment. The underlined areas are the predicted promoter consensus site, the ribosomal binding site (RBS) and the signal sequence (Zheng *et al.*, 1996).

CCTAATCTAACGCTATCACC 3'; antisense) were used to produce a 458 bp product which encodes a region of the gene incorporating the C-terminal region of the β subunit (14 kDa) of urease, a 58 bp intergenic region and the N-terminal region of the α subunit (72 kDa) of urease.

PCR conditions. This PCR reaction was performed in a total volume of 50 μ l and the mixture contained 1 U *Taq* polymerase (Boehringer Mannheim), 10 $\times$ PCR buffer (100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3;

Boehringer Mannheim), 400 μ M each dNTP (Boehringer Mannheim), water, 20 pmol of each primer and DNA template (1 μ l extracted DNA). The thermal cycler (Perkin-Elmer) was programmed for 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 1 min and extension at 72 $^{\circ}$ C for 1 min, followed by a final cycle of 72 $^{\circ}$ C for 5 min. The PCR products were purified for sequencing as described for the *mba* gene. The nucleotide sequences were edited to a 389 bp fragment and aligned with three *U. urealyticum*

urease gene sequences [GenBank accession nos: X51315 (Blanchard, 1990), M36190 (Willoughby *et al.*, 1991) and L40489 (Neyrolles *et al.*, 1996)] with CLUSTAL W (Thompson *et al.*, 1994).

Phylogenetic analysis. Sequence homology was compared and a distance matrix was generated using PHYLIP (version 3.5) (Felsenstein, 1993). The distance matrix was then used to construct an unrooted phylogenetic tree using the neighbour-joining method (Saitou & Nei, 1987). Confidence limits on branching orders were tested by bootstrap analysis (Felsenstein, 1993) using the neighbour-joining and bootstrap applications in CLUSTAL W (Thompson *et al.*, 1994).

RESULTS

mba gene sequencing identifies 11 subtypes of *U. urealyticum*

mba gene fragments were amplified from the five reference serovars tested and 33 clinical samples. The reference serovars 1, 3, 6 and 14 and 31 of the clinical samples produced a 403 or 404 bp fragment, whereas serovar 8 and the other two clinical isolates produced a 448 bp fragment. Alignment of these 38 *mba* gene sequences identified 11 *U. urealyticum* subtypes which, on the basis of their sequence similarities and amplified fragment sizes, could be separated into two major clusters. The phylogenetic tree deduced from this alignment clearly showed these clusters as two widely separated branches (confidence limit 100%) which equate to the parvo biovar and the T960 biovar of *U. urealyticum* (Figs 1 and 2).

Within the parvo biovar there are three major clusters which have been designated *mba* 1, 3 and 6. These distinct types are supported by: (i) the confidence

limits of the branching order, *mba* 1 (confidence limit 98.4%), *mba* 3 (95.9%) and *mba* 6 (98.9%) (Fig. 2) as determined by bootstrapping; and (ii) the similarity matrix (Table 2) which shows that the isolates within each of these types exhibit a homology of $\geq 98\%$ whilst the homology between these types is $< 97\%$. Using these criteria and to minimize subjectivity, an *mba* type has been arbitrarily defined in this study as *U. urealyticum* strains with $\geq 98\%$ homology. A workable nomenclature has also been devised from these data that is as consistent as practicable with serotyping. Consequently, each *mba* type has been numbered according to the serovar that is found within the cluster (Fig. 2). The reference serovar 1 and eight clinical isolates were identified as type *mba* 1; type *mba* 6 contains the reference serovar 6 and six clinical isolates. The third cluster contained two reference serovars (3 and 14) as well as 17 clinical isolates. However, since the sequence identical to serovar 3 was detected most frequently in this study, this cluster was designated type *mba* 3.

The *mba* types 1, 3 and 6 (parvo biovar) could be further subdivided into eight subtypes (Table 1) (Fig. 1). Within type *mba* 3, there were four subtypes (*mba* 3a, 3b, 3c and 3d). *mba* subtypes 3b and 3c each differed from *mba* 3a by only a single bp change. However, there were 3 bp differences between *mba* 3a and *mba* 3d. The sequence of subtype *mba* 3a is identical to that of the reference strain serovar 3 and the published sequence of serovar 3 (Zheng *et al.*, 1995). In this study, serovar 14 was classified as an *mba* 3 subtype (*mba* 3e) rather than as a separate type since there are only 3 bp differences between serovar 14 and type *mba* 3a which equates to a homology of 99%

Fig. 2. Phylogenetic tree of *U. urealyticum* based on the CLUSTAL W alignment of the *mba* gene fragment of the reference serovars 1, 3, 6, 14 and 8 and 33 clinical isolates. Bootstrap values (%) were obtained from 1000 resamplings.

