Proteus mirabilis isolates of different origins do not show correlation with virulence attributes and can colonize the urinary tract of mice

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Proteus mirabilis has been described as an aetiological agent in a wide range of infections, playing an important role in urinary tract infections (UTIs). In this study, a collection of *P. mirabilis* isolates obtained from clinical and non-clinical sources was analysed in order to determine a possible correlation between origin, virulence factors and *in vivo* infectivity. Isolates were characterized *in vitro*, assessing several virulence properties that had been previously associated with *P. mirabilis* uropathogenicity. Swarming motility, urease production, growth in urine, outer-membrane protein patterns, ability to grow in the presence of different iron sources, haemolysin and haemagglutinin production, and the presence and expression of diverse fimbrial genes, were analysed. In order to evaluate the infectivity of the different isolates, the experimental ascending UTI model in mice was used. Additionally, the Dienes test and the enterobacterial repetitive intergenic consensus (ERIC)-PCR assay were performed to assess the genetic diversity of the isolates. The results of the present study did not show any correlation between distribution of the diverse potential urovirulence factors and isolate source. No significant correlation was observed between infectivity and the origin of the isolates, since they all similarly colonized the urinary tract of the challenged mice. Finally, all isolates showed unique ERIC-PCR patterns, indicating that the isolates were genetically diverse. The results obtained in this study suggest that the source of *P. mirabilis* strains cannot be correlated with pathogenic attributes, and that the distribution of virulence factors between isolates of different origins may correspond to the opportunistic nature of the organism.

Received 13 January 2006

Revised 5 April 2006

Accepted 6 April 2006

INTRODUCTION

After *Escherichia coli*, *Proteus mirabilis* is one of the most frequent aetiological agents associated with urinary tract infections (UTIs), particularly in catheterized patients or individuals with structural abnormalities of the urinary tract (Warren *et al.*, 1982). It shows a predilection for the upper urinary tract where it can cause severe histological damage and it is frequently associated with bladder and kidney stone formation (Mobley & Hausinger, 1989).

Several potential *P. mirabilis* virulence factors related to UTI have been described, including fimbrial-mediated adherence to the uroepithelium, swarming motility mediated by flagella, outer-membrane protein (OMP) expression, cell invasiveness, urease production, haemolysin production, and iron acquisition (Coker *et al.*, 2000; Rozalski *et al.*,

1997). Some of these virulence factors are crucial for the successful colonization of the urinary tract and progression of infection (Coker *et al.*, 2000), but the specific role and pathogenic significance of other factors is still the subject of debate (Burall *et al.*, 2004). Although the role of specific virulence factors has been associated with colonization of the urinary tract, the existence of *P. mirabilis* clonal groups related to uropathogenesis has not yet been determined.

E. coli appears as a clear example of the existence of uropathogenic strains that show clonal segregation of specific genes whose expression is associated with pathogenesis (Johnson, 1991). Uropathogenic *E. coli* (UPEC) differ from non-pathogenic *E. coli* and from other *E. coli* pathotypes by the production of specific virulence factors, such as adhesins, toxins, capsules and iron-uptake systems that contribute to virulence and to establishing UTIs (Oelschlaeger *et al.*, 2002a). The presence of different pathogenicity islands containing several genes related to virulence determinants of UPEC strains, and their absence in non-pathogenic *E. coli*, have been recently reported. The acquisition of these pathogenicity islands creates new pathotypes

Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; MR/P, mannose-resistant *Proteus*-like; OMP, outer-membrane protein; UCA, uroepithelial cell adhesion; UPEC, uropathogenic *E. coli*; UPGMA, unweighted pair-group method with arithmetic means; UTI, urinary tract infection.

that are more efficient at establishing a UTI (Oelschlaeger *et al.*, 2002b).

Proteus species are widely distributed in nature and play a significant ecological role. When present in different niches of higher organisms, these species are able to evoke pathological events in diverse regions of the human body (Rozalski *et al.*, 1997). Several authors have investigated *P. mirabilis* virulence factor distribution among strains from different sources, although data are not extensive or conclusive. Mobley & Chippendale (1990) suggest that *P. mirabilis* mannose-resistant, *Proteus*-like (MR/P) haemagglutinin can be significantly more predominant in pyelonephritogenic isolates compared to those from asymptomatic bacteriuria and faecal strains. However, the same authors suggest that expression of urease, a major uropathogenic *P. mirabilis* virulence factor, is present in all isolates from any source (Mobley & Chippendale, 1990). Gaastra *et al.* (1996) assessed the presence and expression of genes of MR/P and uroepithelial cell adhesion (UCA) fimbriae, and production of haemolysin in *P. mirabilis* strains isolated from the urine and faeces of dogs with UTI. These authors observed that all but one of the *P. mirabilis* strains were haemolytic and that most strains produced fimbriae. They

also showed that the UCA fimbrial subunits from dog and human isolates had identical molecular masses and N-terminal sequences, and were immunologically cross-reactive.

