Postgenomic taxonomy of human ureaplasmas – a case study based on multiple gene sequences

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In 2000, the full genome sequence of *Ureaplasma parvum* (previously known as *Ureaplasma urealyticum*) serovar 3 was released. In 2002, after prolonged debate, it was agreed that the former *U. urealyticum* should be divided into two species – *U. parvum* and *U. urealyticum*. To provide additional support for this decision and improve our understanding of the relationship between these two species, the authors studied four 'core' genes or gene clusters in ATCC reference strains of all 14 serovars of *U. parvum* and *U. urealyticum*. These 'core' regions were the rRNA gene clusters, the EF-Tu genes (*tuf*), urease gene clusters and multiple-banded antigen genes (*mba*). The known *U. parvum* genome sequences (GenBank accession no. NC_002162) were used as reference. DNA insertions and deletions (indels) were found in all of the gene regions studied, except *tuf*, but they were found only between, not within, the two species. An incidental finding was that there was inter-copy heterogeneity for rRNA gene cluster sequences. Sequence analysis (sequence heterogeneity and especially indels) of all four selected targets consistently supported the separation of human ureaplasmas into two species. Except for multiple-banded antigen, there was less heterogeneity in amino acid sequences of proteins, between species, than in the nucleic acid sequences of the corresponding genes. The degrees of heterogeneity at the 5' end of the species-specific regions of multiple-banded antigen were almost identical for both amino acid and nucleotide sequences. Analysis of the authors' results provided an interesting case study to help resolve some common problems in the use of sequence data to infer phylogenetic relationships and support taxonomic changes. It is recommended that, to avoid confusion, the new nomenclature be used for human ureaplasmas in future publications.

INTRODUCTION

There have been two recent major developments that affect our understanding of human ureaplasmas. Firstly, the full genome sequence of *Ureaplasma parvum* (previously known

as *Ureaplasma urealyticum*) serovar 3 was released in 2000 (GenBank accession no. NC_002162) (Glass *et al.*, 2000). Secondly, the taxonomy of human *Ureaplasma* species changed in 2002 (Robertson *et al.*, 2002), when the two former *U. urealyticum* biovars were given full species status, as *U. parvum* (previously biovar parvo or biovar 1) and *U. urealyticum* (previously biovar T960^T or biovar 2) (Robertson *et al.*, 2002).

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The GenBank/EMBL/DDBJ accession numbers for the sequences described in this article are: rRNA gene cluster, AF272599–AF272604, AF073446–AF073459, AF059322–AF059335 and AF272605–AF272630; *tuf*, AF270758–AF270770; urease gene clusters, AF085720–AF085733; *mba*, AF055358–AF055367 and AF056982–AF056984.

Comparisons of two *U. parvum* serovar 3 rRNA operons (Fig. A), *U. urealyticum* serovar 8 and *U. parvum* serovar 3 rRNA operon 1 and operon 2 DNA sequences (Fig. B), *U. urealyticum* serovars 8 and 13 and *U. parvum* serovar 3 EF-Tu gene (*tuf*) DNA and amino acid sequences (Fig. C), and *U. urealyticum* serovar 8 (upper line) and *U. parvum* serovar 3 (lower line) urease gene cluster sequences (Fig. D), and a comparison of interspecies heterogeneity of DNA and amino acid sequences of the urease gene clusters of *U. parvum* and *U. urealyticum* (Table A) are available as supplementary material in IJSEM Online.

