The type III secretion system of *Proteus mirabilis* HI4320 does not contribute to virulence in the mouse model of ascending urinary tract infection

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The Gram-negative enteric bacterium *Proteus mirabilis* is a frequent cause of urinary tract infections (UTIs) in individuals with long-term indwelling catheters or with complicated urinary tracts. The recent release of the *P. mirabilis* strain HI4320 genome sequence has facilitated identification of potential virulence factors in this organism. Genes appearing to encode a type III secretion system (TTSS) were found in a low GC-content pathogenicity island in the *P. mirabilis* chromosome. This island contains 24 intact genes that appear to encode all components necessary to assemble a TTSS needle complex, plus at least two putative secreted effector proteins and their chaperones. The genetic organization of the TTSS genes is very similar to that of the TTSS of *Shigella flexneri*. RT-PCR analysis indicated that these genes are expressed at low levels *in vitro*. However, insertional mutation of two putative TTSS genes, encoding the requisite ATPase and a possible negative regulator, resulted in no change in either the growth rate of the mutant or the secreted protein profile compared to wild-type. Furthermore, there was no difference in quantitative cultures of urine, bladder and kidney between the ATPase mutant and the wild-type strain in the mouse model of ascending UTI in either independent challenge or co-challenge experiments. The role of the *P. mirabilis* TTSS, if any, is yet to be determined.

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INTRODUCTION

Proteus mirabilis, a major cause of complicated urinary tract infection (UTI) in humans, primarily infects patients with long-term indwelling urinary catheters (>30 days) or individuals with anatomical abnormalities of the urinary tract (Coker *et al.*, 2000). *P. mirabilis*-induced UTI may be complicated by acute pyelonephritis, fever, kidney or bladder stone formation, catheter encrustation or blockage, and bacteraemia (Coker *et al.*, 2000).

Once this species has gained a foothold in the urinary tract, it raises the local pH by the action of urease (Griffith *et al.*, 1976). Urease catalyses the formation of bladder and kidney stones, which may also encrust or block a urinary catheter. Flagella also play a role in *P. mirabilis* pathogenesis (Mobley *et al.*, 1996); this highly motile member of the *Enterobacteriaceae* is able to convert between short, vegetative swimmer cells and very long, hyperflagellated swarmer cells (Mobley & Belas, 1995). Several fimbriae have been identified in *P. mirabilis*, including MR/P (Adegbola *et al.*, 1983; Bahrani *et al.*, 1994), UCA (Cook *et al.*, 1995; Wray *et al.*, 1986), MR/K (Adegbola *et al.*, 1983; Bahrani *et al.*, 1993) and ATF (Massad *et al.*, 1994).

Other virulence factors include a haemolysin (Mobley *et al.*, 1991; Swihart & Welch, 1990), an IgA protease (Senior *et al.*, 1987; Walker *et al.*, 1999) and capsular polysaccharide (Dumanski *et al.*, 1994).

Type III secretion systems (TTSSs) are involved in both plant and animal pathogenesis or symbiosis by several Gram-negative bacterial species (reviewed by Cornelis, 2006). TTSSs consist of a needle-like structure extending from a channel that spans both the inner and outer bacterial membranes. This needle inserts into the membrane of a target eukaryotic cell and injects effector proteins directly into the cytoplasm. The role of these proteins varies by pathogen, but effects may include host cell attachment (McDaniel & Kaper, 1997), invasion (Menard *et al.*, 1993), antiphagocytosis (Rosqvist *et al.*, 1988), apoptosis (Mills *et al.*, 1997) and modulation of inflammation (Espinosa & Alfano, 2004; Schulte *et al.*, 1996).

Sequencing of the genome of *P. mirabilis* HI4320, a strain isolated from a patient with a long-term urinary catheter, was recently completed. Annotation of the genome facilitated the identification of new potential virulence factors. Several genes appeared to encode components of a previously unknown TTSS. This report details experiments conducted to characterize the nature of this potential *P. mirabilis* TTSS.

Abbreviations: TTSS, type III secretion system; UTI, urinary tract infection.