In order to improve the comprehension of *P. mirabilis* UTI pathogenesis, and to determine a possible correlation between origin, virulence factors and *in vivo* infectivity, in the present study, a collection of clinical and non-clinical *P. mirabilis* isolates was subjected to several *in vitro* assays and was used in the experimental ascending UTI model in mice. In addition, the Dienes test and the enterobacterial repetitive intergenic consensus (ERIC)-PCR assay were performed to evaluate the genetic diversity within the *P. mirabilis* isolate collection.

METHODS

Bacterial strains, media and growth conditions. The collection of *P. mirabilis* isolates used in this study is shown in Table 1. All isolates were stored at -80 °C in Luria-Bertani (LB) broth supplemented with 12 % (v/v) glycerol and grown aerobically at 37 °C. All media were from Difco Laboratories and all chemicals were reagent grade (Sigma). Ovine erythrocytes were from Biokey Laboratory (Uruguay).

Table 1. *P. mirabilis* isolates used in this study

Strain	Main features	Source or reference
Pr2921	Clinical isolate, patient with symptomatic UTI	Zunino <i>et al.</i> (2000)
Pr6515	Clinical isolate, patient with symptomatic UTI	Piccini <i>et al.</i> (1998)
Pr783	Clinical isolate, patient with symptomatic UTI	Zunino <i>et al.</i> (1994)
306	Clinical isolate, patient with symptomatic UTI	Laboratory collection
731	Clinical isolate, patient with symptomatic UTI	Laboratory collection
896	Clinical isolate, patient with symptomatic UTI	Laboratory collection
3028	Clinical isolate, patient with symptomatic UTI	Laboratory collection
UM1	Pr2921 <i>ucaA</i> mutant	Laboratory collection
P2	Pr2921 <i>pmfA</i> mutant	Zunino <i>et al.</i> (2003)
MSD2	Pr2921 <i>mrpA</i> mutant	Zunino <i>et al.</i> (2001)
A4	Pr2921 <i>atfA-C</i> mutant	Zunino <i>et al.</i> (2000)
OPS	Canine clinical isolate, ear infection	Zunino <i>et al.</i> (2003)
4149	Human clinical isolate, breast secretion	Zunino <i>et al.</i> (2003)
EMI1	Human clinical isolate, leg wound	Zunino <i>et al.</i> (2003)
EMI2	Human clinical isolate, leg wound	Zunino <i>et al.</i> (2003)
HE1	Human clinical isolate, arm wound	Zunino <i>et al.</i> (2003)
SEC1	Human clinical isolate, respiratory secretion	Zunino <i>et al.</i> (2003)
SAF1	Human clinical isolate, saphenectomy	Zunino <i>et al.</i> (2003)
XT1	Non-clinical isolate, bacteriological analysis of beef	Zunino <i>et al.</i> (2000)
XT2	Non-clinical isolate, bacteriological analysis of beef	Zunino <i>et al.</i> (2000)
BZ1	Non-clinical isolate, bacteriological analysis of beef	Zunino <i>et al.</i> (2000)
BZ2	Non-clinical isolate, bacteriological analysis of beef	Zunino <i>et al.</i> (2000)
BZ3	Non-clinical isolate, bacteriological analysis of beef	Zunino <i>et al.</i> (2000)
CJ	Murine isolate, normal enteric flora	Laboratory collection
25933	Human isolate, normal vaginal flora	ATCC*
43071	Human isolate, rectum	ATCC

*American Type Culture Collection.

Swarming motility. Swarming motility was examined when 5 µl overnight broth culture was inoculated onto the centre of brain heart infusion agar plates. The plates were pre-dried for 1 h at 37 °C (Belas *et al.*, 1991) and then incubated overnight at 37 °C.

