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Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma parvum* using a mini-transposon plasmid

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Abstract

While transposon mutagenesis has been successfully used for *Mycoplasma* spp. to disrupt and determine non-essential genes, previous attempts with *Ureaplasma* spp. have been unsuccessful. Using a polyethylene glycol-transformation enhancing protocol, we were able to transform three separate serovars of *Ureaplasma parvum* with a *Tn4001*-based mini-transposon plasmid containing a gentamicin resistance selection marker. Despite the large degree of homology between *Ureaplasma parvum* and *Ureaplasma urealyticum*, all attempts to transform the latter in parallel failed, with the exception of a single clinical *U. urealyticum* isolate. PCR probing and sequencing were used to confirm transposon insertion into the bacterial genome and identify disrupted genes. Transformation of prototype serovar 3 consistently resulted in transfer only of sequence between the mini-transposon inverted repeats, but some strains showed additional sequence transfer. Transposon insertion occurred randomly in the genome resulting in unique disruption of genes UU047, UU390, UU440, UU450, UU520, UU526, UU582 for single clones from a panel of screened clones. An intergenic insertion between genes UU187 and UU188 was also characterised. Two phenotypic alterations were observed in the mutated strains: Disruption of a DEAD-box RNA helicase (UU582) altered growth kinetics, while the *U. urealyticum* strain lost resistance to serum attack coincident with disruption of gene UUR10_137 and loss of expression of a 41 kDa protein. Transposon mutagenesis was used successfully to insert single copies of a mini-transposon into the genome and disrupt genes leading to phenotypic changes in *Ureaplasma*

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parvum strains. This method can now be used to deliver exogenous genes for expression and determine essential genes for *Ureaplasma parvum* replication in culture and experimental models.

Keywords

Ureaplasma parvum; *Ureaplasma urealyticum*; transposon mutagenesis; gene disruption; RNA helicase

INTRODUCTION

In the United Kingdom and United States about 1 in 12 pregnancies result in premature birth (<32 weeks out of a normal 40 weeks gestation). Between 25–40% of these preterm births are associated with intrauterine infection and *Ureaplasma* spp. are the organisms most frequently identified. Sixty five percent of very premature newborns (≤ 26 weeks' gestation) were found to have congenital respiratory *Ureaplasma* infection [Sung *et al.* 2011]. These infants often require long-term ventilation and are at an elevated risk of neonatal death and disease. Experimental infection in primates have definitively proven that intrauterine infection of *Ureaplasma parvum*, as a sole pathogen, induces preterm birth and associated neonatal respiratory disease (Novy *et al.*, 2009); however, the ability to study the role of individual bacterial genes in pathogenesis experimentally has been hampered by a lack of tools to deliver or knock-out genes.

Ureaplasma spp. are one of the smallest self-replicating microorganisms identified to date that belong to the class *Mollicutes*. This class possesses a number of bacterial species with unusually small genomes (0.75–0.78 Mbp genomes for *U. parvum* and 0.84–0.95 Mbp genomes for *U. urealyticum*), (Paralanov *et al.*, 2012) that lack a number of genes resulting in this class's characteristic failure to make a bacterial cell wall and deficiencies in a number of metabolic pathway enzymes. As a by-product, these deficiencies mediate an inherent resistance to most antibiotics other than macrolides, fluoroquinolones and tetracyclines (Waites *et al.*, 2005). Ureaplasmas utilise urea conversion to ammonium ions for ~95% of their ATP generation (Romano *et al.*, 1980; Smith *et al.*, 1993) and have a pleomorphic microscopic appearance for organisms of approximately 0.1–1.0 μm in diameter (Robertson *et al.*, 2002). Although initially characterised as a single species separated into 14 serovars, *Ureaplasma urealyticum* was later subdivided into two species (*U. parvum* and *U. urealyticum*). These cluster into two distinctive groups when analysed for patterns of antigenic types or key metabolic gene polymorphisms (such as urease) and show a distinctive phylogenetic divergence when comparing the 16S rRNA and 16S–23S rRNA intergenic regions between *U. parvum* and *U. urealyticum* (Robertson *et al.*, 2002).

There is evidence of transposon gene delivery into the *Ureaplasma* spp. genome that occur in nature. The fully sequenced genome for *Ureaplasma urealyticum* serovar 9 (ATCC strain 33175), which exists as a whole genome shotgun sequence (NZ_AAYQ02000002.1) in the NCBI database, shows the presence of the tetracycline resistance *tetM* gene (UUR9_0151) adjacent to a conjugal transfer protein (UUR9_0147), transposase (UUR9_0146), and integrase (UUR9_0149). The *tetM* gene for this genome is accepted to be part of a *Tn916* a conjugative transposon also called integrative conjugative element (ICE). Tetracycline

resistance for many *U. parvum* and *U. urealyticum* genomes have been reported to be due to transposon-associated *tetM* gene presence in the bacterial genome from isolates from distant countries (Beeton *et al.*, 2009; de Barbeyrac *et al.*, 1996; Govender *et al.*, 2012; Mardassi *et al.*, 2012; Roberts 1990; Taraskina *et al.*, 2002) and donation from transposon-carrying *Enterococcus faecalis* to a close relative to *Ureaplasma* (*Mycoplasma hominis*) during co-culture was demonstrated through mating at a frequency of 10^{-6} to 10^{-7} (Roberts and Kenny 1987).

Transposon-carrying plasmids were first successfully used to insert selectable markers in the genomes of *Mycoplasma pulmonis*, *Mycoplasma hyorhinis* and *Acholeplasma laidlawii* in the late 1980's (Dybvig and Alderete 1988: Dybvig and Cassell 1987; Mahairas and Minion 1989a&b), which showed random genome insertion allowing both investigation of disrupted genes as well as the delivery of exogenous genes. The methods and reagents used to study essential genes and the physiological effects of delivering exogenous genes has continued to expand and be refined (Algire *et al.*, 2009; Paralanov *et al.*, 2012). To date, no report has been made to show successful experimental delivery of transposons or plasmids into *Ureaplasma spp*.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli—One shot Top10 chemically competent *E. coli* cells (Invitrogen; Paisley, Scotland, UK), were used as per manufacturer's instructions. This bacteria has the genotype F-mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 Δlacχ74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-.