METHODS

Bacterial strains and culture conditions. *Proteus mirabilis* HI4320 is a wild-type strain that was isolated from a patient with a long-term indwelling urinary catheter (Mobley & Chippendale, 1990). *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) was used as the host for plasmid construction. All strains and mutants were cultured at 37 °C in non-swarming Luria–Bertani (LB) broth (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 0.5 g NaCl l⁻¹) or on LB solidified with 1.5% agar. Antibiotic supplementation with chloramphenicol (20 µg ml⁻¹), ampicillin (100 µg ml⁻¹) or kanamycin (25 µg ml⁻¹) was provided as necessary.

Identification of TTSS genes. Conserved TTSS genes from *E. coli*, *Shigella flexneri* and *Salmonella typhimurium* were submitted for BLAST analysis (Altschul *et al.*, 1990) against the *P. mirabilis* genome (http://www.sanger.ac.uk/Projects/P_mirabilis/). The extent of the TTSS pathogenicity island was determined by both BLAST analysis and mapping the percentage GC content of the TTSS region.

Construction of a *spa47* mutant. A kanamycin-resistance gene was inserted into the *spa47* gene using the TargeTron method (Sigma) according to the manufacturer's directions. Briefly, a group II intron was reprogrammed to specifically insert into *spa47* by mutagenic PCR using primers *spa47*-IBS, *spa47*-EBS2 and *spa47*-EBS1d (Table 1). The retargeted intron was ligated into plasmid pACD4K-C to create plasmid pMP122 and introduced to *E. coli* DH5 α by electroporation. Correct retargeting of the intron was confirmed by nucleotide sequence analysis using plasmid-based primers pACD4K-C5' and pACD4K-C3' (Table 1). *P. mirabilis* HI4320 was then electroporated with plasmid pMP122 and the T4 helper plasmid pAR1219 (Davanloo *et al.*, 1984). Addition of 0.5 mM IPTG to a chloramphenicol- and ampicillin-resistant isolate induced the intron to jump from pMP122 into the *spa47* gene in the *P. mirabilis* chromosome, an event that was detectable by selection with kanamycin. This mutant, HI4320*spa47*Ωkan, was confirmed by PCR using primers *spa47*-L5 and *spa47*-R3 (Table 1). The single insertion of the kanamycin gene into the *spa47* locus was verified by Southern blotting.

Co-culture of HI4320 and HI4320*spa47*Ωkan. Exponential-phase cultures of *P. mirabilis* HI4320 and HI4320*spa47*Ωkan were

individually cultured at 37 °C with aeration to OD₆₀₀ 1.0. These cultures were diluted to OD₆₀₀ 0.8 and then mixed in a 1 : 1 ratio. This mixture was plated on LB agar and LB agar supplemented with kanamycin to determine the input of each strain into the co-culture. The co-culture was then passaged 1 : 100 into sterile LB twice each day for the next 48 h. Samples were plated using a spiral plater (Autoplate 4000, Spiral Biotech) to determine the proportion of wild-type and mutant bacteria just prior to each culture passage.

Construction of an *exsD* mutant. The *exsD* gene was inactivated using the TargeTron protocol, as described above. Primers *exsD*-IBS, *exsD*-EBS2 and *exsD*-EBS1d (Table 1) were used to retarget the intron. The intron was ligated into pACD4K-C to create plasmid pMP166 and was confirmed by nucleotide sequence analysis. HI4320 was electroporated as described above. The kanamycin-resistant mutant HI4320*exsD*Ωkan was confirmed by PCR using primers *virF*-RT5' and *exsD*-RT3' (Table 1).

RNA isolation and RT-PCR. To measure expression of TTSS genes, *P. mirabilis* strains were cultured to mid-exponential phase (OD₆₀₀ 0.8). RNeasy Protect (2 ml) (Qiagen) was added to 1 ml culture, and RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. DNA was digested using TURBO DNA-free DNase (Ambion). RNA was used as the template for cDNA synthesis using the Superscript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Three genes, located in diverse regions of the TTSS locus, were examined for expression by PCR. Primers used to amplify cDNA are shown in Table 1.