It is now accepted that a decision to create a new species should be based on many independent phenotypic and genotypic characteristics – the theory of 'polyphasic taxonomy' (Vandamme *et al.*, 1996). However, molecular methods and genome-based criteria have become more accessible and attractive (Gürtler & Mayall, 2001; Stackebrandt *et al.*, 2002). DNA–DNA hybridization showing less than 70 % homology between whole genomes is accepted as the most definitive criterion or 'gold standard' for separate prokaryote species (Murray & Schleifer, 1994). The traditional 70 % DNA–DNA hybridization value used to delineate genomic species was found to correspond to genome mispairings in the range 13–13.6 % or 0.097–0.104

nucleotide substitutions per site (Mougel *et al.*, 2002). Similarity of less than 97 % in the 16S rRNA gene is the most widely used practical alternative (Murray & Schleifer, 1994), but these criteria may conflict and additional alternative targets are required (Pettersson *et al.*, 2000; Dellaglio *et al.*, 2004).

The two *Ureaplasma* species discussed herein exhibit many distinct phenotypic and genotypic properties (including DNA–DNA hybridization showing less than 70 % homology), which support the change in taxonomy and fulfil the requirements of the polyphasic theory (Vandamme *et al.*, 1996). However, continued use of the old single-species nomenclature in some recent publications (Daxboeck *et al.*, 2003; Baier *et al.*, 2003) is potentially confusing. To strengthen the case for acceptance and exclusive use of the new ureaplasma taxonomy (Robertson *et al.*, 2002), we studied four ‘core’ genes/gene clusters – the rRNA gene cluster, EF-Tu gene (*tuf*), urease gene cluster and multiple-banded antigen gene (*mba*) – of all 14 human ureaplasma serovars. These four regions were chosen because previous studies have shown that they were promising targets for study of the phylogeny of ureaplasmas and mycoplasmas (Kong *et al.*, 1999b, 2000b; Kamla *et al.*, 1996). In addition, we used this as a case study to help resolve some common problems with the use of sequence data to infer phylogeny and to support the establishment of new taxonomy (Ludwig *et al.*, 1998).

METHODS

Bacterial strains. Reference strains of four *U. parvum* and ten *U. urealyticum* serovars, obtained directly from the American Type Culture Collection (ATCC), were the same as those used in our previous studies (Kong *et al.*, 1999a).

Value of *U. parvum* serovar 3 genome in oligonucleotide primers design. The full genome sequence of *U. parvum* serovar 3 (Glass *et al.*, 2000) greatly facilitated sequencing of the selected genes and gene clusters of the other three *U. parvum* and ten *U. urealyticum* serovars. For this study, we used the following steps. Firstly, to identify conserved regions, we compared known sequences of genes corresponding to our selected genes and gene clusters – rRNA gene cluster, *tuf* and urease gene clusters – in other *Mycoplasma* species and *Ureaplasma* serovars (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996; Neyrolles *et al.*, 1996), in addition to *mba*, which we and others have studied in detail previously (Zheng *et al.*, 1995; Kong *et al.*, 1999a). Based on the results, we designed primers and amplified target regions for sequencing. The target regions sequenced were: (a) the whole rRNA gene cluster, including a short region upstream of the 16S rRNA gene (for *U. parvum* serovars only), the 16S rRNA gene, the 16S–23S rRNA intergenic spacer, the 23S rRNA gene, the 23S–5S rRNA intergenic spacer, the 5S rRNA gene and a short region downstream of the 5S rRNA gene (see supplementary Figs A and B in IJSEM Online); (b) almost the full-length of *tuf* (see supplementary Fig. C in IJSEM Online); (c) the whole urease gene cluster, including short regions upstream of *ureA–ureB–ureC–ureE–ureF–ureG–ureD* and downstream of *ureD* (see supplementary Fig. D in IJSEM Online).

The amplification and sequencing primers used in the study are shown in Table 1. Most primers were used for both amplification and sequencing, but some (as inner sequencing primers) were used only for sequencing.

DNA preparation and PCR. These were performed as described previously (Kong *et al.*, 1999b).

Sequencing and sequence analysis. The PCR products were sequenced as described previously (Kong *et al.*, 2000b). All sites showing unexpected heterogeneity, such as those indicating rRNA gene inter-copy sequence variation and the unique heterogeneity site in serovar 13 *tuf* (see below), were sequenced at least twice, to confirm the results. When necessary, different PCR amplicons and/or inner sequencing primers were used for sequencing.