Ureaplasma spp—Three *U. parvum* strains for serovar 1 (DFK-1, O10, and HPA78), serovar 3 (HPA5, U6, and HPA56) and serovar 6 (HPA2, HPA61, HPA58) were investigated for the capacity to undergo transposon mutagenesis, and these strains were initially isolated from patients, but have been purified through subcloning and have also been previously characterised and used by our group in previous publications (Beeton et al., 2009 & 2012). Various ATCC strains of *U. urealyticum* representing serovar 2 (ATCC 27814), 4 (ATCC 27816), 8 (ATCC 27618), 9 (ATCC 33175), and 10 (ATCC 33699) were generously supplied by Dr. Janet Robertson, (University of Alberta, Canada) and these strains as well as a clinical isolate W11 (serovar 12) (Beeton *et al.*, 2009) were investigated in parallel.

Expression constructs and the mini-transposon plasmid

The *Tn4001*-based mini-transposon plasmid (pMT85) has been previously described by Zimmerman and Herrmann (2005) and was generously provided by Prof. R. Herrmann (Heidelberg University, Germany). The gentamicin resistance gene was not codon-usage optimised for *Ureaplasma* spp. expression.

Transformation of bacteria

Transformation of *E. coli* One shot Top 10 chemically competent bacteria was performed as per manufacturer's instructions using heat shock at 42°C and the plasmid containing bacteria selected with 100 mg/l gentamicin. Transformation of *Ureaplasma* spp. was carried out essentially as outlined for *M. mycoides* in King and Dybvig (1991) with some modifications. The key aspect of *U. parvum* growth is the conversion of urea to ammonium ions, which increase the pH of the growth medium from pH =6.2 (yellow) to pH >9 (dark red). Three 96-well plates containing 10-fold serial dilutions of *Ureaplasma* (200 μl per well, titrated from Rows A–H) were set out the night before the experiment. All of the wells showing pH change consistent with the threshold of detection for phenol red indicator (i.e. last red well, dark orange and first yellow well) were pooled (total volume 10 ml) and utilised for transformation, as these represent *U. parvum* in log phase growth. This 10 ml routinely gave titrations of 5×10^8 CFU. Cells were washed three times (centrifuged at 10000*xg* for 20 min) with 1 ml of 1X Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS) (Invitrogen, Paisley, U.K.) at 4°C. The washed pellet was resuspended in a volume of 375 μl of 100 mM $CaCl_2$ on ice for 30 min, then a volume of 100 μl of bacterial cells containing 10 μg of yeast tRNA, 6 μg of pMT85 and 1 ml of 50% PEG-8000 were added at room temperature for 1 min. Five ml of *Ureaplasma* selective medium (USM; purchased from Mycoplasma experience plc, Surrey, UK) was then added and the cells were allowed to recover at 37°C for 3 h and the growing cells were pelleted by centrifugation at 3600*xg* for 15 min at 4 °C before being resuspended again in 1 ml USM. A volume of 20 μl cell suspension was diluted in USM in a 1/10 dilution series containing 128 mg/L gentamicin in triplicate (as the endogenous MIC_{90} for *U. parvum* is 44 mg/L; unpublished data) and incubated overnight. Control bacteria treated identically (except for the addition of pMT85) were run in parallel and no spontaneous gentamicin resistance was observed. When the first well of transformed bacteria turned the media red, the cells were plated out on *Ureaplasma* selective agar (Mycoplasma Experience ltd) and individual colonies were examined for presence of gentamicin resistance gene by PCR, prior to further characterisation. In some experiments a dilution series of transformed bacteria were directly plated on to *Ureaplasma* selective agar plates containing 128 mg/L gentamicin to determine transformation success relative to *Ureaplasma* selective plates without gentamicin.

Screening of transformed bacteria

Successful transformation of gentamicin resistant bacterial clones was confirmed using PCR and primers designed against the gentamicin resistance gene (*aac-aphD*; 6′-aminoglycoside N-acetyltransferase); forward 5′-ACATGAATTACACGAGGGC-3′, reverse 5′-GTTCTTCTTCTGACATAGTAG-3′; 401 bp amplicon, Tm=54°C, 35 cycles) using standard PCR methods amplified by Promega GoTaq green DNA polymerase. *U. parvum* genes disrupted by transposon insertion into the genome were confirmed using primers against *Ureaplasma* genes: UU390 (hypothetical membrane protein; forward 5′-AGTATTCCCATTGCGACAA-3′, reverse 5′-TATTTATTATCTTTTCTGGAGGTT-3′; 476bp amplicon; Tm=52.4°C); UU450 (hypothetical membrane protein; forward 5′-TTGAATTGAACCCTCAGATCC-3′, reverse 5′-ATTGCTTGATGGAAATGAATCCT-3′ 675bp amplicon; Tm=58°C); UU520 (hypothetical membrane protein; forward 5′-

TCTGGAGGGAGTTTGTCTCC-3′; reverse 5- TTTCGCAAAGGTGCTAAACCA-3′; 730bp amplicon; Tm=58°C), UU582 (RNA helicase; forward 5′-TTACCACGACCACTACGTCC-3′, reverse 5′-TTATTGGCGTTGCACCAACAG-3′; 876bp amplicon, Tm=58°C) intergenic insertion between UU187 and UU188 (forward 5′-AGGTCACGATGTTGTTGCTGA-3′, reverse 5′-CAAATATGGGCAACAGGAGCAG-3′; 600bp amplicon, Tm=58°C). Amplifying the ends of the insertion site was performed using one of the above primers in combination with pMT85-specific primers (designed close to the 5′ and 3′ inverted repeat sequence) 195R (5′-CCGTAATCAAGGTCATAGC-3′, Tm=54.5°C) or 3192F (5- TTTGCTGGCCTTTTGCTCAC-3′, Tm=57°C) at the lowest annealing temperature. Amplicons were purified using the Qiaquick PCR clean up kit (Qiagen, Manchester, U.K.) and submitted to Eurofins MWG Operon (Ebersberg, Germany) for sequencing. Primers specific to pMT85 were also used to determine if transposon insertion only utilised sequences between the inverted repeats (1-3437bp) or whether plasmid sequence containing the *tnp* transposase gene (3438-4820bp) were also present in the genome insertion.