Analysis of concentrated culture supernatants. A 1 ml portion of an overnight *P. mirabilis* culture was collected by centrifugation and resuspended in 1 ml DMEM culture medium (Gibco). This suspension was used to inoculate 100 ml prewarmed DMEM, and the culture was cultured at 37 °C without aeration in an atmosphere of 95% air/5% CO₂. At mid-exponential phase (OD₆₀₀ 0.5) or late-exponential phase (OD₆₀₀ 1.0), the culture was centrifuged at 9000 g for 15 min at 4 °C. The supernatant was filtered (0.2 µm pore size) and concentrated to less than 1 ml using Centrprep YM-10 centrifugal filter units (Millipore). Protein was precipitated overnight using 10% TCA. Samples were separated by SDS-PAGE and stained

Table 1. Oligonucleotide primers used in this study

Primer	Sequence
<i>spa47</i> -IBS	AAAAAAGCTTATAATTATCCTTAAACGACTGGGGAGTGCGCCAGATAGGGTG
<i>spa47</i> -EBS2	TGAACGCAAGTTTCTAATTTTCGGTTTCGTTCCGATAGAGGAAAGTGTCT
<i>spa47</i> -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTGGGGATTAACTTACCTTTCTTTGT
pACD4K-C5'	CCGCGAAATTAATACGACTCACTA
pACD4K-C3'	GGTATCCCCAGTTAGTGTTA
<i>spa47</i> -L5	TAGATAGCATCAATAATC
<i>spa47</i> -R3	TCCTTCTTTGTTGATGAAAC
<i>exsD</i> -IBS	AAAAAAGCTTATAATTATCCTTACGTTTCATTCAAGTGCGCCAGATAGGGTG
<i>exsD</i> -EBS2	TGAACGCAAGTTTCTAATTTTCGGTTAAACGTCGATAGAGGAAAGTGTCT
<i>exsD</i> -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATTCAAGCTAACTTACCTTTCTTTGT
<i>exsD</i> -RT3'	ATCATCTATCACGACGAACCGTGC
<i>mxIA</i> -RT5'	AGATGCCGATGCAGGAGAGATCAT
<i>mxIA</i> -RT3'	TGAGCCATACCTACCGATATGCCA
<i>ipaB</i> -RT5'	GGCGGATTATTAACGCTTGTTGCAGG
<i>ipaB</i> -RT3'	ACCGCTTCAAGTCCATCATTGAGC
<i>virF</i> -RT5'	AACGCTAGGCAATGAGCCTTGT
<i>virF</i> -RT3'	ACCAAACGCGAGCAAACCTGT

with silver (Silver Stain Plus, Bio-Rad) according to the manufacturer's directions. The same protocol was used to examine the culture supernatant of *P. mirabilis* cultured in Minimal A medium (Belas *et al.*, 1991) to mid-exponential phase (OD₆₀₀ 0.5). Alternatively, to attempt to induce secretion of intracellular stores of TTSS effector proteins (Bahrani *et al.*, 1997), *P. mirabilis* was cultured in LB to late-exponential phase (OD₆₀₀ 1.0), collected by centrifugation (9000 g for 15 min at 4 °C), and resuspended in PBS. Congo red was added to a final concentration of 20 µM, and the bacteria were incubated at 37 °C with aeration for 30 min (Bahrani *et al.*, 1997). The suspension was then centrifuged (9000 g for 15 min at 4 °C), and the supernatant was concentrated and analysed by silver staining, as above.

Mouse model of ascending UTI. The CBA/J mouse model of ascending UTI has been described previously (Hagberg *et al.*, 1983; Johnson *et al.*, 1987). Briefly, 6-week-old female CBA/J mice were inoculated transurethrally with a 50 µl suspension of 1×10^8 c.f.u. *P. mirabilis* HI4320 or HI4320spa47Ωkan. At 3 or 7 days post-infection, the urine, bladders and kidneys of the mice were examined for the presence of *P. mirabilis* by plating on LB using a spiral plater (Autoplate 4000, Spiral Biotech). At 7 days, the spleen was also analysed for infection (c.f.u. g⁻¹). Colony counts were enumerated using a QCount (Spiral Biotech).

