The phylogeny of Ureaplasma urealyticum based on the mba gene fragment

THE PHYLOGENY OF *UREAPLASMA UREALYTICUM* BASED ON THE *MBA* GENE FRAGMENT

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Statement of Joint Authorship

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Sequenced the *U. urealyticum* reference serovars and clinical isolates. Aligned the sequences and constructed the phylogenetic tree. Interpreted the data and wrote the manuscript.

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ABSTRACT

Sequencing of *mba* gene fragments of reference strains of *Ureaplasma urealyticum* serovars 1, 3, 6, 14, in addition to 33 clinical *U. urealyticum* isolates is reported. A phylogenetic tree deduced from an alignment of these sequences clearly demonstrates two major clusters (confidence limit 100%), which equate to the parvo and T960 biovars, and five types which we have 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 the 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 sequence of the *mba* 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 procaryotes which are characterised by small genomes with a low G+C content and unusual nutritional requirements (20). 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 (30).

The phylogeny of the *Mollicutes,* the genus *Ureaplasma,* and the species *urealyticum* has been defined by comparison of 16S rRNA gene sequences (10, 15, 23, 32, 38). There are five phylogenetic groups within the *Mollicutes* and the ureaplasmas belong to the *M. pneumoniae - Ureaplasma* clade (15, 38). Six, phylogenetically distinct ureaplasma species, each isolated from a different host (humans and five different animals) have been defined (10, 32). Ureaplasmas isolated from humans are currently classified as a single species *U. urealyticum.* However strains within this species can be clearly separated into two biovars by a variety of techniques including 16S rRNA sequencing (23). Other methods which have been used to distinguish the biovars of *U. urealyticum* include: DNA-DNA hybridisation (5), restriction endonuclease cleavage patterns (21), RFLP analysis (11), one- and two- dimensional gel electrophoresis of cell proteins (16, 33), genome size as determined by PFGE (24), sensitivity to manganese salts (22) and enzyme profiles (6). More recently the biovars have also been distinguished by: PCR of the 16S rRNA gene (27); PCR of the Multiple Banded (MB) antigen gene (*mba*) (34); and by arbitrarily primed PCR (8, 9, 13).

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 (14, 17, 19, 25, 26, 31). 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 characterised. Polymorphisms which differentiate the two biovars have, however, been detected in the urease gene (*ure*) (2, 18), and recently the *mba* gene of serovars 3 and 14 have been compared (41). Whilst the phylogeny of the ureaplasmas has been broadly defined using 16S rRNA (10, 15, 23) 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.

MATERIALS AND 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, Denmark) and serovar 14 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 (12). All ureaplasmas were cultured and stored as previously described (12) using A8 agar medium (modified) (Becton Dickinson, Brisbane, Australia) and Shepard's U9B and U10B liquid broth (30, 31).