Sanger sequencing of genomic DNA to determine insertion site

Gentamicin resistant mutants were scaled up to a 100 ml culture, pelleted at 10,000 *xg* and the genomic DNA extracted utilising the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), as per the manufacturer's instructions. DNA from each preparation was either ethanol precipitated, resuspended in 10 μl of molecular grade water and utilised for Sanger sequencing of the purified genomic DNA utilising pMT85 primers 195R or 3192F, or separated on a 1% agarose gel (in TBE) and utilised for in gel radioactive probe hybridization. The genomic DNA and control pMT85 plasmid digestion were performed at 37°C overnight with HindIII (Promega). The buffers were used according to the manufacturer's recommendations and separated DNA fragments were compared to undigested controls in adjacent lanes. For in gel hybridization: briefly, the gel was dried for 5 h at 50°C and then rehydrated in double-distilled water for 5 min before 30-min incubations in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralizing solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl) at room temperature were performed. The gel was then prehybridized at 65°C using prehybridization solution (20 ml) (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] polyvinylpyrrolidone 400, 0.1% [wt/vol] Ficoll, 0.1% [wt/vol] bovine serum albumin [BSA] [Cohn fraction V], 0.5% [wt/vol] SDS, 150 μg/ml denatured calf thymus DNA). An amplicon containing the *aac-aphD* resistance gene (primers listed above) was purified with a Qiaquick PCR purification kit (Qiagen) and labelled using a random priming kit as per manufacturer's instructions (Agilent technologies Prime-it Random Primer labelling kit 300385) with [^{32}P]-dCTP. Non-incorporated radio-active nucleotides were then removed using by gel filtration using a G50 column (GE Healthcare). Gels were then washed in 2% SSC followed by 0.1% SSC and bands were visualised by autoradiography at −80°C using X-ray film (Fuji Film).

Immunoblot analysis

Strains for investigation were scaled up to an overnight 5 ml culture in USM and pelleted at 17,000 xg for 20 min in a refrigerated centrifuge. The pellet was then solubilised in 50 μl of NuPAGE® LDS Sample Buffer (Life Technologies, Glasgow, U.K.) as per manufacturer's

instructions, and separated by SDS-PAGE. Following electrophoretic transfer to nitrocellulose membrane, blots were blocked in 5% skim milk dissolved in PBS containing 0.05% Tween20 (PBST) and probed with either human high titre anti-*Ureaplasma* sera (as per our previous studies (Beeton *et al.*, 2012) or Virostat plc (Portland, ME) monoclonal anti-multiple banded antigen (MBA) antibody (clone 6525). Bound human and mouse antibodies were detected with appropriate peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch Europe ltd. (Newmarket, U.K.) and Pierce ECL detection reagent (Fisher Scientific, Loughborough, U.K.).

Serum killing assay

Serum killing assays were performed as detailed in our previous studies (Beeton *et al.*, 2012), using the previously characterised sera from healthy volunteers (including seronegative sera F1 and F9 as well as seropositive sera F7, M11 and M12). Following 1 hour challenge with 50% serum diluted in complement fixation buffer (Oxoid plc, Basingstoke, U.K.) at 37°C, surviving bacteria were quantified by titration in USM. Killing was determined as the relative decrease in bacterial titre relative to incubation with heat-inactivated serum (56°C, 30 min) as a control. Surviving bacteria were measured after 48 hour incubation at 37°C to enable maximum growth. All killing assays were performed in triplicate and repeated on at least three separate days with a range of characterised seropositive and seronegative human sera.

RESULTS

Transposon mutagenesis of *Ureaplasma*

Transformation with pMT85 was performed in parallel for eight representative strains of *U. parvum* and *U. urealyticum*. Between 1–5 successful transformants survived per 10^8 *U. parvum* cells used in the transformation reaction for each experiment; however, all parallel transformations of ATCC strains of *U. urealyticum* failed while one clinical isolate of *U. urealyticum* (strain W11; SV12) was successfully transformed. The *U. parvum* gentamicin MIC_{90} was determined to be 44 mg/L and for *U. urealyticum* was 66 mg/L using our previous methods (method detailed in Beeton *et al.*, 2009); therefore, gentamicin selection was performed at 128 mg/L. All resistant clones were found to contain the *aac-aphD* resistance gene from pMT85, which was not found in untransformed controls (Figure 1). Classical insertion into the genome should only insert the genes bordered by the inverted repeats at position 1bp and 3437 bp in pMT85; therefore, PCR was used to investigate the presence of plasmid sequence beyond the second inverted repeat, including the transposase gene (Figure 1). Prototype serovar 3 strain HPA5 was successfully transformed in 16 separate experiments with pMT85 and only transfer of plasmid DNA bordered by the inverted repeats (i.e. no transposase gene sequence) was observed. We also transformed 3 different strains for each of serovar 1, 3 and 6 of *U. parvum*; however, not all of them behaved as HPA5. Probing undigested genomic DNA from these isolates found that the *aac-aphD* gene was located on the chromosome, and probing of HindIII-digested genomic DNA found that with the exception of 3 clones, a single copy was inserted into the genome (Figure 2). Portions of the transposase (trp) gene from pMT85 integrated into the genomes of two strains from serovar 1, one strain from serovar 3 and one strain from serovar 6

