Identification of virulence determinants in uropathogenic *Proteus mirabilis* using signature-tagged mutagenesis

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The Gram-negative bacterium *Proteus mirabilis* causes urinary tract infections (UTIs) in individuals with long-term indwelling catheters or those with functional or structural abnormalities of the urinary tract. Known virulence factors include urease, haemolysin, fimbriae, flagella, DsbA, a phosphate transporter and genes involved in cell-wall synthesis and metabolism, many of which have been identified using the technique of signature-tagged mutagenesis (STM). To identify additional virulence determinants and to increase the theoretical coverage of the genome, this study generated and assessed 1880 *P. mirabilis* strain HI4320 mutants using this method. Mutants with disruptions in genes vital for colonization of the CBA mouse model of ascending UTI were identified after performing primary and secondary *in vivo* screens in approximately 315 CBA mice, primary and secondary *in vitro* screens in both Luria broth and minimal A medium to eliminate mutants with minor growth deficiencies, and co-challenge competition experiments in approximately 500 CBA mice. After completion of *in vivo* screening, a total of 217 transposon mutants were attenuated in the CBA mouse model of ascending UTI. Following *in vitro* screening, this number was reduced to 196 transposon mutants with a probable role in virulence. Co-challenge competition experiments confirmed significant attenuation for 37 of the 93 transposon mutants tested, being outcompeted by wild-type HI4320. Following sequence analysis of the 37 mutants, transposon insertions were identified in genes including the peptidyl-prolyl isomerases *surA* and *ppiA*, glycosyltransferase *cpsF*, biopolymer transport protein *exbD*, transcriptional regulator *nhaR*, one putative fimbrial protein, flagellar M-ring protein *fliF* and hook protein *flgE*, and multiple metabolic genes.

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INTRODUCTION

Proteus mirabilis is a common cause of complicated urinary tract infections (UTIs). These infections are predominant in individuals with urinary tract abnormalities or patients with long-term catheters, such as those in nursing homes or hospitals. Bacteriuria with *P. mirabilis* may lead to fever, pyelonephritis, bacteraemia and even death (Mobley & Warren, 1987; Setia *et al.*, 1984; Warren *et al.*, 1982, 1987). During *P. mirabilis* infection, the urinary tract often becomes obstructed by urinary stones resulting from

urease production (Griffith *et al.*, 1976). Urease hydrolyses urea into carbon dioxide and ammonia; the liberation of ammonia creates an alkaline environment that causes soluble ions to precipitate from urine producing a urinary stone (Mobley & Hausinger, 1989). Whilst urease is critical for uropathogenesis, other potential virulence determinants have also been identified including fimbriae, flagella, haemolysin, IgA protease and an amino acid deaminase (Allison *et al.*, 1992a, b, 1994; Bahrani *et al.*, 1991; Belas & Suvanasuthi, 2005; Belas *et al.*, 1998; Bondarenko *et al.*, 1993; Chippendale *et al.*, 1994; Jones & Mobley, 1988; Liaw *et al.*, 2004; Loomes *et al.*, 1993; Mobley & Chippendale, 1990; Peerbooms *et al.*, 1983; Senior *et al.*, 1987; Tolson *et al.*, 1997; Walker *et al.*, 1999; Wang *et al.*, 2008; Wassif

Abbreviations: MR/P, mannose-resistant/*Proteus*-like; STM, signature-tagged mutagenesis; UTI, urinary tract infection.

et al., 1995; Welch, 1987; Wray *et al.*, 1986; Zunino *et al.*, 2003, 2007). To determine the contribution of these proteins to pathogenesis, isogenic mutants have been constructed to determine their effect on virulence (Bahrani *et al.*, 1994; Dattelbaum *et al.*, 2003; Jacobsen *et al.*, 2008; Johnson *et al.*, 1993; Jones *et al.*, 1990; Li *et al.*, 1999, 2002; Massad *et al.*, 1994b; Mobley *et al.*, 1996; Swihart & Welch, 1990; Zunino *et al.*, 2001). To identify additional genes involved in infection or colonization, global techniques such as signature-tagged mutagenesis (STM) have been employed (Burall *et al.*, 2004; Hensel *et al.*, 1995; Zhao *et al.*, 1999).

The application of STM has been successful in identifying a number of virulence genes in many bacterial organisms (Chiang & Mekalanos, 1998; Mei *et al.*, 1997; Sanschagrin *et al.*, 2008; Schwan *et al.*, 1998; Shea *et al.*, 1996). Using this method, mutants in a mixed population can readily be identified by their unique DNA signature tag located at the end of the transposon. This attribute is particularly desirable following infection within a host in which the population of transposon mutants not recovered from an experimental infection (i.e. attenuated mutants) can be identified from among the input pool of mutants. Thus mutants absent from the recovered population, following infection and host colonization, potentially contain transposon insertions within virulence genes. For a recent and thorough review of this technique, see Mazurkiewicz *et al.* (2006).

Previously, we reported the findings of a non-saturating STM screen in which genes affecting motility, iron acquisition, transcriptional regulation, phosphate transport, urease activity, cell-surface structures and key metabolic pathways were found to be required for *P. mirabilis* UTIs (Burall *et al.*, 2004). However, we estimated that the theoretical coverage of the genome in that study was only 46.5%. To increase coverage to at least 70%, we carried out a second STM screening of 1880 *P. mirabilis* strain HI4320 transposon mutants. Performing co-challenge competition experiments using the CBA strain mouse model of ascending UTI, we identified 37 transposon mutants that were significantly outcompeted by wild-type HI4320. The results of this unbiased study when combined with a previous STM study (Burall *et al.*, 2004) and numerous traditional virulence studies (Allison *et al.*, 1992a, b, 1994; Bahrani *et al.*, 1991, 1994; Belas & Suvanasuthi, 2005; Belas *et al.*, 1998; Bondarenko *et al.*, 1993; Chippendale *et al.*, 1994; Dattelbaum *et al.*, 2003; Jacobsen *et al.*, 2008; Johnson *et al.*, 1993; Jones & Mobley, 1988; Jones *et al.*, 1990; Li *et al.*, 1999, 2002; Liaw *et al.*, 2004; Loomes *et al.*, 1993; Massad *et al.*, 1994b; Mobley & Chippendale, 1990; Mobley *et al.*, 1996; Peerbooms *et al.*, 1983; Senior *et al.*, 1987; Swihart & Welch, 1990; Tolson *et al.*, 1997; Walker *et al.*, 1999; Wang *et al.*, 2008; Wassif *et al.*, 1995; Welch, 1987; Wray *et al.*, 1986; Zunino *et al.*, 2001, 2003, 2007) provide a fairly complete picture of urovirulence properties for this agent of complicated UTIs,