Table 1. Comparison of *mba* and *ure* subtyping schemes with previous subtyping techniques for *U. urealyticum*

Biovar	MB antigen PCR*	16S rRNA PCR†	Serotyping	16S rRNA analysis‡	<i>mba</i> typing (this study)	<i>mba</i> subtyping (this study) [no. clinical isolates]	<i>ure</i> typing (this study) [no. clinical isolates]
Parvo	Primer UM-1* (403 or 404 bp product)	Primer U3† (1300 bp product)	Serovar 3	Serovar 3	<i>mba</i> 3	<i>mba</i> 3a [13]	<i>ure</i> I [3]
						<i>mba</i> 3b [2] <i>mba</i> 3c [1] <i>mba</i> 3d [1] <i>mba</i> 3e (serovar 14)	<i>ure</i> I [1]
			Serovar 14	Serovar 14			
			Serovar 1	Serovar 1	<i>mba</i> 1	<i>mba</i> 1a [7] <i>mba</i> 1b [1]	<i>ure</i> I [1] <i>ure</i> I [1]
			Serovar 6	Serovar 6	<i>mba</i> 6	<i>mba</i> 6a [3] <i>mba</i> 6b [3]	<i>ure</i> I [2]
						<i>mba</i> 8 [1]	
T960	Primer UM-1* (448 bp product)	Primer U8† (1300 bp product)	Serovar 8	Serovar 8	<i>mba</i> 8		<i>ure</i> II [1]
			Serovars 2, 4, 5, 7, 9–13	Serovars 2, 5	<i>mba</i> X	<i>mba</i> X [1]	

* Teng *et al.* (1994).† Robertson *et al.* (1993).‡ Robertson *et al.* (1994).**Table 2.** Similarity matrix of the *mba* subtypes of *U. urealyticum* based on the *mba* fragment sequence comparisons

Strain	Homology (%)										
	<i>mba</i> 1a	<i>mba</i> 1b	<i>mba</i> 3a	<i>mba</i> 3b	<i>mba</i> 3c	<i>mba</i> 3d	<i>mba</i> 3e	<i>mba</i> 6a	<i>mba</i> 6b	<i>mba</i> 8	<i>mba</i> X
<i>mba</i> 1a											
<i>mba</i> 1b	99										
<i>mba</i> 3a	96	96									
<i>mba</i> 3b	96	96	99								
<i>mba</i> 3c	96	96	99	99							
<i>mba</i> 3d	96	96	99	98	98						
<i>mba</i> 3e	96	96	99	98	98	98					
<i>mba</i> 6a	94	94	94	94	94	94	94				
<i>mba</i> 6b	95	94	94	93	93	93	94	99			
<i>mba</i> 8	74	74	73	73	73	73	74	74	74		
<i>mba</i> X	73	72	72	71	71	72	72	72	72	97	

(Table 2). Within type *mba* 1, there were two subtypes (*mba* 1a and 1b) which differed by only a single bp change. Similarly there were two *mba* 6 subtypes that differed by only a single bp change.

The two T960 biovar isolates studied were identified as two separate types, *mba* 8 and *mba* X, using the same criteria. These two isolates differed from each other by 6 bp with a homology of only 97% (Table 2). The *mba* sequences of *mba* 1a, *mba* 6a and *mba* 8 are identical to those of the reference serovars 1, 6 and 8, respectively. Since a serovar with $\geq 98\%$ similarity to *mba* X has not yet been identified, this separate designation has been used.

Urease gene analysis

A fragment of the *ure* gene was also sequenced to confirm the phylogenetic groupings within the *U. urealyticum* species. Partial urease sequences (Fig. 3) were generated for nine clinical isolates (eight parvo biovar isolates and one T960 biovar isolate). These were aligned and compared with the three available sequences in GenBank: accession numbers X51315 (Blanchard, 1990) and M36190 (Willoughby *et al.*, 1991), both serotype 8 *U. urealyticum*; and accession number L40489 (Neyrolles *et al.*, 1996), a serotype 1 *U. urealyticum*. Of the nine clinical isolates studied, eight isolates (of the parvo biovar) had sequences identical