The initial sequencing results were analysed with the program Bestfit in the Comparison program group and then joined together to determine sequences of whole genes/gene clusters. The multiple sequence alignments were performed with the programs Pileup and Pretty from the Multiple Sequence Analysis program group. All of the programs/program groups are available in WebANGIS (<http://www1.angis.org.au/WebANGIS/>), ANGIS (Australian National Genomic Information Service).

RESULTS AND DISCUSSION

Advantages of sequencing multiple strains

In this study, as in our previous study of *mba* (Kong *et al.*, 2000b), we sequenced the three target genes/gene clusters for 13 ureaplasma serovars (excluding serovar 3 sequences from the full genome, which were used as reference). The advantages of this approach are: (i) since the genes/gene clusters are relatively conserved between serovars within each species, the results for different serovars help to confirm the accuracy of sequencing results (Clayton *et al.*, 1995); and (ii) the results help to differentiate interspecies from intraspecies heterogeneity (Mygind *et al.*, 1998).

A previous study showed that there is some intraserovar as well as interserovar/intraspecies and interspecies heterogeneity in *mba* (Knox *et al.*, 1998). However, there is limited intraspecies heterogeneity in the other three gene regions studied and, even in *mba*, intraspecies heterogeneity is much less than between species (Kong *et al.*, 1999b). Therefore, a single reference strain of each serovar provides an example of each species to demonstrate interspecies heterogeneity, which was the focus of this study.

Key characteristics of the ‘core’ genes

Interspecies, intraspecies and inter-copy polymorphisms of the rRNA gene cluster. There was relatively little interspecies heterogeneity in 16S and 23S rRNA genes but considerably more in the two copies of the 5S rRNA genes and the corresponding intergenic spacer regions (Table 2). In common with other targets studied, the intraspecies heterogeneities in these genes were minor and some were assumed to be due to inter-copy differences between duplicate copies (Table 2). Previous studies have shown sequence variation in duplicate copies of rRNA genes of other mollicutes (Pettersson *et al.*, 1996). Analysis of the two copies of the rRNA gene cluster in *U. parvum* serovar 3 genome (GenBank accession no. AE002111 plus AE002112 and AE002127 plus AE002128) showed inter-copy heterogeneity between 16S rRNA genes

Table 1. Sequences of oligonucleotide primers used for sequencing three different genes/gene clusters