P. mirabilis. Newly identified genes include fimbrial, flagellar, metabolic and transport genes.

METHODS

Bacterial strains and plasmids. *P. mirabilis* strain HI4230 (Tet^R) was originally isolated from the urine of an elderly woman presenting with bacteriuria during long-term catheterization (Mobley & Warren, 1987; Warren *et al.*, 1982). For conjugation, 48 *Escherichia coli* S17 λ pir (pUT/mini-Tn5Km2) (Amp^R Kan^R) transformants, each containing a unique DNA signature tag at one end of the mini-Tn5 transposon, were used as donor strains (de Lorenzo *et al.*, 1990; Herrero *et al.*, 1990; Zhao *et al.*, 1999). For identification of *P. mirabilis* transposon mutants, arbitrary PCR products were cloned into pCR2.1-TOPO (Invitrogen) and maintained in either *E. coli* DH5 α or *E. coli* TOP10 (Invitrogen).

Culture media and phenotypic testing. *P. mirabilis* HI4320 was routinely cultured in Luria broth (LB) (1⁻¹: 0.5 g NaCl, 5.0 g yeast extract, 10.0 g tryptone). Both non-swarming agar (1⁻¹: 0.4 g NaCl, 5.0 g yeast extract, 10.0 g tryptone, 15.0 g agar, 5.0 ml glycerol) and Luria agar (1⁻¹: 0.5 g NaCl, 5.0 g yeast extract, 10.0 g tryptone, 15.0 g agar) were used to maintain the transposon mutants and prevent swarming (Belas *et al.*, 1991). Swarming phenotypes of *P. mirabilis* transposon mutants were compared with the wild-type HI4320 swarming phenotype on swarming agar (Burall *et al.*, 2004). Modified urea segregation agar was used to identify the presence of urease activity in the *P. mirabilis* transposon mutants as described previously (Burall *et al.*, 2004; Hu *et al.*, 1990). Minimal A medium [1⁻¹: 10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 0.47 g sodium citrate and 1.0 g (NH₄)₂SO₄] was prepared and autoclaved. After cooling, 1 ml 1 M MgSO₄, 10 ml 20% glycerol and 1 ml 1% nicotinic acid were added per litre and used to screen for auxotrophic *P. mirabilis* transposon mutants (Belas *et al.*, 1991). Minimal A medium agar was made by the addition of 2.5% Bacto Agar to minimal A medium. Antibiotics were added as necessary at the following concentrations: kanamycin, 25 μ g ml⁻¹; tetracycline, 15 μ g ml⁻¹; and ampicillin, 100 μ g ml⁻¹.

Generation of an STM library using bacterial conjugation. *P. mirabilis* transposon mutants were constructed and screened using an STM technique adapted by Hensel *et al.* (1995). Transformation of *E. coli* S17 λ pir with each of the 48 pUT/mini-Tn5Km2 plasmids containing a unique DNA signature tag (provided by C. Tang, Imperial College, London, UK) was performed as previously described (Zhao *et al.*, 1999). Unique DNA signature tags were previously screened and selected based on non-cross-reactivity with one another (Burall *et al.*, 2004). Bacterial conjugation reactions between recipient strain *P. mirabilis* HI4320 and 48 *E. coli* S17 λ pir (pUT/mini-Tn5Km2) (Amp^R Kan^R) donor strains were performed as described previously using a modified protocol (Burall *et al.*, 2004). Overnight cultures of *P. mirabilis* HI4320 (Tet^R) and each of the 48 *E. coli* donor strains (Kan^R Amp^R) were mixed at a ratio of 2:1 or 5:1. Following centrifugation and removal of supernatant, LB medium without antibiotic was added to the pellet and held at room temperature for 5 min. After resuspension of the pellet, 50 or 100 μ l of the suspension was pipetted onto membranes (0.45 μ m pore size) and aseptically positioned on non-swarming agar plates without antibiotic. After drying, plates were incubated at 37 °C for 60–90 min to minimize replication of siblings. Following incubation, membranes were aseptically overlaid with LB medium without antibiotic onto non-swarming agar plates containing kanamycin and tetracycline and incubated at 37 °C overnight. Transconjugants were screened for ampicillin sensitivity to ensure loss of the pUT vector and incorporation of the mini-Tn5 transposon into the genome of *P. mirabilis*, as described previously (Burall *et al.*, 2004). Simultaneously,

screening for resistance to kanamycin and tetracycline was performed to select only *P. mirabilis* transposon mutants containing the kanamycin transposon. In the library, 59/1920 transconjugants selected (3% conjugation rate failure) had to be reselected to create pools in which each one of the 48 unique DNA signature tags was represented only once per pool. Within each pool, one of the 48 mutants served as a negative control. The signature tag of the negative control failed to hybridize and did not cross-hybridize with other tags. For this study, 40 pools (1880 tagged transposon mutants not including negative controls) were created, each with 48 *P. mirabilis* transposon mutants containing a uniquely tagged transposon (Kan^R Tet^R). *P. mirabilis* mutants (Kan^R Tet^R Amp^S) were stored in 96-well plates at -80 °C in 80% glycerol.