	585	β subunit	612
Serovar 1 (strain 7 Genbank Accession No L40489)	<u>AAATTGTGATGAATGAAGGTAGAGAGGC</u>		
Parvo biovar, 8 clinical isolates (this study)	-----		
Serovar 8 (UUUREASE Genbank Accession No.X51315)	-----C-----A--		
Serovar 8 (URELOCAB Genbank Accession No.36190)	-----A-----C-----A--		
Biovar T960, 1 clinical isolate-(this study)	-----C-----A--		
			672
serovar 1	<u>AAAAGTAATTAGTATTAATACTGGGGACCGTCCTATACAAGTTGGATCACATTTTCA</u>		
8 isolates	-----		
Serovar 8	-----C--C-----T-----C-----C--		
Serovar 8	-----C--C-----T-----C-----G--		
1 isolate	-----C--C-----T-----C-----C--		
			732
serovar 1	<u>CTTGTTTGAAGTGAATAGTGCATTAGTATTTTTTGATGAAAAAGGAAATGAAGATAAAGA</u>		
8 isolates	-----		
serovar 8	---A---ACA-----C-----C---C---		
serovar 8	---A---ACA-----C-----C---C---		
1 isolate	---A---ACA-----C-----C---C---		
			792
serovar 1	<u>ACGCAAAGTTGCTTATGGACGACGTTTCGATATCCATCAGGTACTGCTATTCGTTTTGA</u>		
8 isolates	-----		
serovar 8	---T-----T-----		
serovar 8	---T-----T-----		
1 isolate	---T-----T-----		
			852
serovar 1	<u>ACCAGGAGATAAAAAAGAGTTTCAATTATTGATTAGCCGGAACACGC.GAAGTTTGAG</u>		
8 isolates	-----		
serovar 8	-----C-----G-----T-----TT-----		
serovar 8	-----C-----G-----T-----TT-----		
1 isolate	-----C-----G-----T-----T-----		
			868
serovar 1	<u>GTGTAAATGGCTTAGTTAATGGAAAA..CTTAAAAATAAACTCTATTTTACAAGTTTC</u>		905
8 isolates	-----		
serovar 8	-----C-----C-----..-----		
serovar 8	-----C-----C--C--AC-----..-----		
1 isolate	-----C-----C--C--..-----		
			962
		$\rightarrow \alpha$ subunit	
serovar 1	<u>TACTATAGATAAAAAGGGGAACATTATGTTTAAATTTCAAGAAAAATTATTCAGATTT</u>		
8 isolates	-----		
serovar 8	-----CG--..-----C-----C-		
serovar 8	-----CG--..-----C-----C-		
1 isolate	-----CG--..-----C-----C-		
			965
serovar 1	<u>ATA</u>		
8 isolates	---		
serovar 8	---		
serovar 8	---		
1 isolate	---		

Fig. 3. Sequence alignment of the *ure* gene of serovars 1 (*ure* I) and 8 (*ure* II) and the *ure* fragments of 9 clinical isolates of *U. urealyticum*. A dash indicates that the position in the alignment is conserved. The dots indicate spaces between adjacent nucleotides introduced for maximum alignment. The underlined areas are the beta and alpha subunits (Blanchard, 1990).

to that of serotype 1 (L40489) *U. urealyticum* (*ure* I). These eight parvo biovar isolates represented *mba* subtypes 3a, 3b, 1a, 1b and 6a (Table 1). The sequence of the remaining clinical isolate (a T960 biovar isolate, type *mba* 8) differed from the previously published sequences of two different strains of serovar 8, i.e. accession numbers X51315 (by 3 bp differences), and M36190 (by 8 bp differences), both of T960 biovar (*ure* II). Small differences within this fragment in the T960 biovar have previously been reported (Willoughby *et al.*, 1991).

Despite sequencing only a single T960 biovar isolate, these results also clearly demonstrate the two clusters of *U. urealyticum* (Table 1) since there were 28 bp differences between the parvo and T960 biovar isolates.