(Figure 1 and Supplementary Figure 1). Furthermore, in 3 separate experiments with prototype serovar 1 strain DFK-1 only two out of three followed classical integration (Supplementary Figure 1). Sanger sequencing of purified genomic DNA from strains HPA56 and HPA58 confirmed that genomic integration included transposase sequence. Sequencing with primer 195R (designed to sequence across the IR at pMT85 position 1) confirmed genomic integration with interruption of the gene UU047 (predicted ATP/GTP binding protein) at position 390 bp in the coding region for HPA56 and interruption of the gene UU526 (hypothetical open reading frame) at position 543 bp in the predicted reading frame for HPA58 respectively. However, Sanger sequencing of the genomic DNA with primer 3192F (designed to sequence across the IR at position 3437) showed no integration and the intact presence of the pMT85 transposase gene sequence. However, Sanger sequencing of HPA78 mutant genomic DNA (Supplementary Figure 1) with the same primers, did not confirm the presence of transposase gene as both IR were found to interrupt the gene UU440 for this strain.

Interruption of *U. parvum* genes by random genomic insertion

Primer pairs were designed based on the genomic sequence for *U. parvum* ATCC serovar 3 strain 700970 for genes UU390, UU450, UU520, UU582 and intergenic region between UU187 and UU188. These primers successfully amplified these genes by PCR in all parent strains of *Ureaplasma*, while single failure for each primer set to amplify these genes in mutated strains (Supplementary figure 2) was due to transposon integration and disruption of these genes. These disruptions were then, confirmed by sequencing the integration interface (Figure 3) Sequencing of the junctions between transposon insertion for these genes found 8 bp direct repeats that were unique for each clone, adjacent to the inverted repeat from pMT85 (Figure 3). The 8 bp direct repeat for the intergenic insert between UU187 and UU188 replicated the last two bases of the TAA stop codon from UU187 ensuring that UU187 was not disrupted..

Screening *Ureaplasma* mutants for altered expression of the major surface antigen

Immunoblot analysis using monoclonal anti-multiple banded antigen (MBA; UU375) antibody was carried out to examine if any of the mutated strains had altered size or expression of the major surface antigen, MBA, relative to the untransformed parent strain. In 27 transformed clones from four different *Ureaplasma* serovars, MBA-negative clones (phase variation) were never observed, and only one clone showed a small alteration to the MBA mass (Figure). The site of genomic integration for this strain (U6) is known to be at the predicted integral membrane protein gene UU450 (Figure 3), which is not close to the gene encoding the MBA, therefore the observed MBA mass alteration for this strain is not due to direct interference with the coding gene.

Disruption of RNA helicase (UU582) gene altered *Ureaplasma* growth kinetics

Random transposon insertion was found to disrupt the gene UU582 as detailed in figure 3 and supplementary figure 2. UU582 encodes the only copy of an ATP-dependent DeaD-box bacterial RNA-helicase in the *U. parvum* genome and disruption of this gene was found to alter final titre and growth kinetics for the bacteria at a range of incubation temperatures. Of

all the transposon mutated *Ureaplasma* strains, only the disruption of gene 582 (UU582::mTn) had this effect. Maximum bacterial titre was obtained for parent and all other transposon mutated strains by 36 hr at 37°C (Figure 5), while UU582::mTn did not reach maximum titre until 60 hr and had a 3 to 4 log reduction in final bacterial titre. As DEADbox RNA-helicase mutants are reported to be incapable of replication at lower temperatures (Owttrim 2013) we also investigated growth kinetics at 33°C and 25°C (an example of growth for the parent strain at these temperatures is shown in supplementary figure 3). Under these conditions the maximum titre for other *Ureaplasma* strains took longer to attain and UU582::mTn titres were 10^8-fold lower at 25°C.

Altered complement susceptibility for mutated *U. urealyticum* strain

While no alteration in serum killing (either susceptible or resistant) was observed for any of the *U. parvum* strains (data not shown) following transposon mutagenesis, the *U. urealyticum* strain (W11) became significantly more susceptible to complement-mediated attack following transposon mutagenesis (Figure 6). W11 remained resistant to seronegative serum attack (sera that did not show reactivity by immunoblot), but were readily killed by sera containing anti-*Ureaplasma* antibodies. Immunoblot analysis using a high-titre seropositive serum identified a coincident loss of a 41 kDa mass bacterial protein in the mutant strain relative to the serum-resistant parent strain. Genomic DNA analysis identified a single genomic insertion site by in gel hybridisation for the *aac-aphD* gene (data not shown) and direct genomic sequencing of the W11 mutant found that the gene UUR10_0137 (ATCC strain 33699 serovar 10 numbering) was disrupted at amino acid 126 of 231.

DISCUSSION

Here we report the first successful transposon delivery of gentamicin resistance gene 6′-aminoglycoside N-acetyltransferase in *Ureaplasma*. While *U. parvum* and *U. urealyticum* are very closely related, the refined protocol we utilised was consistently successful for *U. parvum*, but failed to deliver the antibiotic selection marker to all ATCC strains of *U. urealyticum* except for one clinical *U. urealyticum* strain. Genomic sequencing of several transposon insertions in *U. parvum* mutants showed that classical inverted repeat-bordered insertion was consistently achieved for some strains, but use of only 1 inverted repeat with retention of the transposase in the insert for a minority of other strains. Investigation of disrupted genes further showed that usually only one insertion of the transposon occurred in the genome at random locations.