CBA mouse model of ascending UTI. To identify attenuated signature-tagged mutants, all 1880 *P. mirabilis* transposon mutants were tested in the CBA/J murine model of ascending UTIs as described previously (Burall *et al.*, 2004; Hagberg *et al.*, 1983; Johnson *et al.*, 1993). For primary screens, each of the 40 pools was transurethraly infected into a total of five mice for a 4-day infection. Subsequent secondary screens of 23 smaller pools containing 20–25 mutants (each DNA signature tag represented only once per pool) were also transurethraly infected into a total of five mice for a 4-day infection. Co-challenge competition experiments between *P. mirabilis* HI4320 and putatively attenuated *P. mirabilis* transposon mutants were performed with a modified protocol (Burall *et al.*, 2004). For each of the 93 competition experiments, a total of five mice were infected with either 1×10^7 or 5×10^7 c.f.u. of a bacterial suspension containing a 1:1 ratio of *P. mirabilis* HI4320 and a *P. mirabilis* transposon mutant. It was not uncommon for mice to die midway through the 7-day infection period; this appeared to be caused primarily by obstruction of urine flow by urease-mediated stone formation. In these instances, experiments were repeated with additional mice to achieve data from a minimum of five mice. Viable counts from plates were expressed in c.f.u. (ml urine)⁻¹ or c.f.u. (g tissue)⁻¹, as described previously (Burall *et al.*, 2004).

In vitro screening using LB and minimal A media. Prior to co-challenge competition experiments, *P. mirabilis* transposon mutants putatively attenuated in both *in vivo* primary and secondary screens were tested *in vitro* to disqualify mutants that may have been attenuated in the mouse model due only to minor growth defects. *In vitro* primary and secondary screening using LB and minimal A media was performed as described previously (Burall *et al.*, 2004). Mutants were placed in pools of 15 for primary screening and then regrouped into pools of five to ten for secondary screening.

Labelling and hybridization screening of STM mutants. For all *in vivo* and *in vitro* screens, DNA signature tags of bacteria in the input inoculum and from recovered bacteria (output sample) were PCR amplified, labelled with digoxigenin-dUTP (Roche) and hybridized to donor DNA dot-blot membranes containing a representative of each DNA signature tag. For *in vivo* screens, recovered bacteria were isolated from the bladder and kidneys. Visual comparison of the hybridized blots between input and output samples was performed to identify putatively attenuated transposon mutants. For *in vivo* primary screens, transposon mutants that lacked a hybridization signal in either or both output samples, had a reduced hybridization signal compared with the input sample or had an intensity equal to or less than that of the negative control were scored anywhere from being slightly attenuated to unrecoverable. Primary *P. mirabilis* transposon mutants attenuated from the primary screen were regrouped into secondary pools of 20–25 mutants each and subjected to a secondary screen. Mutants of the secondary screen in the output sample lacking a hybridization signal compared with the input sample or having an intensity equal to or less than that of the negative control were considered reproducibly unrecoverable. For *in*

vitro primary and secondary screens, putatively attenuated mutants that were able to survive passage in LB and minimal A media, and thus were recovered as seen by hybridization on donor DNA dot blots, were kept as putatively attenuated mutants to be tested in co-challenge competition experiments. The labelling and hybridization procedure was performed for all input and output samples of primary and secondary *in vivo* and *in vitro* screens as described previously with minor modifications (Burall *et al.*, 2004). A two-step PCR was used to amplify the DNA signature tags. A 1:5 dilution of sample was used as template for PCR I. Following PCR I, the product was loaded on a 1.6% (w/v) SeaPlaque agarose gel (Cambrex) and electrophoresed. A 3.5 µl sample of the 80 bp gel slice was used for PCR II. A sample of 10–25 µl of the PCR II digoxigenin-labelled fragments was used for hybridization to donor DNA blots prepared with 10 µl plasmid DNA. The primers used in PCRs I and II are listed in Table 1.

Statistical analysis. Statistical analysis of co-challenge data was performed using a repeated measure of analysis of variance with rank order data (STATA software) as described previously (Bahrani-Mougeot *et al.*, 2002; Burall *et al.*, 2004). Rank order data were used to prevent extreme outliers from skewing the analysis (Bahrani-Mougeot *et al.*, 2002; Burall *et al.*, 2004). The percentage genome saturation for a screen of a given number of transposon mutants was calculated using the formula $n = \ln(1-p)/\ln(1-f)$ derived from Zilsel *et al.* (1992) in which n is the number of transposon mutants, f is equal to 1/number of genes in the genome and p is % probability.

Sequence analysis. Arbitrary PCR and molecular cloning were carried out on *P. mirabilis* transposon mutants that were statistically outcompeted by wild-type HI4320 to identify the gene in which the transposon was inserted. Arbitrary PCR as described by Bahrani-Mougeot *et al.* (2002) was carried out as two sequential amplification steps with primers complementary to the transposon end reading outward and arbitrarily designed primers reading inward from the unknown sequence. Arbitrary PCR 1 primer (Arb 1) and primer 1955 were prepared and used under PCR conditions described previously (Burall *et al.*, 2004). Arbitrary PCR 2 was performed with arbitrary PCR 2 primer (Arb 2) and primer 1954 using 1 µl product from arbitrary PCR 1 as described previously (Burall *et al.*, 2004). Primers used in arbitrary PCRs 1 and 2 are listed in Table 1. Gel-isolated arbitrary PCR product was either directly sequenced or cloned into pCR2.1-TOPO (Invitrogen) and sequenced at the Biopolymer Core Facility at the University of Maryland, MD, USA, or at the Sequencing Core Facility at the University of Michigan, MI, USA. The sequences of primers 1955 and 1954 are both located within the transposon. For

Table 1. Oligonucleotide primers used in this study

Direction of primer	Primer*	Sequence (5'→3')
Forward	P2	TACCTACAACCTCAAGCT
Reverse	P4	TACCCATTCTAACCAAGC
Forward	Arb 1	GGCCACGCGTCGACTAGTCANNNNNN
Reverse	1955	CAGGGCTTTATTGATTCCAT
Forward	Arb 2	GGCCACGCGTCGACTAGTCA
Reverse	1954	ACAGCCGGATCCTCTAGAGT

*Primers P2 and P4 were used in PCRs I and II to amplify the variable region of the DNA signature tags. Primers Arb 1 and 1955 were used in Arbitrary PCR 1 and primers Arb 2 and 1954 were used in Arbitrary PCR 2. Primers 1955 and 1954 amplify outward from the end of the transposon.

all sequencing reactions, primer 1954 was used to determine the interrupted gene sequence.