Urease production. The expression of urease was assessed by placing differentiation disks (Difco Laboratories) aseptically on the surface of cultures grown overnight on LB agar plates and checking the change of colour after 5 min.

Growth rates in urine. Urine supplied by healthy volunteers was collected under aseptic conditions, pooled and filtered immediately after collection using a 0.45 µm pore nitrocellulose filter membrane (Millipore). Bacterial suspensions of similar concentration (OD₆₀₀ 0.015) were prepared in PBS from freshly streaked LB agar plates, and 100 µl of these suspensions was transferred to 10 ml fresh urine and incubated at 37 °C with shaking. The capability to grow in urine was monitored by serial viable bacterial counts on nutrient agar (NA). Three independent assays were performed and the following parameters were used to evaluate growth (Carlberg, 1986): doubling time [$\ln(2t_m)/\ln(R_2/R_1)$] and specific growth rate [$\ln(R_2/R_1)/t$] where R_1 represents the viable bacterial count at time 1 (4 h), R_2 the viable bacterial count at time 2 (7 h), t the interval time in hours, and t_m the interval time in minutes.

Iron-dependent growth assays. To test the ability of *P. mirabilis* to use different iron sources, the procedure described by Sanders *et al.* (1994) was used. Diverse iron compounds, including haemin (5 mg ml⁻¹), haemoglobin (5 mg ml⁻¹), ferric citrate (50 mM) and human transferrin (1 mg ml⁻¹), were evaluated using embedded disks of 5 mm diameter. As a negative control, a disk was soaked in sterile PBS. Three independent assays were performed and the diameter of the growth zones was measured after incubation for 24 h at 37 °C.

Haemagglutination. Isolates were tested for their ability to agglutinate fresh human erythrocytes using the procedure described by Bahrani & Mobley (1994). Bacteria were statically subcultured three times for 48 h at 37 °C in LB broth to enable the expression of MR/ P fimbriae (Old & Adegbola, 1982).

Haemolytic activity. β-Haemolytic activity was determined by plating bacteria onto blood agar base medium supplemented with 5% (v/v) ovine blood. Haemolytic activity was titred using the procedure described by Mobley & Chippendale (1990).

Extraction of OMPs. OMP extraction was performed according to the procedure described by Piccini *et al.* (1998).

SDS-PAGE. Proteins or whole bacteria were suspended in sample buffer, boiled for 10 min and electrophoresed by 12% SDS-PAGE as described by Sambrook *et al.* (1989). The proteins were stained with 0.1% (v/v) Coomassie brilliant blue.

Western blotting. Whole-cell preparations obtained from 48 h static cultures in LB broth were run on SDS-PAGE and proteins were transferred to nitrocellulose membranes (Bio-Rad) as described by Towbin *et al.* (1979). Western immunoblots were performed using a 1:200 dilution in PBS/Tween 20/1% (w/v) skimmed milk of rabbit polyclonal immune sera raised against MrpA (Zunino *et al.*, 2001), PmfA (Zunino *et al.*, 2003) and AtfA (Zunino *et al.*, 2000), and mouse polyclonal immune sera raised against UcaA (Scavone *et al.*, 2004). Isogenic fimbrial *mrpA*, *pmfA*, *atfA-C* and *ucaA* mutants were included as controls in the Western blotting assays (strains MSD2, P2, A4 and UM1 respectively; see Table 1).

DNA amplification by PCR. Genomic DNA of the different isolates was used as a template to amplify the *mrpA*, *pmfA* and *ucaA* genes and for ERIC-PCR analysis. For DNA extraction, bacterial suspensions in MilliQ water were boiled for 10 min, centrifuged (12 096 g, 10 min, 4 °C), and supernatants were recovered for PCR reactions.

The primers used in this study were synthesized by Sigma-Genosys and are described in Table 2. Primers used to amplify *mrpA* were derived from the published *mrp* fimbrial operon sequence from strain HI4320 (GenBank accession no. Z32686; Bahrani & Mobley, 1994). The cycle used was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s, with a final cycle at 72 °C for 7 min. Primers used to amplify *ucaA* were derived from the published *uca* sequence from strain HU1069 (GenBank accession no. U28420; Cook *et al.*, 1995). The cycle used was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, with a final cycle at 72 °C for 7 min. The primers used for ERIC-PCR analysis were derived from those published by Versalovic *et al.* (1991) and the PCR programme was 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 40 °C for 2 min and 65 °C for 8 min, with a final cycle at 65 °C for 16 min.