Primer names*	Target genes/regions*	T _m (°C)†	GenBank accession no.‡	Primer sequences§
113S1	UU113	68.4	AE002111	9814GAA GAA CCC ACC AAA TAC GAG CAG9837
113S2¶	UU113	62.7	AE002111	9862TTG TTG GTG AAC AAA AAT ACA TCA9885
303S1	UU303	64.9	AE002127	5713TTG ATG CAA AAA GAT CAG GTT GTA G5737
303S2¶	UU303	61.7	AE002127	5800GAG AAA CAA GCT GAA CAT AAT GAT C5824
16S10A¶	16S rRNA	66.4	AE002127	6144AAT CCT GAG CCA GGA TCA AAC TC6122
16S23A	16S rRNA	74.3	AE002127	6156GCC GCC AGC GTT AAT CCT GAG C6135
SP1623S1	16S–23S rRNA spacer	65.3	AE002127	7830CTT TCT AAT CAT TGA CAT TAA GTT GTC AGT G7860
SP1623S2¶	16S–23S rRNA spacer	62.8	AE002127	7843GAC ATT AAG TTG TCA GTG AAC AGA AAC7869
23S32S	23S rRNA	63.6	AE002127	7933CTA AGA GCT TAT GGT GA/GA TGC CTT G7957
23S523S	23S rRNA	69.4	AE002127	8424GAA CGG TGA AAA GAA CCC AGA GAT G8448
23S503A¶	23S rRNA	65.7	AE002127	8449CCA TCT CTG GGT TCT TTT CAC C8428
23S516A	23S rRNA	63.7	AE002127	8464GGT TCT ATT TCA CTC CCA TCT CTG8441
23S1104S	23S rRNA	68.7	AE002127	9006GCA AGG ATG TTG GCT TAG AAG CAG9029
23S1082A¶	23S rRNA	68.7	AE002127	9030GCT GCT TCT AAG CCA ACA TCC TTG9007
23S1134S¶	23S rRNA	67.5	AE002127	9034CGT TTA AAG AGT GCG TAA CAG CTC AC9059
23S1117A	23S rRNA	70.8	AE002127	9067CTC GAC AAG TGA GCT GTT ACG CAC TC9042
23S1721S	23S rRNA	68.9	AE002127	9622AAG GAA CTC TGC AAA TTA ACC CCG T9646
23S1698A¶	23S rRNA	68.0	AE002127	9647TAC GGG GTT AAT TTG CAG AGT TCC T9623
23S1729A	23S rRNA	68.5	AE002127	9677TTT TAC AGC GAG CAC CCC TTA TTG9654
23S2257S	23S rRNA	68.5	AE002127	10158GAC AGT GTT AGG TGG GCA GTT TGA C10182
23S2237A¶	23S rRNA	71.9	AE002127	10186CCC AGT CAA ACT GCC CAC CTA ACA C10162
23S2251A	23S rRNA	78.6	AE002127	10198GGA GGC GAC CGC CCC AGT CAA AC10176
23S2578S	23S rRNA	74.6	AE002127	10478GGT TCG GCT GTT CGC CGA TTA AAG AG10503
23S2603A	23S rRNA	55.8	AE002127	10551AGA TAG GGA CCA ACC TGT CTC ACG10528
23S2772S	23S rRNA	65.2	AE002127	10674AAA CGC TGA AAG CAT CTA AGT GTG10697
5S41S	5S rRNA	67.7	AE002128	86GAA ATA CCT GTT CCC ATC CCG A107
5S60S¶	5S rRNA	70.0	AE002128	103CCC GAA CAC AGA AGT CAA GCA CTC126
5S45A	5S rRNA	67.4	AE002128	134CGG CTC TAG AGT GCT TGA CTT CTG111
UU304A1¶	UU304	63.0	AE002128	330TTC TAA TTG CAA TTC TTC AAG ACG307
UU304A2	UU304	71.8	AE002128	400CAC CTT GTT CGC GTG CAT CTT G379
UU114A1¶	UU114	60.1	AE002112	5246A/GTT TAT TGT TTT TGG ATA TAC CAC C5222
UU114A2	UU114	66.2	AE002112	5405CGT CTT CTG GTG TTT GCA TAA TTG5382
TUF5S	<i>tuf</i>	61.3	AE002151	1284TTA ATT TTT AAG GAG ATT TAA AAT GGC1258
TUF32S¶	<i>tuf</i>	62.4	AE002151	1256AAA GCT AAA TTT GAA AGA ACA AAA CC1231
TUF590S	<i>tuf</i>	67.9	AE002151	697TTG ATG AAT TAA TGG ACG CAG TTG A673
TUF646A	<i>tuf</i>	65.3	AE002151	592CGT CCT GAA ATT GTG AAT ACA TCT TC617
TUF874S¶	<i>tuf</i>	66.9	AE002151	414AAA AGA AGA TGT TGA ACG TGG TCA AG389
TUF1132A¶	<i>tuf</i>	65.0	AE002151	107GAC CTA CAG TTT TAC CAC CTT CAC G131
TUF1159A	<i>tuf</i>	59.0	AE002151	77ATT AAT TAC TTG TTT TAA TTA CGC TAC C104
UC424S	<i>ureC</i>	65.6	AE002140	2676CAG CTG GTG GTT TAG ATA CTC ACG2653
UC429S¶	<i>ureC</i>	64.8	AE002140	2672TGG TGG TTT AGA TAC TCA CGT TCA C2648
UC910S	<i>ureC</i>	64.9	AE002140	2181TTT TAC CAG CTT CTA CAA ACC CAA C2157
UC947A¶	<i>ureC</i>	60.6	AE002140	2094AAG TGG TGA CAT ACC ATT AAC ATA TC2119
UC959A	<i>ureC</i>	60.5	AE002140	2083CCT TAG GAT TTA AGT GGT GAC ATA C2107
UC1418S	<i>ureC</i>	68.0	AE002140	1683GG/TG ATC CAA AC/TG CTT CAA TTC CAA C1659
UC1450A	<i>ureC</i>	61.0	AE002140	1601A/GC/TT AGT TAA C/TA/GA ACG TCC ATA A/TGT TCC1624
UE93S	<i>ureE</i>	59.5	AE002140	1147GAA CAT TCA TTT AAC AAG CGA CGA C1126
UE119A	<i>ureE</i>	62.9	AE002140	1073CCA TAT TCA ACA TTT TGA TCT GAT G1097
UE143S¶	<i>ureE</i>	62.9	AE002140	1097CAT CAG ATC AAA ATG TTG AAT ATG G1073
UF217S	<i>ureE</i>	59.5	AE002140	632GCG TTA CTT CTG TAT ATG AAT GAA C608
UF222A¶	<i>ureF</i>	60.0	AE002140	578AAA TTG CC/TA ATA AAT CAC CAT GTA AC603
UF320A	<i>ureF</i>	63.1	AE002140	484CGA GTC TCT CTT GCT AAA CCT TG506
UF721S	<i>ureF</i>	64.7	AE002140	129C/TCT TGA AAT TGC ACA AAT GGA AC107