The co-challenge experiment was conducted by mixing an estimated 2×10^9 c.f.u. ml⁻¹ culture of HI4320 with an estimated 2×10^9 c.f.u. ml⁻¹ culture of HI4320spa47Ωkan according to the method of Li *et al.* (1999). Quantification of the inoculum showed the actual amount of HI4320 to be 1.04×10^9 c.f.u. ml⁻¹ and that of HI4320spa47Ωkan to be 1.43×10^9 c.f.u. ml⁻¹. Mice were inoculated as above with a 50 µl portion (1×10^8 c.f.u. per mouse) of this suspension. After 3 days, the urine, bladders and kidneys of the mice were assessed for bacterial load by plating equal portions onto LB or LB supplemented with kanamycin. Wild-type HI4320 infection was determined by subtracting the number of colonies on the kanamycin plate from the number of colonies on the plain LB plate.

The statistical significance of data obtained in both independent challenge experiments was assessed using the Mann-Whitney test. The co-challenge experiment was analysed using the Wilcoxon matched-pairs test.

RESULTS AND DISCUSSION

Identification of a TTSS locus in *P. mirabilis* HI4320

Annotation of the *P. mirabilis* HI4230 genome sequence is currently under way in collaboration with the Sanger Institute (H. L. T. Mobley and others, unpublished results). A routine BLAST (Altschul *et al.*, 1990) search of draft genome sequences for putative pathogenicity genes identified homologues of several TTSS genes. Beginning with the initial TTSS genes, neighbouring ORFs were submitted for BLAST analysis to find other TTSS homologues. Genes encoding a potential TTSS were found on a low GC-content (30.7 vs 38.9% for the entire genome) 22 kb pathogenicity island. This island contains 24 genes that appear to encode all components necessary to assemble a TTSS needle complex, plus at least two putative secreted effector proteins and their chaperones (Fig. 1a). The ORFs within the pathogenicity island are free of apparent premature termination mutations, deletions or other disruptions. The genes in the island encompass ORFs PMI2681 to PMI2704 in the annotated genome (H. L. T. Mobley and others, unpublished results). The genomic organization and homology of this pathogenicity island are most similar to those of the TTSS regulon from *S. flexneri* (Fig. 1b); thus, the *S. flexneri* gene designations have been

Fig. 1. Schematic representation of the *P. mirabilis* HI4320 TTSS. (a) The TTSS locus of *P. mirabilis* HI4320. Genes are named after their counterparts in the *S. flexneri* TTSS (Buchrieser *et al.*, 2000). The filled arrow indicates the *spa47* gene, which was mutated by insertion of a kanamycin-resistance gene. The predicted percentage similarity between translated *P. mirabilis* genes and *S. flexneri* proteins is listed above each gene. (b) The TTSS locus of *S. flexneri*. Shaded arrows indicate genes that are missing from the *P. mirabilis* TTSS. (c) Potential regulators of the *P. mirabilis* TTSS are found at a chromosomal site distant from the main TTSS locus.

adopted for simplicity. An additional gene (PMI2709), encoding a product homologous to the TTSS effector YopH from *Yersinia enterocolitica*, was found nearby (5 kb) the other TTSS genes.

The *P. mirabilis* TTSS island appeared to lack known regulators of TTSS; therefore, TTSS regulators from other enteric pathogens were aligned to the *P. mirabilis* genomic sequence by BLAST analysis. Two adjacent genes (PMI1962 and PMI1963) with homologies to known TTSS regulators (the positive regulator *virF* from *S. flexneri* and the negative regulator *exsD* from *Pseudomonas aeruginosa*) were identified by this method (Fig. 1c). These genes, with low GC contents of 30.6 and 27.6 %, respectively, are located at a chromosomal locus distant (over 800 kb) from the main TTSS island.

Expression of TTSS genes

To examine whether the *P. mirabilis* TTSS genes are transcribed, RT-PCR was conducted using RNA isolated from HI4320 cultured to exponential phase. Selected TTSS genes were examined for message (Fig. 2a). Although the TTSS genes *mxIA*, *ipaB*, *virF* (Fig. 2a), *mxIG* and *yopH* (data not shown) were expressed in exponential-phase culture, the small amount of amplified message compared to the constitutively expressed *rpoA* gene suggested that the TTSS genes are expressed at a very low level under this growth condition.