DISCUSSION

The detection and typing of *U. urealyticum* has always been difficult. Culture is expensive and time-consuming and, whilst still used routinely, more efficient

alternatives are needed. Previously, *U. urealyticum* has been subtyped into 14 serovars by serotyping (Robertson & Stemke, 1982). However, results may be conflicting due to cross reactivity and also the common expression of epitopes (Cassell *et al.*, 1993), a problem which is exacerbated by the lack of standardized reagents and methods. Serotyping of clinical isolates is particularly difficult since techniques often require the culture of primary isolates prior to serotyping making it difficult to detect serovars present in lower concentrations and those that are more fastidious and grow more slowly (Quinn *et al.*, 1981; Cassell *et al.*, 1993). The immunoperoxidase assay (Quinn *et al.*, 1981) and the modified indirect immunofluorescence test (Naessens & Lauwers, 1987) can both be performed on ureaplasma primary isolation plates. However, again there are no standardized procedures or commercially available products. More recently, DNA-based typing approaches have been used with some success (Teng *et al.*, 1994; Grattard *et al.*, 1995a, b; Kong *et al.*, 1996; Abele-Horn *et al.*, 1997).

Robertson *et al.* (1994) have previously described the relationship between different serovars of *U. urealyticum*. Analysis of a 1422–1430 base region of the 16S rRNA showed clear but minor (98.5–98.9% homology) differentiation of the two biovars of *U. urealyticum* (Robertson *et al.*, 1994). Furthermore, within each biovar the serovars (serovars 2, 5 and 8, T906 biovar; serovars 1, 3, 6 and 14, parvo biovar) could be distinguished on the basis of seven polymorphic base positions. However, this study did not confirm these relationships using a bootstrap analysis.

In the current study, direct sequencing of the *mba* and the *ure* gene fragments was used to reveal the relationship between *U. urealyticum* strains. The *mba* gene was selected for sequencing since the MB antigen, the predominant antigen recognized in human ureaplasma infections (Watson *et al.*, 1990), is present in all reference serovars of *U. urealyticum* (Teng *et al.*, 1994) and since variation in this gene is thought to account for antigenic variation within the species and therefore may be the basis of the current typing schemes (Zheng *et al.*, 1996). Furthermore, the *mba* gene has previously been shown to be variable, with biovar specificity residing in the 5' region (Teng *et al.*, 1994). Sequencing of the *mba* fragments and upstream regions identified sufficient polymorphic base positions for subtyping of *U. urealyticum* and to give significance to phylogenetic branching orders. Analysis of the deduced phylogenetic tree shows two major groups which represent the two *U. urealyticum* biovars (71–74% homology) (Table 2). Within these two biovars five types (*mba* 1, 3, 6, 8 and X, homology $\geq 98\%$) are also clearly distinguishable and confirmed by the confidence limits on the branching order. Direct sequencing of the *mba* gene fragment has further elucidated the relationship between strains of *U. urealyticum* and produced a subtyping technique with higher resolution than other methods previously used for the detection and typing of *U. urealyticum*. Inclusion of the four reference

serovars (1, 3, 6 and 8) in the *mba* typing scheme shows that it is broadly consistent with currently accepted serotyping. However, the scheme has also revealed an inconsistency. Using serotyping and 16S rRNA analysis, serovar 14 (Table 1) is identified as a separate type, whereas using *mba* subtyping, serovar 14 is classified as a subtype of *mba* 3. Presently *mba* subtyping defines the two biovars, five *mba* types and nine *mba* subtypes of *U. urealyticum*. However, it could be extended with further analysis of clinical isolates and other reference serovars of *U. urealyticum*.

Recently ureaplasmas have been detected by PCR in clinical specimens and broadly grouped into two clusters (Kong *et al.*, 1996; Abele-Horn *et al.*, 1997). Although biotyping is easier to perform than serotyping, it does not differentiate the strains sufficiently and is therefore of limited value in epidemiological studies. In contrast, *mba* subtyping is a readily reproducible molecular method and, since it can be used to detect and type *U. urealyticum* in both cultures and original clinical specimens, it will be more useful for epidemiological studies. Furthermore, by exploiting the polymorphic base positions in the *mba* fragment it should be possible to design primers which will selectively amplify all *mba* types directly from clinical specimens and potentially reveal mixed types.

Direct sequencing of the *ure* gene fragment of nine clinical isolates also distinguished the two genomic clusters of *U. urealyticum*. Our results suggest that selection for conservation is greater in the *ure* gene fragment than in the *mba* gene fragment as there is no variation within the fragment in the parvo biovar. Consequently, subtyping based on the *mba* gene fragment is more meaningful and will be more useful for the investigation of the relative pathogenicity of different strains of *U. urealyticum*.

U. urealyticum can now clearly be differentiated into two biovars by a variety of typing techniques (Table 1) including PCR of the *mba* gene and 16S rRNA, and sequence analysis of 16S rRNA. Direct sequencing of both the *mba* and *ure* gene fragments detected only small differences within the biovars and much larger differences in sequence between the biovars. This is further evidence to support the separation of the species *U. urealyticum* into two taxonomic groups.