Table 1. cont.

Primer names*	Target genes/regions*	T_m (°C)†	GenBank accession no.‡	Primer sequences§
UG3A	<i>ureG</i>	62.4	AE002139	11152 CCT ACA CCA ATA ATT AAT GGT CTT TTC 11178
UG475S	<i>ureG</i>	63.4	AE002139	10730 GTT GAT TTA GCT CCT TAT GTT GGT G 10706
UG451A¶	<i>ureG</i>	63.4	AE002139	10706 CAC CAA CAT AAG GAG CTA AAT CAA C 10730
UG499A	<i>ureG</i>	60.6	AE002139	10655 CTT TAT TAC CAC GTG ATT TTA ATG TAT C 10682
UD332S	<i>ureD</i>	59.0	AE002139	10246 CA/TG AA/GC AAC AC/TA CAA ATA TC/TA CA/GT TAG G 10219
UD379A	<i>ureD</i>	62.0	AE002139	10147 TTA AAT TGG/T GCA/G AAC/T TTT CCA TCT TC 10172
UD841A	<i>ureD</i>	62.3	AE002139	9684 CTT C/TTA TGG TTT TCG TAA AAT TAA A/TGG 9710
UU427A1¶	UU427	60.4	AE002139	9446 AAT AAA TTT TGC TAA AAA AGG CAT AC 9471
UU427A2	UU427	56.5	AE002139	9330 GT/CA/T GGT/C TTA AAA T/CTA ACA TCT ACA C 9354
UU427A3	UU427	63.1	AE002139	9175 CAT CAT CAA AAT CTT TAA TAC CAT CAT C 9199

*Primers were named according to their target genes/regions, the 3' end locations (the distance from the beginning of corresponding genes/regions), and directions of primers (sense or antisense). In the target regions designated UU, the numbers indicate the number of the open reading frame (ORF) corresponding to that in the *U. parvum* genome sequence.

†The melting temperature (T_m) values were obtained from the primer synthesizer (Sigma-Aldrich).