Construction of *spa47* and *exsD* mutants

To attempt to find a function for the *P. mirabilis* TTSS, the *spa47* gene was inactivated by insertion of a kanamycin cassette. This gene, encoding a putative ATPase, was chosen because it is essential for TTSS function in other systems (Jarvis *et al.*, 1995; Yuk *et al.*, 1998). The mutant, HI4320spa47 Ω kan, displayed no growth defect *in vitro* compared to HI4320 when the strains were grown independently, and no significant change in growth rate in co-culture (data not shown).

RT-PCR on selected TTSS genes was conducted to determine whether this mutant had a perturbation in TTSS gene expression. However, transcript was detected for all TTSS genes tested: *mxIA*, *ipaB*, *virF* (Fig. 2a), *mxIG*

and *yopH* (data not shown). RT-PCR analysis of the *spa47* gene confirmed the disruption in this gene in HI4320spa47 Ω kan (Fig. 2b), as no message was detected.

TTSS effector proteins have been detected in the culture supernatants of other bacterial species as an index of a functional TTSS (Demers *et al.*, 1998; Gruenheid *et al.*, 2004). A *spa47* ATPase mutant should be unable to secrete TTSS effectors through the needle complex. Therefore, supernatants from wild-type HI4320 and HI4320spa47 Ω kan cultures in DMEM or Minimal A medium were collected and concentrated over 100-fold. These concentrated culture supernatants were separated by SDS-PAGE and stained with silver (Fig. 3a, b, lanes 1 and 2). No differences were observed between the wild-type and mutant secreted protein profiles. The indicator dye Congo red has been used to induce secretion of intracellular stores of TTSS effector proteins in *S. flexneri* (Baharani *et al.*, 1997); therefore, *P. mirabilis* cultures were exposed to Congo red, and the culture supernatants examined. No differences were detected in the wild-type and mutant protein profiles (Fig. 3c, lanes 1 and 2).

The ExsD protein of *P. aeruginosa* is a repressor of TTSS expression (McCaw *et al.*, 2002). ExsD acts as an anti-activator of the TTSS activator ExsA (Hovey & Frank, 1995), which is also homologous to *S. flexneri* VirF. To determine whether the *P. mirabilis* *exsD* homologue controlled TTSS gene expression, a kanamycin cassette was inserted into this gene. The mutant, HI4320exsD Ω kan, was grown in DMEM or Minimal A medium, as described above. The concentrated culture supernatant was examined by SDS-PAGE and silver staining (Fig. 3a, b, lane 3). Again, no difference was detected in the secreted protein profile as compared to that of the wild-type HI4320. Additionally, HI4320exsD Ω kan cultures exposed to Congo red had no difference in the secreted protein profile compared to wild-type cultures (Fig. 3c, lane 3).

Virulence of the *spa47* mutant in the mouse model of ascending UTI

To determine whether the *P. mirabilis* TTSS contributes to pathogenesis in the urinary tract, CBA/J mice were infected transurethrally with either wild-type HI4320 or the *spa47*

Fig. 2. RT-PCR showing expression of selected TTSS genes. RNA was isolated from exponential-phase cultures of either wild-type HI4320 (wt) or the *spa47* mutant. (a) RT-PCR of selected TTSS genes. The gene *rpoA*, encoding RNA polymerase A, was used as a positive control for gene expression. (b) RT-PCR of the *spa47* gene in both HI4320 and HI4320spa47 Ω kan. G, HI4320 genomic DNA template; -, no reverse transcriptase added; +, reverse transcriptase added.