REFERENCES

- Abele-Horn, M., Wolff, C., Dressel, P., Pfaff, F. & Zimmermann, A. (1997). Association of *Ureaplasma urealyticum* biovars with clinical outcome for neonates, obstetric patients, and gynecological patients with pelvic inflammatory disease. *J Clin Microbiol* **35**, 1199–1202.
- Blanchard, A. (1990). *Ureaplasma urealyticum* urease genes; use of a UGA tryptophan codon. *Mol Microbiol* **4**, 669–676.
- Blanchard, A., Gautier, M. & Mayau, V. (1991). Detection and identification of mycoplasmas by amplification of rDNA. *FEMS Microbiol Lett* **81**, 37–42.
- Cassell, G. H., Waites, K. B., Watson, H. L., Crouse, D. T. & Harasawa, R. (1993). *Ureaplasma urealyticum* intrauterine in-

- fection: role in prematurity and disease in newborns. *Clin Microbiol Rev* **6**, 69–87.
- Christiansen, C., Black, F. T. & Freundt, E. A. (1981). Hybridization experiments with deoxyribonucleic acid from *Ureaplasma urealyticum* serovars I to VIII. *Int J Syst Bacteriol* **31**, 259–262.
- Davis, J. W., Jr & Villaneuva, I. (1990). Enzyme differences in serovar clusters of *Ureaplasma urealyticum*, *U. diversum*, and ovine ureaplasmas. *Zentralblatt Bakteriologie Suppl* **20**, 661–665.
- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5c, Department of Genetics, University of Washington, Seattle, WA, USA.
- Grattard, F., Pozzetto, B., De Barbeyrac, B., Renaudin, H., Clerc, M., Gaudin, O. G. & Bebear, C. (1995a). Arbitrarily-primed PCR confirms the differentiation of strains of *Ureaplasma urealyticum* into two biovars. *Mol Cell Probes* **9**, 383–389.
- Grattard, F., Soleihac, B., De Barbeyrac, B., Bebear, C., Seffert, P. & Pozzetto, B. (1995b). Epidemiologic and molecular investigations of genital mycoplasmas from women and neonates at delivery. *Pediatr Infect Dis J* **14**, 853–858.
- Harasawa, R. & Cassell, G. H. (1996). Phylogenetic analysis of genes coding for 16S rRNA in mammalian ureaplasmas. *Int J Syst Bacteriol* **46**, 827–829.
- Harasawa, R., Dybvig, K., Watson, H. L. & Cassell, G. H. (1991). Two genomic clusters among the 14 serovars of *Ureaplasma urealyticum*. *Syst Appl Microbiol* **14**, 393–396.
- Knox, C. L., Cave, D. G., Farrell, D. J., Eastment, H. T. & Timms, P. (1997). The role of *Ureaplasma urealyticum* in adverse pregnancy outcome. *Aust N Z J Obstet Gynaecol* **37**, 45–51.
- Kong, F., Zhu, X. & Zhou, J. (1996). Grouping and typing of *Ureaplasma urealyticum*. *Chung-Hua I Hsueh Tsa Chih* **76**, 138–140.
- Lin, J.-S. L., Kendrick, M. I. & Kass, E. H. (1972). Serologic typing of human genital T-Mycoplasmas by a complement-dependent mycoplasmaicidal test. *J Infect Dis* **126**, 658–663.
- Maniloff, J. (1992). Phylogeny of mycoplasmas. In *Mycoplasmas: Molecular Biology and Pathogenesis*, pp. 549–559. Edited by J. Maniloff, R. N. McElhaney, L. R. Finch & J. B. Baseman. Washington, DC: American Society for Microbiology.
- Mouches, C., Taylor-Robinson, D., Stipkovits, L. & Bove, J. M. (1981). Comparison of human and animal ureaplasmas by one- and two-dimensional protein analysis on polyacrylamide slab gel. *Ann Inst Pasteur Microbiol* **132B**, 171–196.
- Naessens, A. & Lauwers, S. (1987). Modified indirect immunofluorescence test for serotyping large numbers of *Ureaplasma urealyticum* clinical isolates. *J Clin Microbiol* **25**, 191–192.
- Neyrolles, O., Ferris, S., Behbahani, N., Montagnier, L. & Blanchard, A. (1996). Organization of *Ureaplasma urealyticum* urease gene cluster and expression in a suppressor strain of *Escherichia coli*. *J Bacteriol* **178**, 647–655.
- Quinn, P. A., Arshoff, L. U. & Li, H. C. S. (1981). Serotyping of *Ureaplasma urealyticum* by immunoperoxidase assay. *J Clin Microbiol* **13**, 670–676.