‡All GenBank sequences were from the related sections of the full genome sequences of *U. parvum* serovar 3 (Glass *et al.*, 2000).

§Numbers represent the numbered base positions at which primer sequences start and finish (and correspond with those in GenBank). Letters behind a solidus (/) indicate alternative nucleotides in different species/serovars, which were based on the comparison with the other related GenBank sequences.

¶Primers used only for sequencing; all the other primers were used for both PCR and sequencing.

(one site), 16S–23S rRNA intergenic spacer regions (two sites), 23S rRNA genes (four sites) and 5S rRNA genes (one site) but none in the 23S–5S rRNA intergenic spacer regions (Fig. A in IJSEM Online). In sequences of the corresponding genes of the other 13 serovars the result was 'N' (i.e. unknown or unidentifiable nucleotide) rather than 'A, T, C or G', at several sites, even after repeat sequencing or use of different amplification and sequencing primers. We assumed that most, if not all, of these

were due to inter-copy polymorphisms (see Table 2 and supplementary Figs A and B in IJSEM Online) (Ueda *et al.*, 1999). If these inter-copy polymorphisms were ignored, the intraspecies heterogeneity in rRNA gene clusters between the two human *Ureaplasma* species was very low. In future, the design of primers or probes or study of the phylogenetic relationships should take account of polymorphisms between multi-copy rRNA gene clusters (Gürtler, 1999).

Table 2. Comparison of interspecies, intraspecies and inter-copy heterogeneity in rRNA gene clusters of *U. parvum* and *U. urealyticum*

Genes/regions	DNA length	Heterogeneity sites: N (%)		
		Interspecies*	<i>U. parvum</i> intraspecies	<i>U. urealyticum</i> intraspecies
16S rRNA gene	1513†	14 (0.93)	2 (1‡)	2 (1‡)
16S–23S rRNA gene spacer	302	13 (4.3)	2 (2‡)	0
23S rRNA gene	2903	26 (0.90)	8 (7‡)	10 (9‡)
23S–5S rRNA gene spacer	71	3 (4.2)	0	0
5S rRNA_1 gene	115	7 (6.1)	1 (1‡)	3 (3‡)
5S rRNA_2 gene	115	8 (7.0)	–	–
5S rRNA_1 gene–UU114 spacer§	136	31 (3.8)	–	–
5S rRNA_2 gene–UU304 spacer§	105	20 (19.0)	–	–

**U. parvum* and *U. urealyticum* interspecies heterogeneity sites were determined independently of intraspecies heterogeneity sites.

†Length modification was based upon two other mollicutes 16S rRNA genome annotations (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996), especially considering *Mycoplasma pneumoniae* annotation (Himmelreich *et al.*, 1996).

‡Numbers in parentheses were assumed numbers to contain inter-copy heterogeneity (see text for further explanation).

§The rRNA gene cluster (or 5S rRNA gene) downstream external spacer regions.

***U. urealyticum* serovar 13 EF-Tu gene (*tuf*).** Previous studies have shown that differences in *tuf* can distinguish species and may reflect some phenotypic relationships better than the 16S rRNA gene (Kamla *et al.*, 1996). EF-Tu gene (*tuf*) DNA sequences were the same in serovars within each species, except for that of serovar 13 of *U. urealyticum*. It contains two base differences (but the same amino acid sequences) compared with the other nine *U. urealyticum* serovars (Fig. C in IJSEM Online). This difference is of interest in view of another reported difference between serovar 13, which gives an intermediate response in the Mn^{2+} (manganese)-inhibition test, and all other *U. urealyticum* serovars, which are fully inhibited (Robertson & Chen, 1984).

Intraspecies and interspecies heterogeneity of urease gene clusters. There were 21, nine and one intraspecies heterogeneity sites in the urease gene cluster DNA sequences for *U. parvum* serovars 1, 6 and 14 (compared with serovar 3), respectively. There were seven heterogeneity sites (or 12 bp – one site has 6 bp difference) in *U. urealyticum* serovar 2, compared with all other *U. urealyticum* serovars. These results show greater heterogeneity between urease gene clusters of *U. parvum* serovars than between those of *U. urealyticum* serovars, as we found previously for *mba* (Kong *et al.*, 1999a).

