Virulence properties and integron-associated antibiotic resistance of *Klebsiella mobilis* strains isolated from clinical specimens

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This study examined *Klebsiella mobilis* isolates cultured from clinical specimens for virulence-associated properties and antibiotic resistance. The strains produced a number of siderophores, including enterobactin, aerobactin and yersiniabactin. All isolates were able to adhere to and invade epithelial cells. They had cytotoxic activity, which caused destruction of human laryngeal epithelial HEP-2 cells and evoked lysis of murine macrophage J774 cells. Analyses of HEP-2 and J774 cellular morphology and DNA fragmentation in the cells showed features typical of cells undergoing apoptosis. Some *K. mobilis* strains harboured class 1 integrons carrying the *aadA1* gene encoding an aminoglycoside adenyltransferase.

INTRODUCTION

Klebsiella mobilis is an opportunistic pathogen reported to cause nosocomial infections. Strains of *K. mobilis* occur in water, sewage, soil and dairy products, and in the faeces of humans and animals (Grimont & Grimont, 2005). In the literature, *K. mobilis* is often referred to as *Enterobacter aerogenes*, as it was initially included in the genus *Enterobacter* (Hormaeche & Edwards, 1960). However, DNA relatedness studies (Steigerwalt *et al.*, 1976) and numerical classification have showed that this species is closer to *Klebsiella* than to *Enterobacter*, and it was proposed that *Enterobacter aerogenes* was transferred to the genus *Klebsiella* under the name of *K. mobilis* (Bascomb *et al.*, 1971).

Despite the clinical significance of *K. mobilis* strains, their pathogenic mechanisms have not been identified. They are probably multifactorial, with the involvement of a number of putative virulence factors. To multiply in infected organisms, bacteria have to acquire iron that is bound to host proteins such as transferrin, lactoferrin or ferritin. This is often carried out by siderophores, low-molecular-mass chelators that specifically bind Fe^{3+} outside the bacterial cell and transport it into the cytoplasm. It has been demonstrated that siderophores contribute to the virulence of a wide variety of bacterial pathogens (Wooldridge & Williams, 1993; Lawlor *et al.*, 2007).

In some pathogenic bacteria, colonization of the epithelial cells is followed by invasion of the underlying tissues. These

processes are associated with the initiation of infection and are therefore considered as essential virulence factors. After adhesion and invasion to epithelial cells, pathogenic strains may produce a number of toxins that cause the clinical manifestation of infection. Some strains produce enterotoxins, α -haemolysin and a thiol-activated leukotoxic haemolytic toxin, which requires activation with 2-mercaptoethanol (2-ME) (Barnes *et al.*, 2001; Paraje *et al.*, 2005).

The major defence mechanism of host non-specific immunity is cell-mediated killing. Phagocytes, either resident in tissues or circulating in blood, contribute to the primary line of innate defence against bacterial pathogens by providing their removal and destruction at the level of the epithelial barrier. Some bacterial pathogens have developed strategies for avoiding the antimicrobial effects of phagocytes and have evolved mechanisms that kill the immune cells. Some studies have attempted to define the role of apoptosis during bacterial infection. In some settings, apoptosis may contribute to bacterial virulence and pathological tissue injury, but in others, it may be of benefit to the host by enhancing the killing of the bacteria and controlling the inflammatory response (Navarre & Zychlinsky, 2000).

In addition to the multifactorial mechanism of pathogenesis, the treatment of *K. mobilis* infections is often complicated by multidrug resistance. Recent focus on the spread of antibiotic resistance among pathogenic bacteria has been on integrons, genome platforms that are responsible for integration and rearrangements of resistance gene cassettes. The integrated gene cassettes are expressed and the strain thus acquires antibiotic resistance (Nemergut *et al.*, 2008).

Abbreviations: 2-ME, 2-mercaptoethanol; AI, apoptosis index; ERIC, enterobacterial repetitive intergenic consensus; HPI, high-pathogenicity island; NI, necrosis index; p.i., post-infection; TTSS, type III secretion system.