Sequence analysis and translation were performed using online search engines at <http://expasy.org>. Homology searches (BLAST) of nucleotide and protein sequences (Altschul *et al.*, 1990) were completed using the public database GenBank (<http://www.ncbi.nlm.nih.gov>). All nucleotide sequences of identified transposon mutants were examined using the Sanger Institute's database of the sequenced and annotated *P. mirabilis* HI4320 genome at http://www.sanger.ac.uk/Projects/P_mirabilis/ (Pearson *et al.*, 2008). The nucleotide sequence was analysed for presence of the transposon and placed in the Sanger Institute's *P. mirabilis* BLAST server to query exact sequence matches. By examining matches of unknown nucleotide sequence at the end of the transposon to known *P. mirabilis* sequence, a transposon insertion could be determined at the exact nucleotide within the genome. The correlating *P. mirabilis* nucleotide number where the transposon inserted and that of the Sanger Institute's annotation of *P. mirabilis* was used to identify the gene.

SDS-PAGE and Western blot analysis. Attenuated transposon mutants of *P. mirabilis* confirmed by co-challenge competition experiments were analysed by Western blotting for the presence of flagellin (Mobley *et al.*, 1996) and the MrpA fimbrial subunit (Burall *et al.*, 2004; Li *et al.*, 1999). Bacterial suspensions were standardized at OD₆₀₀=0.8 and protein was precipitated using TCA. Samples were denatured for 5 min by boiling in sample buffer, separated by 15 % SDS-PAGE gel and transferred to a PVDF membrane (Millipore). In Fig. 1(a), samples were standardized using a 2-D Quant kit (Amersham) and 11 µg protein was loaded onto the gel. After Western blotting, membranes were incubated with rabbit polyclonal antiserum against either flagellin or MrpA followed by a secondary goat anti-rabbit IgG conjugated to horseradish peroxidase. Membranes were developed using ECL Plus Western blotting detection reagents (GE Healthcare).

RESULTS AND DISCUSSION

Isolation of attenuated *P. mirabilis* mutants using STM

The primary *in vivo* screen of 1880 *P. mirabilis* transposon mutants resulted in 570 mutants being characterized as slightly attenuated to unrecoverable. In a secondary *in vivo* screen of these 570 transposon mutants, 217 mutants were reproducibly unrecoverable. The further reduction in number of transposon mutants eliminated following the second *in vivo* screen was a result of an individual mutant's fitness relative to the population being screened.

Elimination of auxotrophic mutants or mutants with growth defects

The 217 transposon mutants identified as attenuated in both primary and secondary *in vivo* screens were tested *in vitro* to identify those with minor growth defects that may decrease generation time in rich LB medium and to identify mutants with auxotrophic mutations by lack of growth in minimal A medium. Following passage of the 217 transposon mutants *in vitro*, 141 mutants were not outcompeted in either LB or minimal A medium. The remaining 76 transposon mutants were placed in secondary

Fig. 1. (a) Flagella and mannose-resistant/*Proteus*-like fimbriae production by wild-type *P. mirabilis* HI4320 and selected STM transposon mutants shown by Western blotting of standardized whole-cell preparations reacted with rabbit anti-FlaA and anti-MrpA, respectively. The molecular masses of the FlaA and MrpA subunit proteins are indicated in kDa. Mutant 2E2, in which metabolic gene *cyaA* is disrupted, appeared to be deficient in both FlaA and MrpA expression. As expected, mutant 8A6, which disrupted *flhF*, lacked FlaA but appeared to have increased MrpA production compared with wild-type HI4320. Likewise, mutant 21B2, which disrupted *flgE*, lacked FlaA but did not appear to have an increase in MrpA. Interestingly, the putative adhesin disrupted in mutant 36D2 did not produce MrpA. Metabolic mutant 10E4 and unidentified mutants 15B4 and 17A4 did not produce MrpA. Mutant 38E2, which disrupted a multicopy oxidase, did not produce FlaA. (b–g) Swarming phenotypes of *P. mirabilis* (b) and selected transposon mutants (c–g) after spotting overnight cultures onto swarming agar and incubating for 18 h. Mutants showed deficient to aberrant swarming patterns in comparison with wild-type *P. mirabilis* HI4320 (WT) with vertical lines depicting the edge of the outer swarming raft.

pools and each was retested in both LB and minimal A media. Of these transposon mutants, 21 were outcompeted after performing morning and afternoon passages for 4 days in LB and minimal A media. Thus a total of 196 transposon mutants were not outcompeted in LB or

minimal A medium and displayed no growth defects after primary and secondary *in vitro* screens.

Co-challenge and identification of attenuated mutants

CBA mice were co-challenged with wild-type *P. mirabilis* HI4320 and each of the 93 transposon mutants that were considered most attenuated out of the 196 transposon mutants based on hybridization intensity results from the dot blots. Of the 93 transposon mutants tested, 37 were statistically outcompeted by wild-type HI4320. Attenuation by at least 100-fold in urine, bladder or kidney samples was observed in 15 of the 37 statistically significant transposon mutants outcompeted in the co-challenge experiment (Table 2).