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 ^{0}C until an alkaline colour change was observed. Broths were boiled for 20 minutes, then centrifuged for 5 minutes at 15 000 x g (Beckman Microfuge E). The supernatant was collected and then used for *mba* gene PCR. More recently we have used another previously described method (3) with greater success, i.e. (ii) Stored clinical samples (U9B broth) of *U. urealyticum* were thawed and 250 μL of the sample was centrifuged at 15,900 x g (Beckman Microfuge E) for 20 min at 4 ^{0}C. The pellet was resuspended in 50 μL of Solution A (10 mM Tris HCl pH 8.3, 100 mM KCl, 2.5 mM $MgCl_2$) and 50 μL Solution B (10 mM Tris HCl pH 8.3, 2.5 mM $MgCl_2$, 1% Tween 20, 1% Triton X-100) with 120 μg/mL of proteinase K. The suspension was incubated for 1 hour at 60 ^{0}C, then 10 min at 94 ^{0}C . It was then cooled and 5 μL of each sample was used for *mba* gene PCR or stored at -20 ^{0}C until PCR analysis. (iii) Stored clinical samples and the reference strain serovars 1, 3, 6, 14 and 8, were inoculated into 25 mL of 10B broth and incubated at 37 ^{0}C until an alkaline colour change was observed. The broth was then centrifuged at 24,000 x g (Beckman J2-21M/E) for 1 hour. The pellet was washed in 2 mL of cold TE buffer and re-centrifuged for 15 mins at 15,900 x g (Beckman Microfuge E) at 4 ^{0}C. The pellet was resuspended in equal volumes (250 μL) of Solution A and Solution B with 120 μg/mL of proteinase K and then incubated for 1 hour at 60 ^{0}C. Samples were cooled and the DNA was extracted (36) with equal volumes of (a) phenol, (b) phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol), and (c) chloroform/isoamyl alcohol (24:1, vol/vol) and then precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The DNA pellet was resuspended in 25 μL of 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 (34). This gene codes a surface exposed protein (MB) and contains a 5', 450 bp region plus a 3' region from position 451 - 1206 of identical 18 nucleotide repeats (40). Primers UMS-125 (5'-GTATTTGCAATCTTTATATGTTTTCG-3') and UMA226 (5'-CAGCTGATGTA-AGTGCAGCATTAAATTC-3') (34) 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 1U of *Taq* polymerase (Boehringer Mannheim), 10x PCR buffer (100 mM Tris HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3, Boehringer Mannheim), 200 μM of each deoxynucleotide triphosphate (Boehringer Mannheim), water, 0.25 μM of each primer and DNA template (5 μL of prepared clinical specimen or 1 μL of extracted DNA). Amplification was performed in a thermal cycler (Perkin-Elmer) programmed for 35 cycles-denaturation at 94 ^{0}C for 30 s, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 1min, followed by a final cycle of 72 ^{0}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 excised and purified using a Wizard PCR Preps DNA Purification System (Promega). Nucleotide sequencing was performed using the dideoxy method (29) 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 311bp fragment (parvo biovar) or a 315 bp fragment (biovar T960) and then aligned with the *U. urealyticum mba* gene sequence of the serovar 3 reference strain, GenBank accession number L20329 (40), using Clustal W (35).

Urease Gene PCR and sequencing

Primers

For the amplification of the urease gene we used the previously published (39) primers 14b, (5'-CCAGGTAAATTAGTACCAGG-3'-sense) and 72c, (5'-CCTAATCTAACGCTATCACC-3'-antisense) to produce a 458 bp product which

encodes a region of the gene incorporating the C-terminal region of the β subunit (14 kD) of urease, a 58 bp intergenic region and the N-terminal region of the α subunit (72 kD) of urease.

PCR Conditions

This PCR reaction was performed in a total volume of 50 μL and the mixture contained 1 U of *Taq* polymerase (Boehringer Mannheim), 10x PCR buffer (100 mM Tris HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3, Boehringer Mannheim), 400 μM of each deoxynucleotide triphosphate (Boehringer Mannheim), water, 20 pmol of each primer and DNA template (1 μL of extracted DNA). The thermal cycler (Perkin-Elmer) was programmed for 35 cycles - denaturation at 94 ^{0}C for 30 s, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min, followed by a final cycle of 72 ^{0}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 numbers: X51315 (2), M36190 (39) and L40489 (18) using Clustal W (35).

Phylogenetic analysis

Sequence homology was compared and a distance matrix generated using Phylip (version 3.5) (7). The distance matrix was then used to construct an unrooted phylogenetic tree using the neighbour-joining (NJ) method (28). Confidence limits on branching orders were tested using bootstrap analysis (7) using the NJ and bootstrap applications in Clustal W (35).

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 demonstrated 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).