In the current study, we investigated the interactions of *K. mobilis* human isolates with human epithelial cells and macrophages. We examined the adherence and invasion ability of these strains to human laryngeal epithelial (HEp-2) cells, and their cytotoxic activity towards epithelial cells and murine macrophages. Moreover, we examined the apoptotic effects of *K. mobilis* on epithelial cells and macrophages originating from a well-established macrophages cell line, J774. Adhesion and invasion of the strains to the host cells, cytotoxic activity and the ability to induce apoptosis may be related to the development of disease. We also investigated siderophore-based iron-acquisition systems of *K. mobilis* human isolates and determined their susceptibility to antibiotics with a focus on integron-based resistance. Therefore, this study is important for revealing *K. mobilis* pathogenicity mechanisms essential for the establishment of infection and antimicrobial resistance crucial for survival and growth during antibiotic therapy.

METHODS

Bacterial strains. Five strains of *K. mobilis* were used in this study. The strains were isolated from samples taken from hospitalized patients: MPU E5 from faeces, MPU E10 from aspirate, MPU E61 from ulceration, and MPU E29 and MPU E36 from urine. The isolates were identified using the API 20E system (bioMérieux) and further confirmed by sequencing the *hsp60* gene (Morand *et al.*, 2009). Strains were maintained at -75°C in brain heart infusion broth (bioMérieux) containing 50% (v/v) glycerol.

Clonal analysis. To determine whether the isolates were genetically similar, enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting was applied to amplify ERIC sequences of genomic DNA. The primers used were ERIC 1 (5'-ATGTAAGCTCCTGGG-GATTAC-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). The PCR was carried out as described by Versalovic *et al.* (1991). Electrophoretic patterns of the products were analysed using Gel Compar II version 3.5 software (Applied Maths) with the Dice coefficient and UPGMA method.

Siderophore production. Production of siderophores was determined in cross-feeding assays, which test the ability of bacteria to promote the growth of siderophore-deficient receptor-proficient indicator strains grown under iron starvation. *Salmonella typhimurium* TA 2700 (enterobactin indicator), *Escherichia coli* LG 1522 (aerobactin and rhodotorulic acid indicator), *Aureobacterium flavescens* JG-9 (indicator strain for hydroxamate siderophores distinct from aerobactin) and *Morganella morganii* SBK 3 (rhizoferrin and α -keto acids indicator) were used for the detection of siderophore production (Reissbrodt & Rabsch, 1988). In addition, *Yersinia enterocolitica* 5030, a strain that is unable to produce siderophores but uses exogenous yersiniabactin, was used to detect the production of yersiniabactin (Haag *et al.*, 1993).

PCR analysis of the *Yersinia* high-pathogenicity island (HPI). The presence of the *Yersinia* HPI was determined by PCR analysis with primers targeting the *irp1* gene and DNA regions that encompass the *asnT/int* and *int/ybtS* genes (Karch *et al.*, 1999). The PCR conditions were as follow: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 5 min for the *irp1* gene; 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 8 min for the *asnT/int*

region; and 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 5 min for the *int/ybtS* region. Amplification was carried out in a 25 μl volume with 2.5 μl $10\times$ PCR buffer, 0.25 μM each primer (Oligo.pl), 200 μM dNTP mix, 2.5 mM MgCl_2 , 0.5 U Hi-Fi *Taq* polymerase (Novazym) and 0.5 μg genomic DNA.

Cell cultures. The murine macrophage cell line J774 was maintained in RPMI 1640 (Biomed) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), gentamicin (5 $\mu\text{g ml}^{-1}$) and 2 mM L-glutamine (Sigma). HEp-2 cells were cultured in Eagle's minimum essential medium (EMEM; Biomed) supplemented with 5% FCS, 2 mM glutamine, 80 IU penicillin ml^{-1} , 80 μg streptomycin ml^{-1} and 1 mg nystatin ml^{-1} . The eukaryotic cells were seeded with 100 μl of a suspension of 2×10^5 bacterial cells per well and incubated at 37°C in an atmosphere with 5% CO_2 (Keller *et al.*, 1998).