We utilised a PEG-based transformation protocol when more recent transposon mutagenesis of *M. bovis, M. agalactiae, M. mobile, M, pneumoniae, and M. hyopneumoniae* have favoured electroporation (Maglennon *et al*. 2013, Baranowski *et al*. 2014, Shimizu *et al*. 2014, Sharma *et al*. 2014). However, Voelker and Dybvig (1996) found similar efficiencies in comparing transposon delivery via conjugation, PEG-based transformation and electroporation of *M. arthritidis*, including one strain where PEG-based transformation was successful when electroporation failed. However, they also found a wide variation in efficiency between strains. Our attempts at transferring tetracycline resistance to

Ureaplasma spp. using conjugation with a *tetM*-positive Tn916-positive *E. faecalis* failed (data not shown) and we did not attempt electroporation methods for comparison.

Large scale analysis of random genome integration has been used for *Mycoplasma* spp. to determine the essential genes of a minimum genome, the underlying presumption being that essential genes cannot be disrupted. *M. genitalium* has the smallest genome and saturating Tn mutagenesis on this organism led to the proposal of a between 265-350 genes as being the minimum required to sustain self-replicating independent life (Hutchison *et al.*, 1999). Further refinement of this method identified all 43 RNA-coding genes to be essential and 382 of 482 *M. genitalium* protein-coding genes to be essential for culture growth of *M. genitalium* (Glass *et al.*, 2006). These studies were hugely influential in the construction of the minimum synthetically assembled genome based on *M. genitalium* in 2008 (Gibson *et al.*, 2008). However, comparison of the smallest *Mycoplasma* and *Ureaplasma spp.*, which vary in their use of glycolysis, arginine-metabolism and urea-metabolism for survival, showed *U. parvum*, *M. hominis* and *M. genitalium* only have an overlap of 247 coding sequences (Pereyre *et al.*, 2009). These observations suggest that, much is yet to be learned from minimum genome analysis of the other mollicutes.

Comparison of 19 sequenced genomes for human *Ureaplasma* spp. have identified an average of 608 predicted genes for each *U. parvum* genome and 664 for *U. urealyticum* (Paralanov *et al.*, 2012). A total of 1020 possible predicted protein coding genes, including singletons, were identified with a core conserved genome of 515 genes. Now that transposon mutagenesis is routinely successful with *U. parvum*, determination of non-essential genes in a microbe that does not use the glycolysis pathway will add empirical verification to the composition of a hypothetical minimum gene set. However, we acknowledge that our studies have not examined whether transposon insertion into the genome had altered gene expression. Two of the transposon-mutated strains showed phenotypic alteration. Further, we would predict that all of the other identified open-reading frame disruptions that we identified should result in a failure to express fully functional proteins, as the predicted open reading frames were disrupted between 150–704bp into the expected coding region. However, as they are hypothetical open reading frames of unknown function, they may not be expressed in parent strains. The one exception may be the integration between UU187(*rpoB*)) and UU188 (*rpoC*), both of which are predicted to be homologues of DNA-directed RNA polymerase subunit beta. The stop codon of UU187 and the intergenic region between the genes was conserved, thus expression of both of these genes may be conserved. In any event, no phenotypic alteration was observed for this mutant. Most of the disrupted genes were of unknown function and only listed as predicted open-reading frames. Genes UU390, UU450, and UU520 may encode membrane proteins and UU047 is predicted to encode a conserved hypothetical ATP/GTP-binding protein. UU526 is predicted to encode an MBA paralogue that should be expressed as a surface-associated membrane lipoprotein, as is UU440, which is also predicted to be a hypothetical membrane lipoprotein. While associated with an alteration in serum resistance, the gene UUR10_0137 is a predicted protein of unknown function.

We have also identified an intermediate level of gene class here, where disruption of the only annotated RNA helicase in the *U. parvum* genome (UU582) resulted in a significant

physiological growth alteration that would likely affect the capacity of the resultant strain to survive *in vivo*. Removal of the DEAD RNA helicase gene from Mycoplasma mycoides subspecies capri also results in a viable but slow growing phenotype (jglass data not shown). No mutant in the orthologous gene was found for M, genitalium, but one was found in M. pneumoniae and M. pulmonis transposon bombardment studies. RNA helicases largely belong to superfamily two of the six families of nucleic acid helicases. The *U. parvum* RNA helicase in particular belongs to the DEAD-box family based on the signature sequence, Asp-Glu-Ala-Asp (Fairman-Williams *et al.*, 2010). While many bacteria encode a few RNA helicases, a substantial number of sequenced bacterial genomes only contain a single DEAD-box helicase (Lu *et al.*, 1999). Although *E. coli* encodes 5 RNA helicases, assessment following individual disruption of each found that the Δ*deaD* mutation was primarily responsible for observed growth defects at 37°C including increased doubling time. The Δ*deaD* (as well as the Δ*srmB*) mutation in *E. coli* also exhibited a cold sensitive phenotype (Jagessar and Jain 2010), very similar to our observations for *U. parvum*.

We were unable to successfully deliver the mini-transposon to a range of *U. urealyticum* strains despite being performed in parallel with the same conditions and reagents used to successfully mutagenise *U. parvum*. The only exception was a single experiment where a clinical strain (W11) was successfully transformed resulting in delivery of the gentamicin resistance gene. This mutant exhibited our second observed phenotypic change: altered survival following serum challenge. Previously we have characterised the complement sensitivity of *U. parvum* strains and found that some strains (such as HPA5) are very sensitive and killed by seronegative serum, some strains are readily killed only by serum containing anti-*Ureaplasma* antibodies (such as HPA2 and DFK-1), and others are inherently resistant (Beeton *et al.*, 2012). However, we did not observe any alteration of serum sensitivity or resistance of the mutagenised *U. parvum* strains compared to the parent strains in this study (data not shown). While we have not finished extending our studies of serum resistance of all *U. urealyticum* serovars as yet, characterisation of the W11 strain found it to be completely resistant to all previously characterised anti-*Ureaplasma* antibody containing (seropositive) sera. However, following transposon integration into the W11 genome, the resultant strain was sensitive to killing by seropositive sera. The altered phenotype was co-incident with loss of a 41 kDa protein detected by the human high titre anti-*Ureaplasma* serum used to challenge the *Ureaplasma* strains and the single transposon insertion site was found to disrupt the hypothetical gene UUR10_0137 (ATCC 33699 serovar 10 gene annotation numbering). This is a predicted membrane protein that is highly conserved in all *U. parvum* and *U.u realyticum* serovars. Further, there are highly homologous predicted open-reading frames in all the other non-haemoplasma members of the *Mycoplasma pneumoniae* group of mycoplasmas. The predicted mass of this open reading frame is 27 kDa, but the size may be increased by post-translational modification. It is also possible that the disruption of this gene may not have any direct bearing on the loss of the expression of the 41 kDa protein or alteration of serum resistance. Further experiments are required to determine if isolated expression of this gene is capable of solely mediating serum resistance.