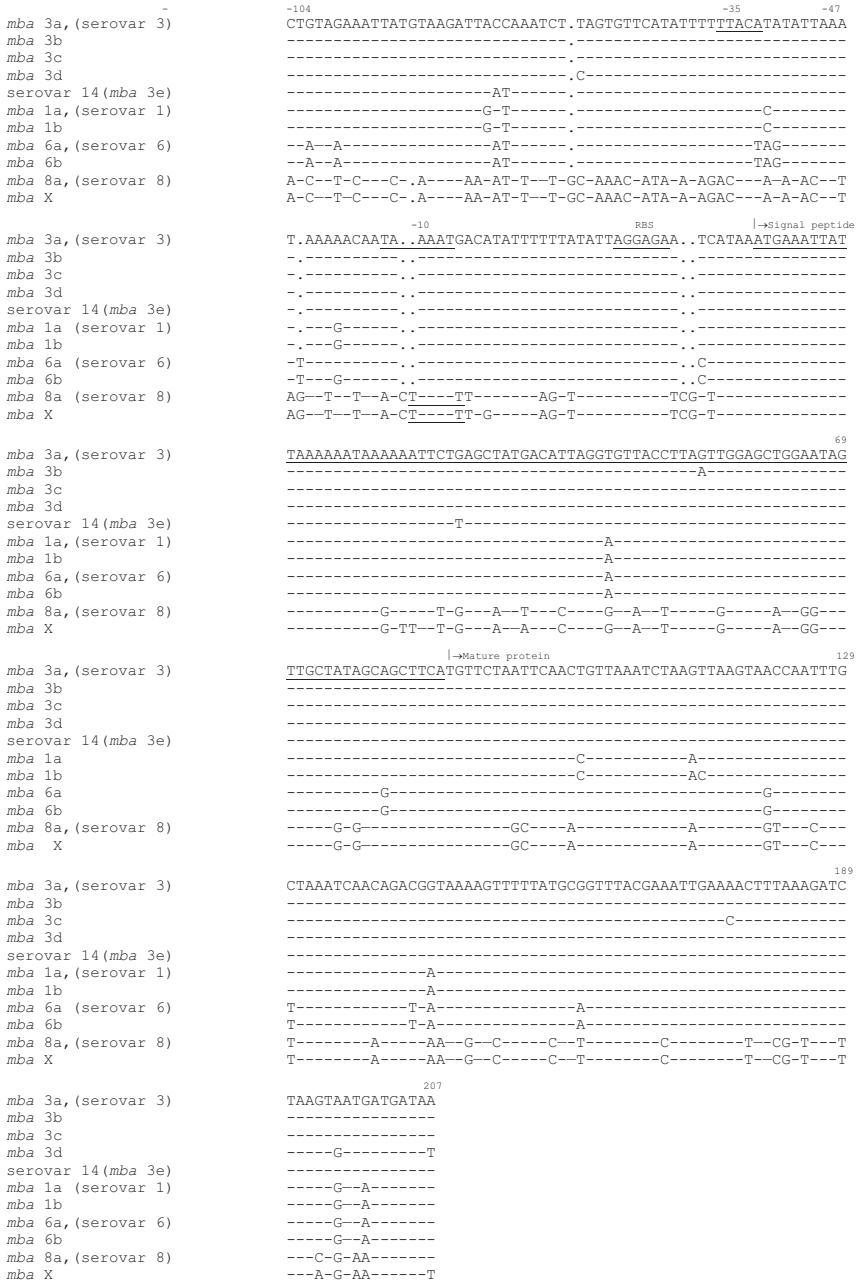

FIG. 1. Nucleotide sequence alignment of the *mba* fragments of the *mba* subtypes and the reference serovars 1, 3, 6, 8 and 14. A dash indicates that the position in the alignment is conserved. The dots indicate spaces between adjacent nucleotides introduced for maximum alignment. The underlined areas are the predicted promoter consensus site, the ribosomal binding site (RBS) and the signal sequence (41).

Within the parvo biovar there are three major clusters which we have designated as *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 an homology of ≥98% whilst the homology between these types is <97%. Using this criteria and to minimise subjectivity, an *mba* type has been arbitrarily defined in this study as *U. urealyticum* strains with ≥98% homology. A workable nomenclature has also been devised from these data that is as consistent as practicable with serotyping.