Interspecies heterogeneity between urease gene clusters of the two species was greater in the intergenic spacer regions (where it varies from 16.8 to 31.8%) than in the genes themselves (where the range of heterogeneity is 5.9 to 9.7%). Variation in amino acid sequences, between species, is less (range 0.97 to 6.7%) than in nucleic acid sequences of urease genes (see supplementary Table A in IJSEM Online).

The 'molecular clock' is different for different *mba* regions. As we described previously, different *mba* regions apparently evolve at different rates, i.e. according to different 'molecular clocks' (Bromham & Penny, 2003). The upstream regions are more heterogeneous than *mba* itself (Kong *et al.*, 1999a) and the repetitive regions are more heterogeneous than the 5' end regions. This should be taken into account when using different regions to infer the phylogeny (Kong *et al.*, 1999a).

Indels. Analysis of insertions and deletions (indels) is a very useful tool with which to study bacterial phylogeny (Britten *et al.*, 2003; Gupta & Griffiths, 2002). We compared the distribution of indels in the four 'core' genes/gene clusters of four *U. parvum* serovars with those of ten *U. urealyticum* serovars (Figs B, C and D in IJSEM Online) (Kong *et al.*, 2000b). All indels were consistent between serovars within each species, which strongly supports the separation of *Ureaplasma* species based on indels. The rRNA gene cluster of *U. parvum* differed from that of *U. urealyticum* as follows: (a) a TGTG insertion in the 16S rRNA gene; (b) an AT (for operon 1) or A and C

(for operon 2) deletion and AT insertion (for operons 1 and 2) in the 16S–23S rRNA intergenic spacer region; (c) a G insertion in 23S–5S rRNA intergenic spacer region; and (d) a TTAGG (for operon 1) or AAAAA (for operon 2) deletion in the 5S rRNA gene (Fig. B in IJSEM Online). There were no indels in the EF-Tu gene (*tuf*) (Fig. C in IJSEM Online). In the urease gene clusters, there were: (a) a TCAAT deletion in the *ureA*–*ureB* spacer; (b) AAC, T and CTA insertions in the *ureB*–*ureC* spacer; (c) a CA deletion in the *ureC*–*ureE* spacer; and (d) an ACATT insertion in the *ureF*–*ureG* spacer (Fig. D in IJSEM Online).

Despite these specific differences, the numbers of insertions or deletions, sites and total number of bases in these three genes were not significantly different between the two species. In *mba*, there were no indels in species-specific sites, but there was an AAATT insertion, an AA deletion, a 45 bp deletion and a TC deletion in *U. parvum* upstream of *mba* (Kong *et al.*, 2000b).

Genes, intergenic spacers or gene clusters?

Many studies have shown that intergenic spacer regions are more heterogeneous than the neighbouring genes (Garcia-Martinez *et al.*, 1999; Kong *et al.*, 1999b). Our study confirmed this by showing greater heterogeneity in the intergenic spacers, especially the external spacer regions, of both the rRNA gene clusters and the urease gene cluster compared with the corresponding genes (see Table 2 and supplementary Figs A, B and D and Table A in IJSEM Online) (Jung *et al.*, 2003). In addition, for urease gene clusters and *mba*, indels only existed in the gene spacer regions. Because the gene cluster as a whole is a functional group, we suggest that there are a couple of advantages in considering them as a unit in basic and applied research. (i) Whole gene clusters contain both conserved and variable sequences and phylogenetic data derived from them are stable and discriminatory (Gürtler, 1999), which is valuable in solving taxonomic problems (Harasawa, 1999). (ii) Species-specific primer pairs based on whole gene clusters are generally more specific and easier to design than primers based on any single component (Kong *et al.*, 2000a).