- Razin, S. & Freundt, E. A. (1984). The Mycoplasmas. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 740–773. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Razin, S., Harasawa, R. & Barile, M. F. (1983). Cleavage patterns of *Mycoplasma* chromosome, obtained by using restriction endonucleases, as indicators of genetic relatedness among strains. *Int J Syst Bacteriol* **33**, 201–206.
- Robertson, J. A. & Chen, M. H. (1984). Effects of manganese in the growth and morphology of *Ureaplasma urealyticum*. *J Clin Microbiol* **19**, 857–864.
- Robertson, J. A. & Stemke, G. W. (1979). Modified metabolic inhibition test for serotyping strains of *Ureaplasma urealyticum* (T-strain Mycoplasma). *J Clin Microbiol* **9**, 673–676.
- Robertson, J. A. & Stemke, G. W. (1982). Expanded serotyping scheme for *Ureaplasma urealyticum* strains isolated from humans. *J Clin Microbiol* **15**, 873–878.
- Robertson, J. A., Pyle, L., Stemke, G. W. & Finch, L. R. (1990). *Ureaplasma urealyticum* shows diverse genome size by pulse-field electrophoresis. *Nucleic Acids Res* **18**, 1451–1455.
- Robertson, J. A., Vekris, A., Bebear, C. & Stemke, G. W. (1993). Polymerase chain reaction using 16S rRNA gene sequences distinguishes the two biovars of *Ureaplasma urealyticum*. *J Clin Microbiol* **31**, 824–830.
- Robertson, J. A., Howard, L. A., Zinner, C. L. & Stemke, G. W. (1994). Comparison of the 16S rRNA genes within the T960 and parvo biovars of ureaplasmas isolated from humans. *Int J Syst Bacteriol* **44**, 836–838.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Shepard, M. C. & Lunceford, C. D. (1976). Differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T mycoplasmas) in primary cultures of clinical material. *J Clin Microbiol* **3**, 613–625.
- Shepard, M. C. & Lunceford, C. D. (1978). Serological typing of *Ureaplasma urealyticum* isolates from urethritis patients by an agar growth inhibition method. *J Clin Microbiol* **8**, 566–574.
- Stemke, G. W. & Robertson, J. A. (1996). *Ureaplasma gallorale*, an isolate from chickens, is most closely related to the human isolate, *U. urealyticum*. *Int J Syst Bacteriol* **46**, 1183–1184.
- Swenson, C. E., VanHamont, J. & Dunbar, B. S. (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.
- Teng, L.-J., Zheng, X., Glass, J. I., Watson, H. L., Tsai, J. & Cassell, G. H. (1994). *Ureaplasma urealyticum* biovar specificity and diversity are encoded in multiple-banded antigen gene. *J Clin Microbiol* **32**, 1464–1469.
- Thompson, J. D., Higgins, D. J. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Van Kuppeveld, F. J. M., van der Logt, J. T. M., Angulo, A. F., van Zoest, M. J., Quint, W. G. V., Niesters, H. G. M., Galama, J. M. D. & Melchers, W. J. G. (1992). Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl Environ Microbiol* **58**, 2606–2615.
- Watson, H. L., Blalock, D. K. & Cassell, G. H. (1990). Variable antigens of *Ureaplasma urealyticum* containing both serovar-specific and serovar-cross-reactive epitopes. *Infect Immun* **58**, 3679–3688.
- Weisburgen, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). A phylogenetic analysis of the Mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.

Willoughby, J. J., Russell, W. C., Thirkell, D. & Burdon, M. G. (1991). Isolation and detection of urease genes in *Ureaplasma urealyticum*. *Infect Immun* **59**, 2463–2469.

Zheng, X., Teng, L.-J., Watson, H. L., Glass, J. I., Blanchard, A. & Cassell, G. H. (1995). Small repeating units within the *Ureaplasma urealyticum* MB antigen gene encode serovar specificity

and are associated with antigen size variation. *Infect Immun* **63**, 891–898.

Zheng, X., Lau, K., Frazier, M., Cassell, G. H. & Watson, H. L. (1996). Epitope mapping of the variable repetitive region within the MB antigen of *Ureaplasma urealyticum*. *Clin Diagn Lab Immunol* **3**, 774–778.