Bacterial adhesion to and invasion of HEp-2 cells. Adhesion and invasion were examined in quantitative assays following a method modified from that of Keller *et al.* (1998). The epithelial cell monolayer was infected with bacteria at an m.o.i. of 50 (2×10^5 HEp-2 cells were incubated with approximately of 1×10^7 bacterial cells) for 2 h. During the infection period, bacteria adhered to and invaded the HEp-2 cells. In samples where adhesion and invasion were analysed, 200 μl EMEM with 2% FCS and 2% D-mannose were added for 4 h. Associated and internalized bacteria were released by treatment with 100 μl 0.01% Triton X-100 in PBS for 5 min at room temperature and the number of bacteria was calculated by plate counting on tryptic soy agar (Difco). For quantification of internalized bacteria, infected cells were washed in PBS and incubated with EMEM containing gentamicin (100 $\mu\text{g ml}^{-1}$) for 2 h at 37°C to kill extracellular bacteria. After three washes in PBS, the cells were incubated in medium without gentamicin for 2 h. The number of intracellular bacteria was determined by plating the lysates onto tryptic soy agar. The number of attached bacteria was determined by subtracting the number of intracellular bacteria from the total count. The results were expressed as an adhesion index, i.e. the mean number of associated bacteria per 100 HEp-2 cells. The invasion index was expressed as the percentage of the number of internalized bacteria per 100 HEp-2 cells compared with the number of adhering bacteria (Superti *et al.*, 2005). As controls, an invasive strain of *Y. enterocolitica* O:8/1B (pYV⁺) and non-pathogenic *E. coli* K-12 C600 were included. The adhesion and invasion indices shown in Table 1 represent two independent experiments performed in triplicate.

Cytotoxic activity towards epithelial cells. The assay was performed as described by Krzymińska *et al.* (2009b). To prepare bacterial filtrates, bacteria were incubated in tryptic soy broth in a shaking incubator with agitation at 300 r.p.m. at 37°C for 18 h. Next, 0.18 M 2-ME was added for 30 min at 37°C (Paraje *et al.*, 2005). Cytotoxic activity was examined for filtrates from cultures grown in tryptic soy broth and those activated with 2-ME. The cultures were centrifuged at 3000 r.p.m. in a microfuge for 20 min and the supernatants were sterilized through 0.22 μm pore size membrane filters (Millex-GV; Millipore). Twofold serial dilutions (1:2–1:512) of culture filtrates in PBS were added to the wells of a tissue culture plate containing a confluent HEp-2 monolayer and incubated for 24 h at 37°C . As a negative control, filtrates of the non-pathogenic *E. coli* K-12 C600 were added. The cytotoxic titre of each isolate was calculated by determining the reciprocal of the highest dilution of culture filtrate that produced a cytopathic effect. The results were observed under an inverted microscope (Nikon Eclipse TE-2000). All tests were performed in duplicate.

Cytotoxic activity towards murine macrophage J774 cells in a neutral red retention assay. Neutral red is a biomarker of cellular stress as a supravital dye taken up in the lysosomes of viable cells. The

Table 1. Adhesion, invasion and cytotoxic activity of *K. mobilis* strains

Strain	$10^{-5} \times \text{AI}^*$	Invasion index (%)†	Cytotoxic titre	
			HEp-2‡	J774§
MPU E5	18.4 ± 4.6	13.7 ± 4.8	1	39
MPU E10	4.2 ± 0.9	9.7 ± 3.6	1	67
MPU E29	27.9 ± 5.7	25.3 ± 9.1	32	45
MPU E36	7.9 ± 2.7	1.1 ± 0.3	1	48
MPU E61	62.4 ± 15.7	15.8 ± 7.9	16	53
<i>E. coli</i> K-12 C600	0.04 ± 0.01	0.05 ± 0.03	—	—
<i>Y. enterocolitica</i> O:8/1B	15.4 ± 7.2	40.15 ± 11.4	128	76

*Mean number of associated (c.f.u.) bacteria per 100 HEp-2 cells.