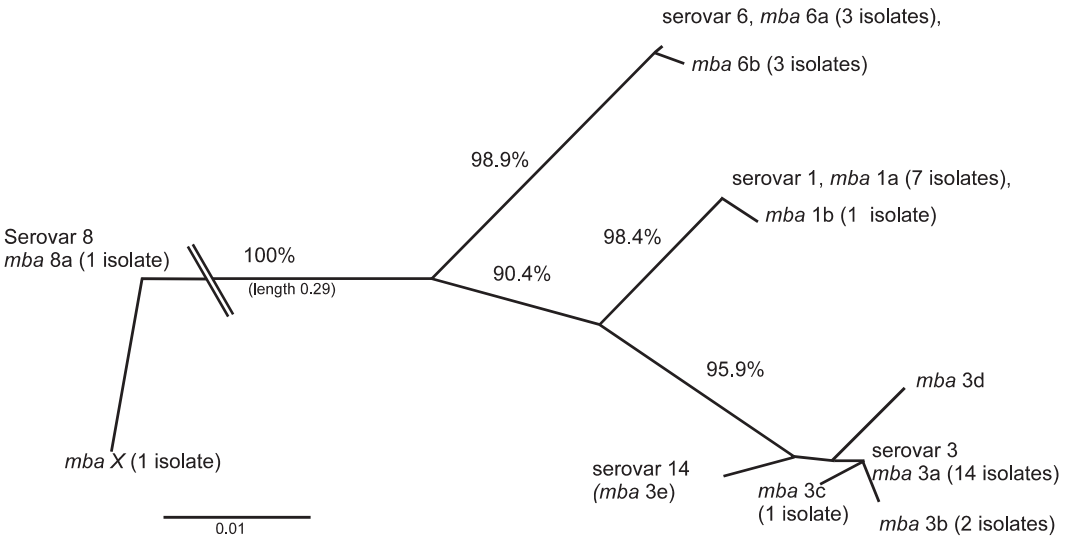

FIG. 2. Phylogenetic tree of *U. urealyticum* based in the Clustal W alignment of the *mba* gene fragment of the reference serovars 1, 3, 6, 14, and 8 and 33 clinical isolates. Bootstrap percentage values were obtained from 1000 resamplings.

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 we designated this cluster as 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, 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 the reference strain serovar 3 and the published sequence of serovar 3 (40). 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 an homology of 99% (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.

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

Biovars	MB antigen PCR Teng *et al*. (34)	16S Rrna PCR Robertson *et al* (27)	Serotyping	16S rRNA analysis Robertson *et al* (23)	*mba* typing (this study)	*mba* subtyping (this study) [no. clinical isolates]	*ure* typing (this study) [no. clinical isolates]
parvo	Primers UM-1 (34)	Primers U3 (27)	serovar 3	serovar 3	3	3a (13)	I (3)
						3b (2)	(1)
	403 or 404 bp product	1300 bp product				3c (1)	
						3d (1)	
			serovar 14	serovar 14		3e(serovar 14)	
			serovar 1	serovar 1	1	1a (7)	(1)
						1b (1)	(1)
			serovar 6	serovar 6	6	6a (3)	(2)
						6b (3)	
T 960	Primers UM-1 (34)	Primers U8 (27)	serovar 8	serovar 8	8	8 (1)	II (1)
	448bp product	1300bp product	serovars 2, 4, 5, 7, 9-13	serovars 2, 5	X	X (1)	

TABLE 2. Similarity matrix of the *mba* subtypes of *U. urealyticum* based on the *mba* fragment sequence comparisons.

	% homology										
Strains	*mba* 1a	*mba* 1b	*mba* 3a	*mba* 3b	*mba* 3c	*mba* 3d	*mba* 3e	*mba* 6a	*mba* 6b	*mba* 8	*mba* X
*mba*1a											
mba 1b	99										
mba 3a	96	96									
mba 3b	96	96	99								
mba 3c	96	96	99	99							
mba 3d	96	96	99	98	98						
mba 3e	96	96	99	98	98	98					
mba 6a	94	94	94	94	94	94	94				
mba 6b	95	94	94	93	93	93	94	99			
mba 8	74	74	73	73	73	73	74	74	74		
mba x	73	72	72	71	71	72	72	72	72	97	

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 an 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: Genbank Accession No. X51315 (2) and Genbank Accession No. M36190 (39), both serotype 8 *U. urealyticum*; and Genbank Accession No. L40489 (18), a serotype 1 *U. urealyticum.* Of the nine clinical isolates studied, eight isolates (of the parvo biovar) had sequences identical 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).