The major surface antigen MBA (gene UU375 in ATCC strain 700970) has also previously been shown to be susceptible to phase variation following bacterial stress (Zimmerman *et al.*, 2009 & 2011) or alteration of size (Robinson *et al.*, 2013). We only observed one mutant with an alteration in MBA size and no mutants with loss of MBA expression. Therefore, the temperature-shock and selection in gentamicin associated with transformation do not appear to trigger phase variation and we would expect that loss of MBA expression would require gene disruption.

We have succeeded in developing a methodology that is capable of delivering a mini-transposon to the *U. parvum* genome, which results in random gene disruption. This report shows that analysis of transposon mutated *Ureaplasma* strains is now possible to determine non-essential genes, especially important for the numerous hypothetical open-reading frames identified in the analysis of 19 sequenced *Ureaplasma* spp. genomes (Paralanov *et al.*, 2012). This methodology can also now be utilised to determine the minimal genome contingent for a bacterial class that do not utilise glycolysis to survive and shed further light on core essential genes. Characterisation of disrupted gene mutants with pathogenesis studies in experimental *in utero* model infections will also be a key to identifying pathogenic markers within the *U. parvum* genome, as it has been shown to initiate preterm labour and chronic lung disease in preterm neonates experimentally as a sole pathogen (Novy *et al.*, 2009). Transposon mutagenesis will also be valuable in enabling delivery and expression of exogenous genes to *U. parvum* for in vivo tracking and possibly as a mucosal vaccine delivery tool of the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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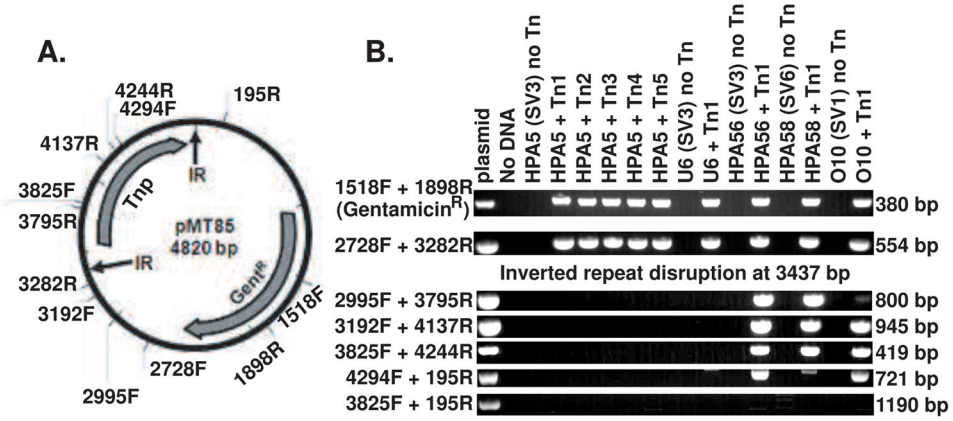

Figure 1.

PCR mapping of regions of mini-transposon plasmid pMT85 (A) present in the genomes of gentamicin resistant selected clonal strains (B). Presence of the gentamicin resistance gene (primers 1518F to 1898R) was only found in transposon mutated strains. PCR probing for different regions of the plasmid identified that strains such as HPA5 and U6 only contain plasmid DNA from between the inverted repeat (IR) regions. Whereas, other transposon mutated strains (HPA56, HPA56 and O10) from different serovars (SV) contain mini-transposon plasmid DNA that include some of the transposase (Tnase) gene. No Tn indicates parental strain, while different numbers after Tn indicate clones from separate experiments. Expected amplicon size is indicated to the right of the figure.