†Mean percentage of number of internalized bacteria $\pm$ SD per 100 HEp-2 cells compared with the number of adhering bacteria from two separate experiments performed in triplicate.

‡Reciprocal of the highest dilution of culture filtrate that produced a cytopathic effect.

§Percentage of cytotoxicity was determined 24 h after infection by a neutral red retention assay.

assay was performed in microtitration plates as described by Carbonell *et al.* (1997) with slight modifications. The macrophage monolayer was incubated with bacterial culture filtrates, obtained as described above, at 37 °C for 24 h. For negative and positive controls, the monolayers were infected with non-pathogenic *E. coli* K-12 C600 and invasive *Y. enterocolitica* O:8/1B filtrates, respectively. Next, the medium was removed and the cultures were washed with PBS, and 200 μ l neutral red (50 μ g ml⁻¹) was added to each well and incubated for 3 h at 37 °C. After incubation, the dye solution was aspirated and the cells were rinsed in PBS before being fixed with formalin in calcium chloride solution (40 % formaldehyde, 10 % anhydrous calcium chloride), which was then removed. The incorporated dye was eluted from the cells by adding ethanol/acetic acid mixture (50 % ethanol and 1 % acetic acid) and the absorbance at 540 nm was measured using a plate reader. The absorbance was corrected against blank wells containing sterile growth medium processed as described above. Cell viability was determined by comparison with the absorbance values obtained from the blank wells (taken as 100 % viability). Cytotoxic activity was calculated by subtracting the percentage of viable cells from 100.

Assessment of apoptosis of HEp-2 and J774 cells. Two different methods were used for the detection of apoptotic epithelial cells and macrophages. Cell cultures were evaluated morphologically for the characteristics of apoptotic cell death. The infected cell monolayer was detached using 0.25 % trypsin and 0.25 % EDTA in PBS, and the suspension was stained with acridine orange (100 μ g ml⁻¹) and ethidium bromide (100 μ g ml⁻¹) (Superti *et al.*, 2005) and examined under a Nikon Eclipse TE-2000 fluorescence microscope. Each sample was analysed in triplicate by counting at least 100 or more cells for each replicate. The cells were classified, according to the colour and structure of the chromatin, into three different groups: viable, apoptotic and necrotic cells. The percentage of apoptotic and necrotic cells, represented as apoptosis indices (AIs) and necrosis indices (NIs), were determined by counting a minimum of 100 cells selected at random from three preparations (Falcón *et al.*, 2001). The AI and NI data are presented as means $\pm$ SD, and represent two independent experiments performed in triplicate.

Analysis of intranucleosomal fragmentation was carried out as described previously (Krzyżmińska *et al.*, 2009a). Infected cells were lysed with hypotonic buffer [100 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 % SDS, 200 mg proteinase K ml⁻¹] for 16 h at 37 °C. The lysates were extracted with an equal volume of

phenol:chloroform (1:1) and with an equal volume of chloroform:isoamyl alcohol (24:1) before precipitation with ethanol. The precipitates were dried, solubilized in TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA] and digested with 2 mg RNase ml⁻¹. Electrophoresis was performed in a 1.5 % agarose gel (Basica LE GQT; Prona) at 120 V for 3 h. The DNA was stained with ethidium bromide, visualized under UV light and digitalized using a Bio-Print V.99 system (Vilbert-Lourmat).