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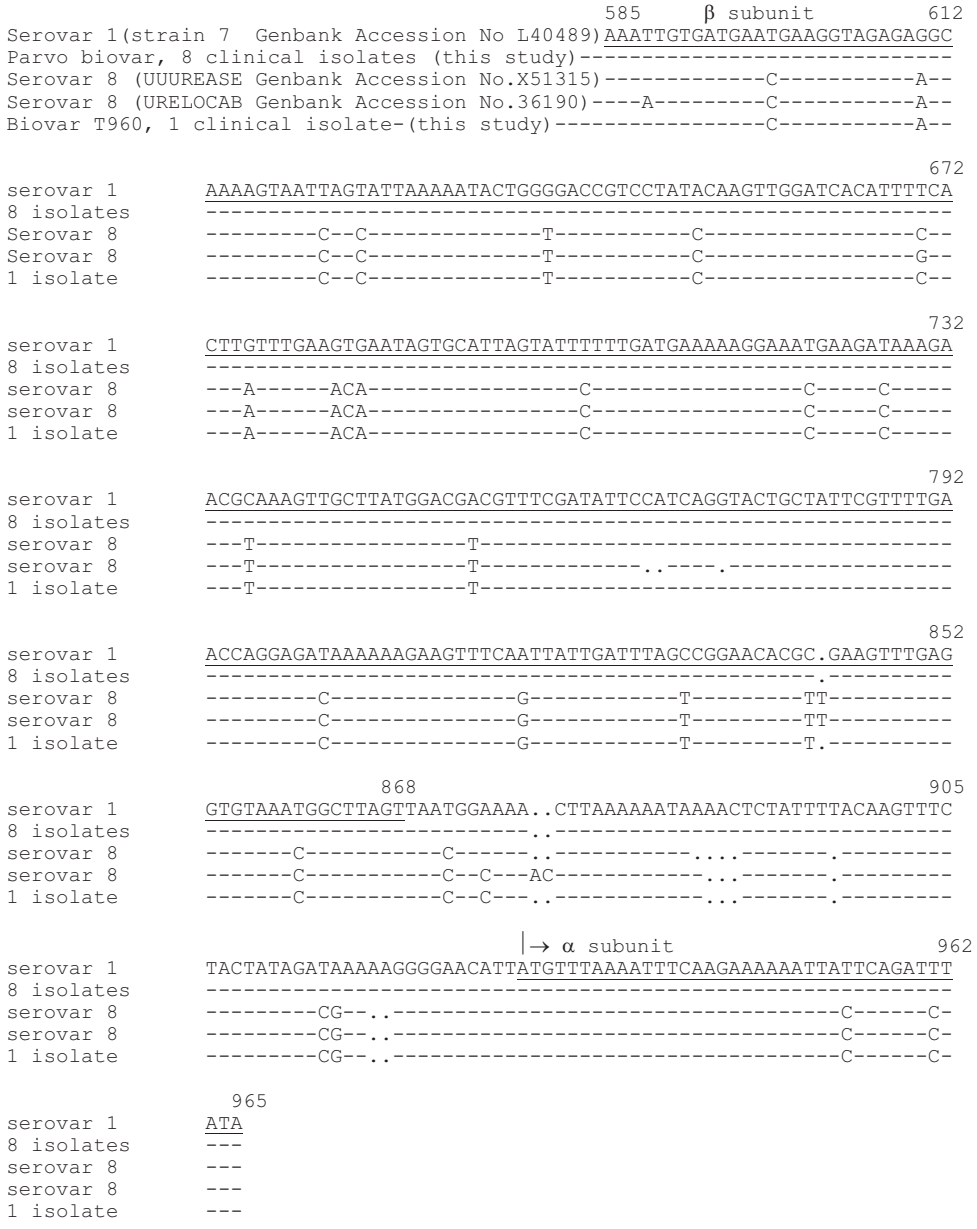

FIG. 3. Sequence alignment of the *ure* gene of serovars 1 (*ure* I) and 8 (*ure* II) and the *ure* fragments of nine clinical isolates of *U. urealyticum.* A dash indicates that the position in the alignment is conserved. The dots indicate spaces between adjacent nucleotides introduced for maximum alignment. The underlined areas are the beta and alpha subunits (2).

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 Genbank Accession Nos: 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 (39).

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 T 960 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 (4) however results may be conflicting due to cross reactivity and also the common expression of epitopes (4), a problem which is exacerbated by the lack of standardised 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 (4, 19). The immunoperoxidase assay (19) and the modified indirect immunofluorescence test (17) can both be performed on ureaplasma primary isolation plates however again there are no standardised procedures or commercially available products. More recently DNA based typing approaches have been used with some success (1, 8, 9, 13, 34).

Robertson *et al.* (1994) (23) 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* (23). 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 recognised in human ureaplasma infections (37) is present in all reference serovars of *U. urealyticum* (34) 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 (40). Furthermore the *mba* gene has previously been shown to be variable, with biovar specificity residing in the 5'

region (34). 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 ≥98%) are also clearly distinguished, 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 (1, 13). While 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 within 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.

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