All assays were done at least in duplicate, using independently prepared lysates, with any discrepancies investigated further as needed. A negative control without the addition of DNA was included in the reactions. PCR products were electrophoresed in 0.8% (w/v) agarose gels at 110 V and stained with ethidium bromide for 15 min.

Animals and experimental ascending UTI. All animal experiments were conducted in accordance with procedures authorized by Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), Montevideo, Uruguay. Female CD-1 6–8-week-old mice from the breeding facilities at IIBCE were used and provided with food pellets and tap water *ad libitum*. The ascending UTI model in mice (Zunino *et al.*, 2000) was used to assess the virulence of different *P. mirabilis* isolates. Groups of 9 to 11 mice were challenged with each isolate.

Table 2. Names and sequences of the primers used for amplification of *mrpA*, *pmfA* and *ucaA* genes and ERIC analysis

Assay	Primer name	Oligonucleotide sequence (5'–3')
<i>mrpA</i> amplification	mr5	ATTTCAGGAAACAAAAGATG
	mr3	TTCTTACTGATAAGACATTG
<i>pmfA</i> amplification	pm1	GGATCATCTATAATGAACTG
	pm2	CTGATAATCAACTTGGAAGTT
<i>ucaA</i> amplification	U1	GTAAGTTGTTGCGCAAAC
	U2	TTGAGCCACTGTGGATACA
ERIC	ERIC 1R	ATGTAAGCTCCTGGGGATTAC
	ERIC 2	AAGTAAGTGACTGGGGTGAGCG

Dienes test. The Dienes test was performed as described by Bale & Hollis (1992). Each isolate was tested against all other isolates within the collection and the assays were performed in duplicate.

Data analysis. The results of doubling time and specific growth rate were assessed using one-way analysis of variance (ANOVA). Numbers of c.f.u. per organ were compared by Kruskal–Wallis non-parametric analysis. In all cases, the differences were considered significant at $P < 0.05$. For ERIC-PCR analysis, each amplified product revealed by electrophoresis was recorded as binary data presence (1) and absence (0), and an initial matrix of 0 and 1 was constructed. A dendrogram was created by using the Pearson product–moment correlation coefficient and the unweighted pair-group method with arithmetic means (UPGMA) algorithm.

RESULTS AND DISCUSSION

Swarming motility

P. mirabilis possesses some distinctive characteristics within the Enterobacteriaceae family, such as swarming motility, a coordinate multicellular behaviour dependent on flagella that occurs when cells grow on solid rich media or on viscous surfaces (Mobley & Belas, 1995; Williams & Schwarzhoff, 1978). Some reports have indicated that *P. mirabilis* swarming motility occurs *in vivo*, promoting UTI (Mobley *et al.*, 1996; Allison *et al.*, 1994; Pazin & Braude, 1974). However, other studies indicate that this property is not essential in *P. mirabilis* UTI (Jansen *et al.*, 2003; Legnani-Fajardo *et al.*, 1996; Zunino *et al.*, 1994). In the present study, typical *P. mirabilis* swarming motility was exhibited by all isolates when grown on agar plates.

Urease production

Urease, a nickel metalloenzyme that catalyses the hydrolysis of urea, resulting in an elevation of pH, contributes to the formation of bladder and kidney stones in *P. mirabilis* UTI (Mobley & Hausinger, 1989). In this study, all *P. mirabilis* isolates showed strong production of urease. However, although urease can be associated with stone formation and serious kidney damage, it cannot be used as an index of virulence, since nearly all *P. mirabilis* strains produce this enzyme (Mobley & Chippendale, 1990).

Growth in urine

Although urine may prevent bacterial growth, not only by its continuous flow, but also because of its osmolarity, urea concentration and low iron concentration (Robledo *et al.*, 1990), uropathogenic micro-organisms have developed the ability to grow in this medium (Russo *et al.*, 1996). Gordon & Riley (1992) suggest that *E. coli* strains isolated from the urinary tract have significantly higher *in vitro* growth rates in urine than strains isolated from other areas. Therefore, we tested the ability of *P. mirabilis* isolates to grow in human urine, assessing the doubling time and the specific growth rate (Table 3). All but two *P. mirabilis* isolates (EMI2 and SAF1) were able to grow and showed no significant differences in doubling time or in specific growth rate ($P > 0.05$). Six isolates showed a large standard deviation in growth rate,

which may suggest a lack of significant differences between bacterial generation times.