Of the 37 significantly attenuated transposon mutants, a total of 29 were identified and were grouped into five categories, including cellular processes, transport, regulation, cell-surface structures and motility, and metabolism (Table 3). The transposon insertions of 24 of these identified mutants were located within the open reading frame of the gene listed for each mutant (Table 3). The five remaining mutants (6D1, 11C4, 32E1, 13D1 and 38E2) had transposon insertions that were located upstream of the gene (Table 3).

Cellular processes

Mutants 11C4 and 32E1, each containing unique tags, had an identical site of transposon disruption just prior to the start codon of the open reading frame of *surA*. Mutant 6D1 had a transposon disruption after a predicted promoter sequence and before the start of the open reading frame of *ppiA*. Both *surA* and *ppiA* encode periplasmic peptidyl-prolyl isomerases, which are ATP-independent enzymes that catalyse the *cis-trans* conversion of peptidyl proline bonds that facilitate proper protein folding. Genes that encode these proteins have been shown to have a role in uropathogenesis (Hunstad *et al.*, 2005; Justice *et al.*, 2005, 2006).

Transposon mutants 11B2 and 20C4, also containing unique tags, had identical disruptions within *cpsF*, which encodes a glycosyltransferase required for lipopolysaccharide core modification (Klena *et al.*, 1992; MacLachlan *et al.*, 1991; Parker *et al.*, 1992). Previously, this mutant has also been found to compromise urinary tract virulence (Allison *et al.*, 1994). The aberrant swarming phenotype of mutant 20C4 was similar to that of a *P. mirabilis* U6450 *TnphoA* insertion mutant of *cpsF* unable to assemble capsular polysaccharide (Gygi *et al.*, 1995). Upon further analysis, mutant 11B2 produced a swarming phenotype like that of HI4320 and not of 20C4. It is possible that there was a compensatory mutation in transposon mutant 11B2 restoring the swarming phenotype to that of HI4320 or that the difference in the tag sequence itself somehow governed this phenotype.

Transport

The disrupted gene of transposon mutant 7A1, *exbD*, encodes a biopolymer transport protein. ExbD works in conjunction with TonB and ExbB to form an energy transducing system that uses the proton motive force of the cytoplasmic membrane to transport micronutrients across the outer membrane into the cell. The TonB–ExbB–ExbD system is essential for internalization of iron–siderophore complexes across outer-membrane receptors (Braun *et al.*, 1996; Fischer *et al.*, 1989). Thus these mutants would be outcompeted in the iron-limited urinary tract (Alteri & Mobley, 2007).

Gene regulation

Transposon mutant 4B6 was identified as disrupting the transcriptional activator protein NhaR, a DNA-binding protein of the LysR family of transcriptional regulators, and was attenuated in the urine ($P=0.001$) and kidneys ($P=0.004$) and overall ($P<0.0005$) *in vivo*. NhaR expression has been shown to be induced in response to alkaline conditions and activates transcription of the *pgaABCD* operon required for poly- β -1,6-*N*-acetyl-D-glucosamine production (Goller *et al.*, 2006); the latter promotes *E. coli* fitness by aiding cell-to-cell adhesion, attachment to surfaces and stabilization of biofilm structures (Itoh *et al.*, 2005). Unlike uropathogenic *E. coli*, *P. mirabilis* produces urease, which creates an alkaline microenvironment (Jones *et al.*, 1990). When *P. mirabilis* infects the urinary tract, urease-mediated urea hydrolysis increases environmental pH, which may cause activation of NhaR; this could lead to increased expression of genes that may ultimately contribute to the persistence of infection. Presently, gene targets of the *P. mirabilis* NhaR regulator have yet to be identified. The finding of *nhaR* in our STM screening provides evidence that the urinary tract is alkaline in the presence of *P. mirabilis* and supports the premise that *P. mirabilis* HI4320 responds to alkaline conditions by producing virulence factors to aid in UTIs.

Cell-surface structures and motility

In our previous STM study (Burall *et al.*, 2004), only one motility-related gene, *cheW*, was identified. Here, we report findings of three additional motility genes important in *P. mirabilis* HI4320 colonization. Two genes of the flagellum regulon, *fliF* and *flgE*, were identified in our STM screen. *fliF* encodes protein subunits that make up the MS ring of the flagellum, which is the first synthesized substructure and is crucial for flagellar assembly (Grunenfelder *et al.*, 2003; Kubori *et al.*, 1992; Wu & Newton, 1997). The other flagellar transposon mutant had a disruption in the hook protein encoded by *flgE*. This mutant can form the PL ring complex and distal rod, but cannot assemble a complete flagellum (Komoriya *et al.*, 1999). Both Western blot and swarming results of the *flgE* and *fliF* transposon mutants were identical; no flagellin protein was detected and a

Table 2. Co-challenge with *P. mirabilis* HI4320 and attenuated STM mutants in the CBA mouse model of ascending UTI

Five female CBA/J mice were inoculated transurethrally with 10^7 c.f.u. (approx. equal numbers of wild-type and mutant). After 7 days, mice were euthanized and organs were harvested to determine the number of c.f.u. g^{-1} or c.f.u. ml^{-1} for wild-type and mutant strains. NA, Not applicable (ratios were not statistically significantly different from 1); NS, not statistically significant.