Fig. 3. Silver staining of concentrated culture supernatants. HI4320, the *spa47* mutant and the *exsD* mutant were grown to late-exponential phase in DMEM medium (a) or mid-exponential phase in Minimal A medium (b). Mid-exponential cultures in DMEM were also examined (data not shown). (c) *P. mirabilis* strains were cultured in LB before exposure to Congo red. The culture supernatants were collected and concentrated before separation by SDS-PAGE on a 10% gel. Lanes: 1, wild-type HI4320; 2, *spa47* mutant HI4320*spa47*Ωkan; 3, *exsD* mutant HI4320*exsD*Ωkan.

mutant HI4320*spa47*Ωkan. After 3 days, the bacterial load in the bladder and kidneys was enumerated (Fig. 4a). There was no significant difference between the c.f.u. (g tissue)⁻¹

values for HI4320 and the *spa47* mutant. To determine whether the TTSS played a role in a more advanced stage of UTI, CBA/J mice were infected with HI4320 or

Fig. 4. Independent and co-challenges of CBA/J mice comparing *P. mirabilis* HI4320 with the *spa47* mutant. For independent challenges at (a) 3 days and (b) 7 days, mice were inoculated with either wild-type HI4320 or the *spa47* mutant. Each data point represents $\log_{10}[\text{c.f.u. (ml urine)}^{-1}]$ or $\log_{10}[\text{c.f.u. (g tissue)}^{-1}]$ collected from one mouse. For the 3 day co-challenge (c), each mouse was infected with a 1:1 mixture of HI4230 and the *spa47* mutant. For both independent and co-challenge experiments, horizontal bars denote the median value of a population. The limit of detection of this assay was 10^2 c.f.u. per millilitre or per gram. ■, Wild-type HI4320 (WT); ▲, HI4320*spa47*Ωkan mutant. No statistically significant differences were found between groups.

HI4320spa47 Ω kan as before. The infection was allowed to progress for 7 days before examination of the urine, bladder, kidneys and spleen of the mice. One mouse infected with HI4320spa47 Ω kan died on day 6, and one mouse infected with HI4320 died on day 7; these were not included in the data analysis. However, there were no significant differences in bacterial numbers between wild-type HI4320 and the spa47 mutant (Fig. 4b). The presence of bacteria in the spleens of some mice indicated that *P. mirabilis* had crossed from the kidneys into the bloodstream of these mice.

To test whether the spa47 mutant had a more subtle defect in UTI pathogenesis, mice were co-infected with a mixed culture of wild-type HI4320 and the spa47 mutant HI4320spa47 Ω kan. After 3 days of co-challenge, bacteria were enumerated from the urine, bladder and kidneys of the mice (Fig. 4c). Again, there were no significant differences between HI4320 and the spa47 mutant.

Conclusions

TTSS have been consistently implicated in direct interactions with eukaryotic hosts (Alfano & Collmer, 2004; Mota & Cornelis, 2005). However, TTSS have not yet been found to play a role for any species in urinary tract pathogenesis. *P. mirabilis* appears to possess all the machinery necessary to produce a functional TTSS, but if this structure is made, it does not contribute to virulence in the mouse model of ascending UTI. This bacterium is believed to gain access to the urinary tract following contamination of the periurethral area, and has been isolated from the intestinal microbiota of humans (Senior, 1983). Since several intestinal pathogens employ a TTSS, it is possible that *P. mirabilis* uses its TTSS while living in the gastrointestinal tract but not in the urinary tract. Another possibility is that the *P. mirabilis* TTSS does not play any role in human disease, but rather is used while the bacterium is living in soil or water (C. W. Penn, personal communication).

Alternatively, the *P. mirabilis* TTSS may not be functional. Although a functional TTSS has been well described in enterohaemorrhagic and enteropathogenic *E. coli* (EHEC and EPEC), an additional cryptic TTSS has also been reported in EHEC O157:H7 (Hayashi *et al.*, 2001; Perna *et al.*, 2001). This TTSS, called ETT2, is present to varying degrees in diverse *E. coli* strains (Ren *et al.*, 2004). However, in contrast to the *P. mirabilis* TTSS (which appears to be intact), the cryptic *E. coli* TTSS is marked by multiple deletions and frameshift mutations (Ren *et al.*, 2004). Notably, TTSS regulators encoded within ETT2 have been shown to affect the expression of genes within the functional TTSS of EHEC (Zhang *et al.*, 2004). Thus, even if *P. mirabilis* does not produce a TTSS needle complex, the genes within the TTSS cluster may have other functions.

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