Iron-dependent growth assays

It has been suggested that *P. mirabilis*, like other pathogenic Enterobacteriaceae, could obtain iron from haem-containing compounds by the action of haemolysins that produce lysis of host cells. Piccini *et al.* (1998) suggest that *P. mirabilis* expresses iron-regulated OMPs that could be involved in haem uptake and may have a role in pathogenesis. When iron-dependent growth was assessed, all *P. mirabilis* isolates were able to use ferric citrate, haemin and haemoglobin as unique iron sources when cultured on iron-free solid media [NA supplemented with ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA)]. Diameters of growth zones around the disks were similar between every isolate (21–31 mm, 19–25 mm and 11–21 mm for ferric citrate, haemin and haemoglobin, respectively). However, none of the isolates was able to grow on NA/EDDHA supplemented only with transferrin.

OMP patterns

OMPs are potential virulence factors with a wide range of possible functions related to the development of UTI (Moayeri *et al.*, 1991). When OMP profile was analysed using SDS-PAGE, all isolates showed similar patterns. A predominant 39 kDa band that corresponded to OmpA and a 36 kDa band that may have represented a peptidoglycan-associated matrix protein were observed. Both proteins have been described by Bub *et al.* (1980) in *P. mirabilis* strains.

Haemolysin production

Haemolysin production by uropathogens has usually been correlated with *in vitro* cytotoxicity (Peerbooms *et al.*, 1983). In this study, haemolysin expression by *P. mirabilis* isolates was initially evaluated on blood agar base medium plates supplemented with ovine blood. All isolates except OPS, SEC1 and SAF1 generated β -haemolysis haloes around bacterial growth areas. When haemolytic activity was titred, we observed that titres varied between isolates, but all produced measurable haemolytic activity that peaked during the late-exponential phase of growth. However, differences of more than twofold were found in haemolytic titres between different isolates (Table 3). No correlation between haemolytic activity and origin of isolates could be established. Our results are in accordance with those reported by Mobley & Chippendale (1990), who did not find significant differences between the haemolytic activity of *P. mirabilis* strains isolated from pyelonephritis, and that of catheter-associated and faecal strains.

Haemagglutination assay

Haemagglutination, a property that reveals specific adhesin–receptor interactions, is associated with expression of *P. mirabilis* MR/P fimbriae (Old & Adegbola, 1985, 1982; Adegbola *et al.*, 1983). When haemagglutination was

Table 3. Bacterial features potentially associated with virulence of *P. mirabilis* isolates

+, Presence of the assessed property; –, absence of the assessed property; NG, no growth.

Isolates	Doubling time in urine (min)*	Specific growth rate in urine (h ⁻¹)†	Haemolytic activity titre‡	<i>ucaA</i> PCR	Protein expression	
					UCA	ATF
Pr2921	38.76 ± 11.53	1.12 ± 0.03	1/32	+	+	+
Pr6515	58.02 ± 35.25	0.88 ± 0.53	1/16	+	+	–
Pr783	39.36 ± 3.41	1.06 ± 0.09	1/16	+	+	+
306	44.69 ± 10.25	0.97 ± 0.24	1/8	+	+	+
731	46.72 ± 6.60	0.90 ± 0.12	1/32	+	+	+
896	54.18 ± 0.10	0.77 ± 0.01	1/4	+	+	+
3028	37.35 ± 1.46	1.11 ± 0.04	1/8	+	+	+
OPS	58.22 ± 8.24	0.72 ± 0.10	NG	+	+	–
4149	61.75 ± 39.95	0.85 ± 0.55	1/32	+	+	–
EMI1	33.90 ± 1.32	1.23 ± 0.05	1/4	+	+	+
EMI2	NG	NG	1/16	+	+	+
HE1	51.33 ± 22.16	0.89 ± 0.39	1/32	+	+	+
SEC1	58.40 ± 41.65	0.96 ± 0.68	NG	+	+	+
SAF1	NG	NG	NG	+	+	–
XT1	42.10 ± 9.82	1.02 ± 0.24	1/64	–	–	+
XT2	31.26 ± 1.95	1.33 ± 0.08	1/8	–	–	+
BZ1	40.42 ± 26.52	1.31 ± 0.86	1/16	+	+	+
BZ2	54.39 ± 25.27	0.86 ± 0.40	1/8	+	+	+
BZ3	30.13 ± 9.83	1.46 ± 0.48	1/16	–	–	+
CJ	34.77 ± 0.81	1.20 ± 0.03	1/64	+	+	+
25933	24.96 ± 0.95	1.67 ± 0.06	1/16	+	+	+
43071	33.85 ± 11.02	1.30 ± 0.42	1/16	+	+	+

*Data expressed as mean ± SD of three independent assays.