Attenuated mutant	No. of mice	Mutant : wild-type ratio (<i>P</i> value)*			
		Urine	Bladder	Kidneys	Overall†
11C4‡	5	4.49×10^{-3} (0.003)	1.48×10^{-2} (<0.0005)	1.40×10^{-2} (<0.0005)	9.76×10^{-3} (<0.0005)
32E1	5	NA (NS)	4.51×10^{-2} (0.003)	NA (NS)	NA (NS)
11B2	5	NA (NS)	2.25×10^{-1} (0.003)	1.44×10^{-1} (0.002)	1.26×10^{-1} (0.006)
20C4‡	6	1.55×10^{-3} (0.001)	1.75×10^{-3} (<0.0005)	6.52×10^{-4} (<0.0005)	1.21×10^{-3} (<0.0005)
6D1‡	4	6.75×10^{-3} (0.045)	NA (NS)	1.28×10^{-3} (<0.0001)	4.02×10^{-3} (0.013)
9C6	3	NA (NS)	NA (NS)	3.51×10^{-1} (0.036)	NA (NS)
12E4	5	4.33×10^{-1} (0.021)	NA (NS)	NA (NS)	NA (NS)
13D1	5	2.69×10^{-2} (0.013)	NA (NS)	NA (NS)	8.74×10^{-2} (<0.0005)
38E2	5	1.52×10^{-1} (0.021)	NA (NS)	1.01×10^{-2} (<0.0005)	NA (NS)
1F6	4	1.15×10^{-2} (0.027)	NA (NS)	5.39×10^{-2} (<0.0001)	5.40×10^{-2} (0.011)
23D5‡	5	7.14×10^{-3} (<0.0001)	NA (NS)	1.94×10^{-3} (<0.0001)	1.33×10^{-2} (<0.0001)
7A1‡	5	2.47×10^{-2} (0.004)	8.42×10^{-3} (0.019)	2.03×10^{-2} (0.017)	1.57×10^{-2} (0.001)
4B6	4	3.95×10^{-1} (0.001)	NA (NS)	1.47×10^{-1} (0.004)	3.45×10^{-1} (<0.0005)
4A3	4	2.33×10^{-1} (0.004)	NA (NS)	NA (NS)	NA (NS)
8A6	8	NA (NS)	2.57×10^{-2} (0.002)	NA (NS)	NA (NS)
21B2	7	NA (NS)	NA (NS)	1.72×10^{-1} (<0.0005)	NA (NS)
36D2	5	NA (NS)	2.63×10^{-1} (0.032)	NA (NS)	NA (NS)
32C4	6	NA (NS)	4.17×10^{-2} (0.003)	NA (NS)	NA (NS)
6F6	5	2.02×10^{-1} (0.039)	NA (NS)	NA (NS)	3.15×10^{-1} (0.029)
2E2‡	5	5.46×10^{-6} (<0.0001)	5.34×10^{-5} (<0.0001)	5.03×10^{-5} (<0.0001)	2.45×10^{-5} (<0.0001)
5F1‡	5	2.00×10^{-2} (0.025)	3.94×10^{-2} (<0.0001)	9.13×10^{-3} (<0.0001)	1.93×10^{-2} (<0.0001)
11D4	4	5.57×10^{-1} (0.028)	NA (NS)	9.37×10^{-2} (0.015)	NA (NS)
11G3‡	5	4.56×10^{-3} (0.012)	NA (NS)	1.23×10^{-1} (0.053)	4.17×10^{-2} (0.098)
8B5‡	3	NA (NS)	1.03×10^{-4} (<0.0001)	2.42×10^{-4} (<0.0001)	6.07×10^{-4} (<0.0001)
10E4	5	1.60×10^{-2} (<0.0001)	1.06×10^{-2} (<0.0001)	7.47×10^{-2} (0.025)	2.33×10^{-2} (<0.0001)
15C3	4	4.27×10^{-1} (0.016)	NA (NS)	NA (NS)	NA (NS)
17A4‡	5	NA (NS)	2.47×10^{-3} (0.007)	2.33×10^{-2} (0.004)	1.46×10^{-2} (0.002)
16F1	5	NA (NS)	NA (NS)	1.05×10^{-1} (0.043)	NA (NS)
40A6‡	8	1.01×10^{-2} (0.003)	6.97×10^{-3} (0.001)	2.93×10^{-2} (<0.0005)	1.25×10^{-2} (<0.0005)
8H4‡	5	6.10×10^{-3} (0.040)	NA (NS)	NA (NS)	8.76×10^{-2} (0.003)
11A4	5	3.71×10^{-2} (0.032)	NA (NS)	8.07×10^{-2} (0.007)	3.65×10^{-2} (0.008)
15A6‡	5	3.72×10^{-3} (<0.0005)	5.99×10^{-4} (0.002)	3.81×10^{-4} (<0.0005)	9.45×10^{-4} (<0.0005)
15B4‡	4	11.6 (0.014)	494 (<0.0001)	1.89 (0.001)	22.1 (<0.0001)
17F5	5	2.20×10^{-1} (<0.0005)	NA (NS)	NA (NS)	3.72×10^{-1} (0.022)
22F6	4	3.15×10^{-2} (0.024)	NA (NS)	NA (NS)	1.03×10^{-1} (0.043)
33B5	5	NA (NS)	NA (NS)	6.31×10^{-1} (<0.0005)	NA (NS)
37D5‡	5	5.48×10^{-4} (0.002)	2.64×10^{-2} (<0.0005)	NA (NS)	1.56×10^{-2} (<0.0005)

*Ratio of the number of mutant c.f.u. g^{-1} or c.f.u. ml^{-1} to the number of wild-type c.f.u. g^{-1} or c.f.u. ml^{-1} for significantly attenuated mutants as determined using the method described by Bahrani-Mougeot *et al.* (2002).

†Overall *P* values were calculated in a similar way to the other values, but all numbers were pooled without regard to tissue.

‡Mutants in which there was attenuation by at least 100-fold in the urine, bladder or kidney samples.