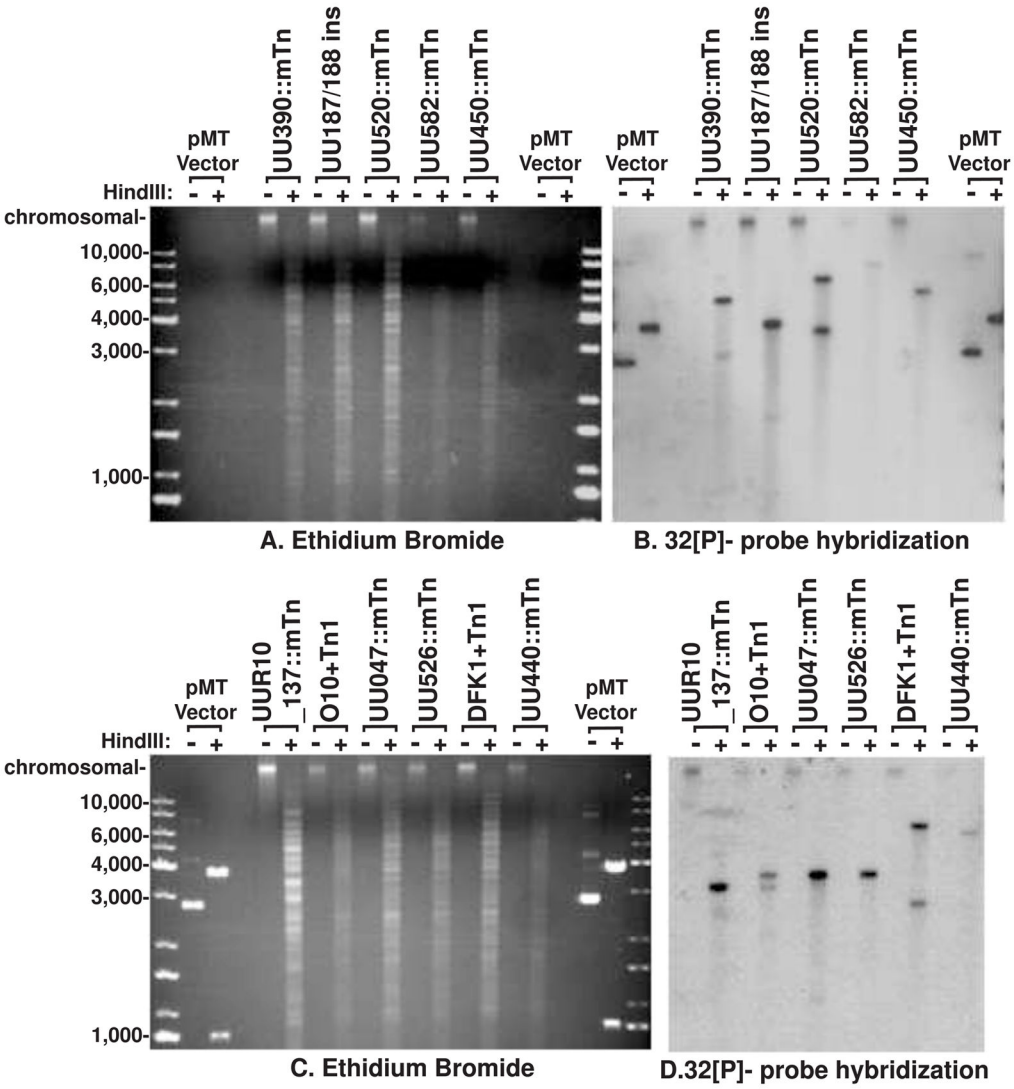

Figure 2.

In gel hybridization detection of gentamicin selection gene. Agarose gel separation of total genomic DNA extracted from transposon mutated Ureaplasma strains. Comparing HindIII digested and undigested genomic DNA. A and C show ethidium bromide visualisation of the DNA prior to probing with the gentamicin resistance gene (visualised by autoradiography in B and D). Three mutants (UU350::mTn, DFK1+Tn1, and O10+Tn1) show 2 bands suggesting a mixed colony or 2 insertion sites. No undigested samples show any extra-chromosomal plasmid DNA. All the remaining examined isolates show a single insertion site into the genome. HindIII-digested and undigested pMT85 vector is shown along with the KAPA Universal DNA ladder for fragment size comparison.

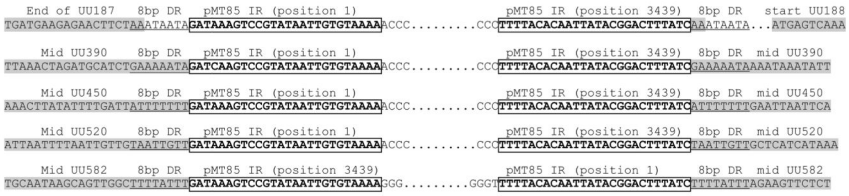

Figure 3.

Sequence alignment of transposon insertion boundaries for HPA5 187/188 intergenic insertion, UU390::mTn, UU450::mTn, UU520::mTn, and UU582::mTn. Inverted repeats are highlighted in green, coding regions of genes are highlighted in grey. The Tn insertion sites are bordered by 8-basepair direct repeats, with 100% identity intra-strain, but unique when compared inter-strain (except being very AT-rich). The direct repeat for the 187/188 insertion shows that the direct repeat replicates the stop codon for UU187 (TAA), therefore no disruption of UU187 coding region occurred.

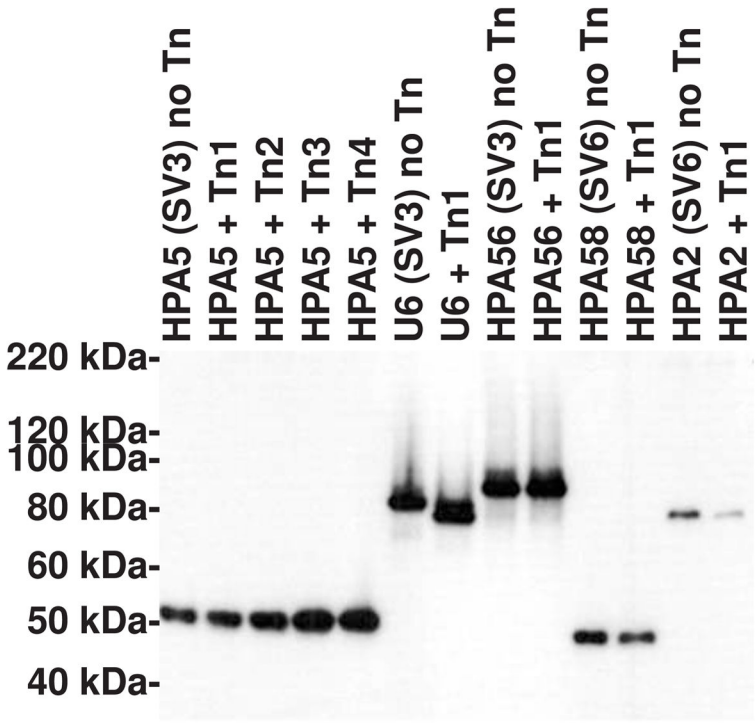

Figure 4.