†Data expressed as mean ± SD of three independent assays.

‡Values express the highest haemolytic titre observed in the exponential phase of growth. The haemolytic titre was defined as the highest dilution at which no visible erythrocyte pellet was observed.

evaluated within our isolate collection, it was observed that every tested isolate was able to agglutinate fresh human erythrocytes. As expected, the reaction was not inhibited by 50 mM mannose.

PCR amplification and expression of fimbrial genes

P. mirabilis represents a particular case in which various types of fimbriae can be expressed simultaneously by the same cell (Mobley & Belas, 1995). Several authors suggest that *P. mirabilis* fimbriae are implicated in the colonization of the urinary tract (Coker *et al.*, 2000; Rozalski *et al.*, 1997).

In order to detect *mrpA*, *pmfA* and *ucaA* fimbrial genes, PCR was performed using specific primers and genomic DNA from each isolate. PCR amplification of fimbrial genes indicated that all isolates carried *mrpA* and *pmfA*. However, *ucaA* was not detected when genomic DNA of isolates BZ3, XT1 and XT2 was tested. In all cases, *P. mirabilis mrpA*,

pmfA and *ucaA* fimbrial genes amplified by PCR exhibited the predicted sizes (565, 563 and 580 bp, respectively).

The ability of the different isolates to synthesize MR/P, UCA, PMF and ATF fimbriae was assessed by Western blotting using immune polyclonal antisera. We determined that all isolates expressed MR/P and PMF fimbriae. These two particular types of fimbriae are frequently related to *P. mirabilis* UTI pathogenesis (Zunino *et al.*, 2001, 2003; Bahrani *et al.*, 1994; Massad *et al.*, 1994).

A different situation was observed when expression of UCA and ATF fimbriae was assessed, since these types of fimbriae were not expressed by all isolates. Nineteen isolates were able to express UCA fimbriae, except XT1, XT2 and BZ3, which also lacked the *ucaA* gene, as observed after PCR amplification (Table 3). The isolates that expressed UCA fimbriae exhibited bands whose molecular masses ranged from 23 to 29 kDa. These results confirm those reported by Tolson *et al.* (1995), who suggest that the molecular mass of the structural subunit of *P. mirabilis* non-agglutinating fimbriae (NAF, an alternative name proposed for UCA

fimbriae) is also in this range. Interestingly, we also observed that the two isolates Pr783 and 43071 showed two bands of ~ 23 and ~ 26 kDa, respectively, which specifically reacted with the anti-UcaA immune serum. This could be explained by fimbrial glycosylation or the existence of fimbrial antigenic variation, among other hypotheses (Power & Jennings, 2003).

Finally, 18 isolates expressed ATF fimbriae, while no reactive bands were seen in the lanes corresponding to Pr6515, OPS, 4149 and SAF1 when Western blotting was performed. We

could not observe any correlation between expression of UCA or ATF fimbriae and isolate sources.

Experimental ascending UTI in mice

Although the experimental ascending UTI model in mice has been extensively used to evaluate the role of different *P. mirabilis* virulence factors (Zunino *et al.*, 2000, 2001, 2003; Legnani-Fajardo *et al.*, 1996; Bahrani *et al.*, 1994; Massad *et al.*, 1994; Jones *et al.*, 1990; Swihart & Welch, 1990), so far, it has not been used to assess the potential infectivity of

Fig. 1. Virulence of the different *P. mirabilis* isolates tested in the UTI ascending model in mice. (A) Colonization of kidneys by different *P. mirabilis* isolates; (B) colonization of bladders by different *P. mirabilis* isolates. Each dot represents the $\log_{10}$ (c.f.u.) recovered from each organ. Each horizontal bar represents the median $\log_{10}$ (c.f.u.) recovered from the kidneys (A) and bladders (B). The limit of detection was 10^2 c.f.u. per kidney or bladder.