DNA or protein sequence? Which protein or gene region?

To fulfil polyphasic theory requirements (Vandamme *et al.*, 1996), DNA sequences and protein amino acid sequences should be considered together (Agosti *et al.*, 1996). However, DNA sequences often reflect the phylogeny more accurately and have greater (about double) discriminatory power (Simmons *et al.*, 2002). Our study showed that the *mba* species-specific region (5' end) DNA (67/430 = 15.6%) and the corresponding amino acid sequences (24/147 = 16.3%) have nearly identical levels of heterogeneity (Kong *et al.*, 2000b). However, urease gene subunit (Table A in IJSEM Online) and EF-Tu gene DNA sequences are more heterogeneous than their corresponding protein

amino acid sequences (Fig. C in IJSEM Online). For example, for the ureaplasma EF-Tu gene, DNA sequence heterogeneity was 54 out of 1185 (4.6%) bases compared with 2 out of 394 (0.5%) differences in amino acids between the two species. Presumably, genetic changes that significantly alter the structure, and therefore the function, of proteins such as enzymes are incompatible with survival. On the other hand, genetic variation that causes antigenic variation in multiple-banded antigens is not only consistent with survival but also an advantage if it helps the organism to evade the host immune response.

Many surface protein antigen genes are used to study the phylogeny of different microbes and to develop practical identification and typing schemes (Bush & Everett, 2001; Stackebrandt *et al.*, 2002). Sometimes, the gene or even the gene region selected can significantly affect the results (Bromham & Penny, 2003). For example, ureaplasma *mba* contains both species-specific (5' end region) and serovar definition sites (repetitive regions or 3' end). Thus, the 5' end region would be the appropriate region for studying species-level phylogeny, rather than the repetitive regions (Zheng *et al.*, 1995). If different bacterial species share almost identical protein antigens as a result of lateral gene transfer (Lawrence, 2002) – for example, the *Streptococcus agalactiae* Alp3 protein and *Streptococcus pyogenes* R28 protein (Stalhammar-Carlemalm *et al.*, 1999) – the corresponding genes lose their value for studying species-level taxonomy (Thornton, 2002).

Why 'core' genes?

Of the two *Ureaplasma* species discussed herein – *U. parvum* and *U. urealyticum* (Robertson *et al.*, 2002) – a full genome sequence was available only for *U. parvum* (Glass *et al.*, 2000). In future, our understanding of human ureaplasmas would be significantly improved by availability of the full genome sequence of *U. urealyticum* also. In particular, it would help to elucidate the nature and significance of the >80 kbp size difference between the two human *Ureaplasma* species (Robertson *et al.*, 1990; Fraser *et al.*, 2000) in reverse evolution (Rocha & Blanchard, 2002) and pathogenesis (Povlsen *et al.*, 2002). Meanwhile, alternative strategies such as analysis of selected 'core' genes or gene clusters (as in this study) can be used to infer the phylogenetic relationship between species (Daubin *et al.*, 2002). The rationale for the choice of these four genes was that the rRNA gene cluster (Stackebrandt *et al.*, 2002) and *tuf* (Kamla *et al.*, 1996) have been widely accepted targets for phylogenetic/taxonomic studies and the urease gene cluster and *mba* are unique determinants (Stackebrandt *et al.*, 2002) of ureaplasma metabolism (Neyrolles *et al.*, 1996) and antigenicity (Zheng *et al.*, 1995).

Conclusion

Analysis of four 'core' genes/gene clusters further supported the establishment of two separate human *Ureaplasma* species – *U. parvum* and *U. urealyticum*. Significant

differences between genes/gene clusters in the degree of heterogeneity between and within species sheds further light on the relationships between them and makes a useful case study to help understand common problems in the use of sequence data to infer phylogeny and support taxonomic change (Ludwig *et al.*, 1998).

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