Immunoblot analysis of SDS solubilised total bacterial protein probed with monoclonal anti-multiple banded antigen antibody. Of 26 individual transformation experiments only one strain (U6) showed altered mobility of the MBA following transposon mutagenesis (+Tn). Representative gel of several experiments is shown showing 3 strains of serovar (SV) 3 and 2 strains of serovar 6.

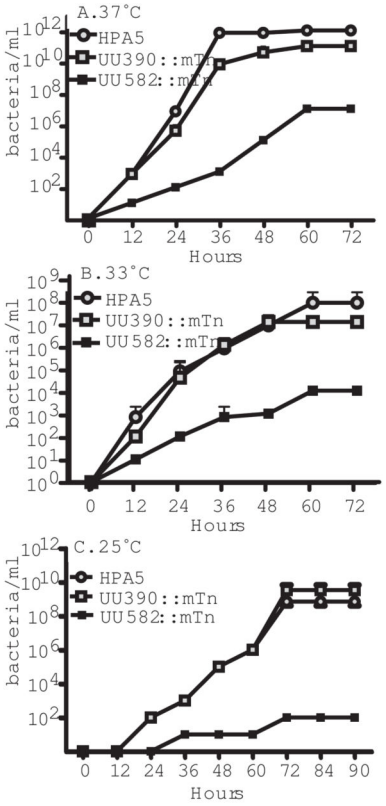

Figure 5.

Growth kinetics for *U. parvum* parent strain (HPA5) compared to membrane protein disruption (UU390::mTn) or DEADbox RNA-helicase gene disruption (UU582::mTn) when incubated at 37°C (A.), 33°C (B.), or 25°C (C.). Strains were titrated out in a 10-fold dilution series and growth measured at time points indicated by urease conversion of urea to ammonium ions. *Ureaplasma* growth is shown as colour (pH indicator) changing units per ml. Mean and standard deviation of dilutions performed in triplicate. Results were consistent through three repeated experiments.

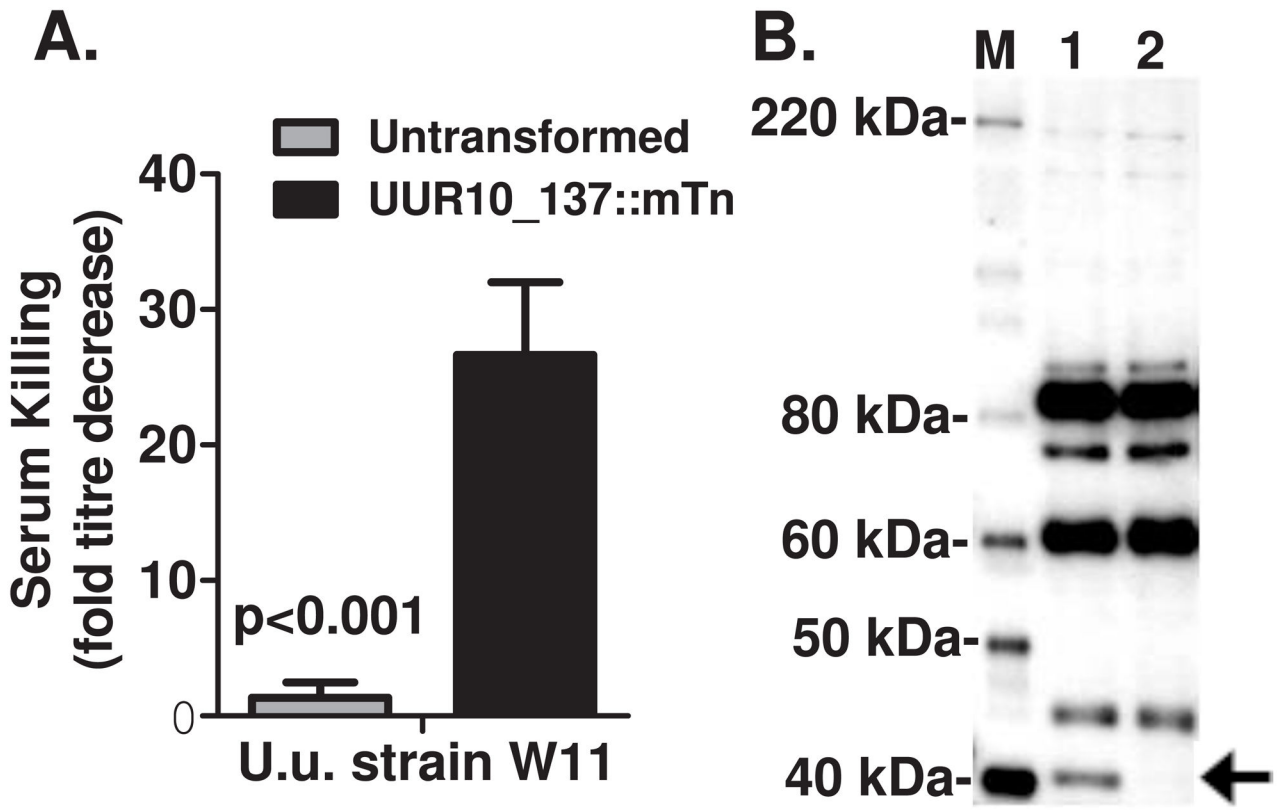

Figure 6.

Serum killing (A) and immunoblot analysis using human high titre seropositive serum (B) for parental *U. urealyticum* strain W11 and following successful transposon mutagenesis (+Tn). Serum killing of increases significantly following 1 h challenge with human serum containing anti-*Ureaplasma* antibodies and analysis of this serum shows the serum-sensitive transposon mutated strain has lost a 41 kDa band that was immunoreactive with the challenging serum. Bar graph shows mean +/− SEM of experiments performed in triplicate. Representative immunoblot from three repeat experiments shown.