P. mirabilis isolates from diverse sources. Different groups of mice were transurethrally challenged with each isolate. After sacrifice, the bladder and kidneys were homogenized and viable bacterial counts were performed. The numbers of c.f.u. recovered from bladders and kidneys of the different groups of mice are shown in Fig. 1. All isolates from our collection were able to infect the urinary tract of mice and no significant differences were observed among the number of bacteria recovered from the kidneys and bladders of the different groups of animals ($P > 0.05$).

Genetic diversity

In the present study, we evaluated the genetic diversity of our *P. mirabilis* collection. Firstly, we performed a simple Dienes test, which requires only an agar plate and an incubator. This test is based on the mutual inhibition of different strains as they swarm towards one another on an agar surface. The presence of Dienes lines between two isolates indicates with a high degree of confidence that they are genetically distinct (Pfaller *et al.*, 2000). If two strains are related or identical, there is no mutual inhibition and no lines of demarcation are formed. This assay was highly effective for differentiating the diverse isolates, and we could determine that all isolates tested, except BZ1-BZ2 and XT1-XT2, were genetically unrelated (Dienes lines were present).

Secondly, we analysed the genetic profile of the isolates using ERIC-PCR. ERIC sequences, 126 bp elements which contain a highly conserved, central inverted repeat and are located in extragenic regions of the bacterial genome, have

been successfully used for DNA typing (Olive & Bean, 1999). All *P. mirabilis* isolates from our collection were typable by ERIC-PCR. Fingerprints consisted of 4–17 amplification bands, ranging in size from 400 to 4000 bp (Fig. 2). All isolates showed unique ERIC-PCR patterns, even the BZ1–BZ2 and XT1–XT2 pairs that were not differentiated by the Dienes test.

These four strains, recovered from routine microbiological analysis of beef, showed the highest degree of similarity among *P. mirabilis* isolates. However, a fifth isolate that shared a similar origin (BZ3) was more phylogenetically distant than BZ1, BZ2, XT1 and XT2, compared to several clinical urinary and non-urinary isolates. In general, genetic diversity analysis did not show that groups of isolates were related to their sources or to virulence factor distribution. The results obtained from the Dienes test were highly consistent compared to the ERIC-PCR results, although using ERIC-PCR we were able to differentiate BZ1 from BZ2 and XT1 from XT2 at the genetic level.

Conclusions

P. mirabilis has been described as an aetiological agent of a wide range of infections, both in humans and in diverse animal species. Moreover, *P. mirabilis* is a ubiquitous organism that can be found in different environments outside the host, such as soil, water and sewage (Rozalski *et al.*, 1997).

In the present study, we used *in vitro* and *in vivo* assays to characterize a collection of *P. mirabilis* isolates recovered

Fig. 2. ERIC-PCR profiles and dendrogram of *P. mirabilis* isolates from different sources. Upper panel: ERIC-PCR agarose gel of the collection of *P. mirabilis* isolates, showing fingerprints as generated using ERIC 1R and ERIC 2 primers. Lower panel: dendrogram for the *P. mirabilis* isolate collection, based on ERIC fingerprints of all *P. mirabilis* strains using Pearson product-moment correlation coefficient and the UPGMA algorithm.

from clinical (urinary and non-urinary) and non-clinical sources. The main goal of our investigation was to assess a possible correlation between origin, presence of potential urovirulence factors, and infectivity of the different isolates, using the experimental ascending UTI model in mice. For this purpose, we characterized a collection of *P. mirabilis* isolates from different sources with regard to several potential urovirulence factors, infectivity in mice and genetic diversity.

Our results support the hypothesis that clinical and non-clinical *P. mirabilis* isolates represent overlapping populations that show similar capacities for causing UTI in mice. The distribution of virulence factors between strains of different origin may reflect the opportunistic nature of the organism, and also indicates that *P. mirabilis* strains cannot be distributed into clonal pathogenic groups.

Our data also indicate that uropathogenesis of *P. mirabilis* may be a multifactorial process in which different virulence factors can act in a concerted or even redundant fashion, since absence of different factors did not abolish the colonization of the lower and upper urinary tract in mice (Zunino *et al.*, 2000, 2001, 2003; Legnani-Fajardo *et al.*, 1996; Bahrani *et al.*, 1994; Massad *et al.*, 1994).

The considerable similarities between virulence features of clinical and non-clinical *P. mirabilis* isolates have potentially important implications for disease prevention, antibiotic resistance avoidance and pathogenesis studies.

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