Table 3. Identification of disrupted genes in attenuated *P. mirabilis* transposon mutants

Classification	Mutant	Site of attenuation*	PMI no.†	Gene	Annotation	Western analysis‡		Swarming§
						FlaA	MrpA	
Cellular processes	11C4	U, B, K, O	PMI2332	<i>surA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase	+	+	Normal
	32E1	B	PMI2332	<i>surA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase	+	+	Normal
	11B2	B, K, O	PMI3190	<i>cpsF</i>	Glycosyltransferase	+	+	Normal
	20C4	U, B, K, O	PMI3190	<i>cpsF</i>	Glycosyltransferase	+	+	Short
	6D1	U, K, O	PMI2823	<i>ppiA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase A	+	+	Normal
	9C6	K	PMI0283		Putative metalloprotease	+	+	Normal
	12E4	U	PMI1598	<i>yidA</i>	Putative phosphatase	+	+	Normal
	13D1	U, O	PMI3359		Putative antitoxin	+	+	Normal
Transport	38E2	K, O	PMI2342	<i>sufI</i>	Putative multicopy oxidase	–	+	No raft
	1F6	U, K, O	PMI3705		Putative ABC transporter; ATP-binding protein	+	+	Normal
	23D5	U, K, O	PMI2259	<i>metN</i>	D-Methionine ABC transporter; ATP-binding protein	+	+	Normal
Regulation	7A1	U, B, K, O	PMI0030	<i>exbD</i>	Biopolymer transport protein	+	+	Normal
	4B6	U, K, O	PMI0012	<i>nhaR</i>	Transcriptional activator	+	+	Normal
	4A3	U	PMI3001		Putative fimbrial protein	+	+	Normal
Cell surface structures and motility	8A6	B, K	PMI1630	<i>fliF</i>	Flagellar M-ring	–	+	No raft
	21B2	K	PMI1651	<i>flgE</i>	Flagellar hook protein	–	+	No raft
	36D2	B, O	PMI2575		Putative adhesin	+	–	Normal
Metabolism	32C4	B	PMI2014		Putative membrane protein	+	+	Normal
	6F6	U, O	PMI1448		Putative lipoprotein	+	+	Normal
	2E2	U, B, K, O	PMI3333	<i>cyaA</i>	Adenylate cyclase	–	–	Star-shaped
	5F1	U, B, K, O	PMI1546	<i>guaB</i>	Inosine-5'-monophosphate dehydrogenase	+	+	Normal
	11D4	U, K	PMI2760	<i>edd</i>	6-Phosphogluconate dehydratase	+	+	Normal
	11G3	U, K, O	PMI2378	<i>cbbC</i>	Probable molybdopterin-containing oxidoreductase	+	+	Normal
	8B5	B, K, O	PMI2046	<i>aceE</i>	Pyruvate dehydrogenase E1 component	+	+	Star-shaped
	10E4	U, B, K, O	PMI0565	<i>sdhC</i>	Succinate dehydrogenase cytochrome <i>b</i> ₅₅₆ subunit	+	–	Elongated
Others	15C3	U	PMI1607	<i>sdaA</i>	L-Serine deaminase 1	+	+	Normal
	16F1	K	PMI1184		Putative exported protein	+	+	Normal
	40A6	U, B, K, O	PMI1193		Putative exported protein	+	+	Normal
	8H4	U, O	PMI1000		Putative phage-related protein	+	+	Normal
Unknown¶	17A4	B, K, O	NA			+	–	Normal
	11A4	U, K, O	NA			+	+	Normal
	15A6	U, B, K, O	NA			+	+	Normal
	15B4	U, B, K, O	NA			+	–	Normal
	17F5	U, O	NA			+	+	Normal
	22F6	U, O	NA			+	+	Normal
	33B5	U, K	NA			+	+	Normal
	37D5	U, B, O	NA			+	+	Short

*Tissues in which there was a statistically significant reduction in the number of mutant c.f.u. following co-challenge of individual STM mutants and wild-type. U, urine; B, bladder; K, kidneys; O, overall significance in U, B and K.

†*P. mirabilis* HI4320 gene number in the complete genomic sequence.

‡Western blots were assessed for detection of flagella and MR/P fimbriae proteins.

§Mutants were tested for swarming, urease activity and growth on minimal A medium agar. After 18 h, the pattern on swarming agar was compared with that of wild-type. After 24 h, all mutants had urease enzymic activity on urea segregation agar and grew on minimal A medium. All mutants were urease-positive.

||Mutant in which transposon disruption was located upstream of the start codon of the gene listed.

¶Mutant not identified.

swarming-deficient phenotype was observed, probably due simply to lack of flagella synthesis. In addition, mutant 8A6, in which the transposon disrupts *fliF*, appeared to be overproducing *mrpA* (Fig. 1a).

The five known *P. mirabilis* fimbriae are mannose-resistant/*Proteus*-like fimbriae (MR/P) (Sareneva *et al.*, 1990), *P. mirabilis* fimbriae (Massad & Mobley, 1994), ambient-temperature fimbriae (Massad *et al.*, 1994a), non-agglutinating fimbriae (Tolson *et al.*, 1995; Wray *et al.*, 1986) and *P. mirabilis* P-like pili (Bijlsma *et al.*, 1995). The completed annotation of the *P. mirabilis* HI4320 genome has revealed an additional 12 putative fimbrial operons (Pearson *et al.*, 2008). Mutant 4A3 contained a disruption of the gene that encodes PMI3001, a putative fimbrial protein, which is located within one of these newly identified fimbrial operons. PMI3001 is located in fimbrial operon 14 (Pearson *et al.*, 2008). Mutant 4A3 was attenuated and outcompeted by wild-type HI4320 in the murine model of ascending UTI, suggesting that this newly identified fimbrial operon may play a role in the pathogenesis of UTIs, as has been shown for the *mrp* operon (Baharani *et al.*, 1994).

Transposon mutant 36D2 was outcompeted *in vivo* and contained a disruption in PMI2575, which encodes a putative adhesin-type autotransporter. Autotransporters have been associated with virulence functions such as adhesion, aggregation, invasion, biofilm formation and toxicity (Alamuri & Mobley, 2008; Wells *et al.*, 2007). By Western blot analysis, it appeared that mutant 36D2 was negative for synthesis of the MrpA fimbrial subunit (Fig. 1). There is no clear explanation, however, as to why a mutation in this adhesin would result in lack of MrpA expression.

Metabolism

A total of seven metabolic genes were identified in our STM screen including those involved in nucleotide, carbohydrate and amino acid metabolism. Mutation of these genes did not interfere with growth of *P. mirabilis* *in vitro* in rich medium, although disruption of *guaB* and *aceE* resulted in an increased lag phase compared with wild-type HI4320 in minimal A medium, probably due to defects in nucleotide metabolism and central metabolism, respectively. Transposon mutants 5F1 and 2E2 had disruptions in genes involved in purine metabolism. Mutant 5F1 disrupted *guaB* encoding inosine 5'-monophosphate dehydrogenase, which catalyses the first step of GTP synthesis. The *cyaA* gene disrupted in transposon mutant 2E2 encodes adenylate cyclase, which catalyses the breakdown of ATP to 3',5'-cyclic adenosine monophosphate (cAMP). Interestingly, this mutant was unable to produce either FlaA or MrpA. Four genes were involved in carbohydrate metabolism. Mutant 11G3 was disrupted in *cbbC*, a probable molybdopterin-containing oxidoreductase; this is similar to formate dehydrogenase, which is involved in glyoxylate, dicarboxylate and methane

metabolism. Transposon mutant 11D4 disrupted *edd*, encoding 6-phosphogluconate dehydratase, which is involved in the Entner–Doudoroff pathway. Mutant 10E4 disrupted *sdhC*, which encodes the succinate dehydrogenase cytochrome *b*₅₅₆ subunit, which is part of the citric acid or TCA cycle. This mutant was also found by Western blot analysis not to produce MrpA, the major fimbrial subunit of MR/P fimbria. Mutant 8B5 had a transposon disruption in *aceE* encoding pyruvate dehydrogenase, which is involved in glycolysis. Mutant 15C3 had a disruption in *sdaA* encoding L-serine deaminase 1, which catalyses L-serine to pyruvate and ammonia and is involved in amino acid metabolism of glycine, serine, threonine and cysteine.

Three additional mutants identified as PMI1184, PMI1193 and PMI1000 encoded putative exported proteins and a putative phage-related protein, respectively (Table 3). A total of seven mutants (11A4, 15A6, 15B4, 17F5, 22F6, 33B5 and 37D5) were unable to be identified due to limited or poor DNA sequencing results despite persistent attempts. An additional mutant, 17A4, had a transposon insertion in the intragenic region between PMI1420 and PMI1421.

Phenotypic characterization

All 37 attenuated transposon mutants were examined for synthesis of known *P. mirabilis* virulence factors including flagella and fimbriae. Production of flagella and MR/P fimbriae was analysed using rabbit serum raised against flagellin and the MrpA subunit, respectively, and analysed by Western blotting. Of the 37 attenuated mutants, a total of eight had a defect in producing either of these proteins: four were unable to produce FlaA and five were unable to produce MrpA (Table 3; Fig. 1). This suggests the possibility that pleiotropic effects resulted from certain sites of transposon insertion. Modified urea segregation agar was used to identify urease-negative mutants in the library of *P. mirabilis* transposon mutants. Of 1880 transposon mutants tested, however, none of the STM mutants were deficient for urease enzymic activity. Screening on swarming agar was performed to identify mutants with aberrant or deficient swarming phenotypes compared with the swarming phenotype of wild-type HI4320. Visual comparison of the swarming differentiation patterns such as the number and length of swarming rafts was made between HI4320 and each transposon mutant. Of 1880 transposon mutants tested, eight had swarming patterns distinct from that of the wild-type HI4320. Swarming patterns varied from no swarming rafts, multiple short swarming rafts and a small star-shaped raft to elongated swarming rafts (Table 3; Fig. 1). The growth phenotypes of the 37 attenuated transposon mutants were verified on minimal A medium agar and also by independent growth curves in LB and minimal A media. After 24 h, all mutants grew as well as wild-type HI4320 on minimal A medium and in independent LB growth curves (data not shown). Two transposon mutants with

disruptions in *aceE* and *guaB* demonstrated an increased lag phase (12 and 23 h, respectively) relative to wild-type HI4320 (2.5 h) but displayed identical exponential growth rates to the wild-type in independent liquid cultures of minimal A medium.

Conclusions

For this STM study, a total of 1880 *P. mirabilis* HI4320 transposon mutants were screened for virulence following transurethral challenge of approximately 500 CBA mice. A total of 40 pools, each containing 48 mutants, were tested in a primary *in vivo* challenge. A total of 23 groups of approximately 20–25 mutants were tested in a secondary murine screen. All attenuated mutants were tested for growth defects in LB and minimal A media. Mutants outcompeted by the wild-type strain *in vitro* were eliminated from further consideration. In total, 37/1880 STM transposon mutants were identified as being attenuated *in vivo*.

In comparison with our previous STM study (Burall *et al.*, 2004), many mutants had transposon disruptions in genes belonging to similar classifications, although no single gene was identified in both studies. Further analysis revealed that genes identified in both studies did not belong to similar putative operons. The total number of transposon mutants created in both STM studies (3968) predicts a theoretical coverage of 69.6% of the *P. mirabilis* HI4320 genome. The incomplete saturation of the genome may explain why the same gene was not identified in both STM studies. Together with the numerous virulence studies in which targeted mutants have been assessed for virulence (see Introduction), we are approaching a complete cataloguing of urovirulence genes for *P. mirabilis*, a prominent cause of urolithiasis, catheter-associated UTIs and other complicated UTIs.

P. mirabilis HI4320 expresses virulence factors that facilitate infection of the bladder and ascension of the ureters to the kidneys to cause pyelonephritis. Using STM, we identified 37 *P. mirabilis* HI4320 mutants that were significantly attenuated or exhibited reduced fitness in the murine model of ascending UTIs. Virulence factors identified by STM included genes associated with cellular processes, transport, regulation, cell-surface structures and motility, and metabolism, which contribute to the uropathogenesis of *P. mirabilis*. To satisfy molecular Koch's (Falkow's) postulates for these putative virulence genes, it will be necessary to construct specific mutants in the discovered genes, assess their attenuation and complement the mutants with the cloned determinants.

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