Detection of a type III secretion system (TTSS). TTSS genes were detected by PCR. Genomic DNA was isolated with a genomic mini kit (A&A Biotechnology). We used two sets of primers (Oligo.pl): ESCV1 (5'-TAACCTCTTCCCCACAATC-3') and ESCV2 (5'-TATCCCCA-ACAGGCAAAC-3') that target the *escV* gene (Kyaw *et al.*, 2003), and ASCV-F (5'-GTAARCAGATGAGTATCGATGG-3') and ASCV-R (5'-GAGACSCGGGTGACGATAAT-3') for detection of the *ascV* gene (Yu *et al.*, 2004). PCR analyses were carried out in a C1000 thermal cycler (Bio-Rad) in a 50 μ l volume with 1 μ g genomic DNA, 5 μ l 10 $\times$ PCR buffer with NH₄(SO₄)₂, 0.25 mM each primer, 200 mM dNTPs, 2.5 mM MgCl₂ and 2 U Hi-Fi *Taq* polymerase. The PCR products were separated in a 1.5 % agarose gel.

Samples for scanning electron microscopy were prepared as described by Krzyżmińska *et al.* (2009b). Bacteria were cultured overnight in brain heart infusion with 2 % agar at 37 °C. After incubation, the bacteria were harvested and suspended in PBS to a McFarland scale no. 1 and diluted 1:100 in EMEM. The bacterial suspension was incubated with a monolayer of Vero cells for 45 min at 37 °C and passaged into the next well containing Vero cells. The passages were repeated four times. The bacteria were then fixed in 1 % formaldehyde for 1 h on ice, washed in PBS and placed on a glass coverslip. The samples were fixed in 2 % glutaraldehyde for 5 min at room temperature, washed in TE buffer and dehydrated in a series of acetone solutions. The samples were critical-point dried, coated with gold and observed under an EVO 40 (Zeiss) scanning electron microscope.

Antimicrobial susceptibility. Antibiotic resistance patterns were determined according to the standard disc diffusion method (CLSI, 2009). Isolates were tested for susceptibility to 16 antibiotics representing 8 classes: aminoglycosides, amphenicols, carbapenems, penicillins, cephalosporins, quinolones, sulfonamides and tetracyclines. The production of extended spectrum β -lactamases was assayed by the double-disc synergy test with ceftazidime, cefotaxime

and amoxicillin/clavulanic acid. The production of AmpC β -lactamases was checked by a three-dimensional assay with cefoxitin and *E. coli* ATCC 25922 as a control (Coudron *et al.*, 2000). All antibiotic discs were from Oxoid.

Identification of integrase genes. The sequences of primers chosen were as described by Dillon *et al.* (2005). Primers Int1F and Int1R were used for the detection of class 1 integrase (*intI1*; 160 bp product), Int2.F and Int2.R for class 2 integrase (*intI2*; 788 bp product), and Int3.F and Int3.R for class 3 integrase (*intI3*; 979 bp product). PCRs were performed in a 25 μ l volume with 2.5 μ l 10 $\times$ PCR buffer with $\text{NH}_4(\text{SO}_4)_2$, 0.25 μ M each primer (Oligo.pl), 200 μ M dNTP mix, 2.5 mM MgCl_2 , 2 U *Taq* DNA polymerase and 100 ng genomic DNA. Amplification involved initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 8 min.

Analysis of the variable region of the class 1 integron. The sequences of primers complementary to the 5' and 3' conserved regions have been published elsewhere (Lévesque *et al.*, 1995). PCRs were conducted as follow: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 5 min, with a final elongation at 72 °C for 8 min. The nucleotide sequences of the amplicons were determined in a 3130xl genetic analyser (Applied Biosystems). A sequence similarity search was carried out using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Conjugation assay. The transfer capability of integrons was determined in a conjugation assay with an *E. coli* J-53 (Rif^R) recipient strain in a broth mating procedure. The donor and recipient strains were cultured for 18 h at 30 °C, mixed in a 1:1 ratio and incubated for 3 h at 37 °C. Samples (20 μ l) were spread on agar plates containing rifampicin and streptomycin and incubated overnight at 30 °C. The presence of class 1 integrase among the transconjugants was confirmed by the multiplex PCR described above.

RESULTS AND DISCUSSION

Clonal analysis

The clonal relatedness of the strains was established by ERIC-PCR typing. The ERIC fingerprints consisted of 3 to 17 bands ranging in size from 160 to 5400 bp. All strains were genetically unrelated, with the highest similarity between two isolates reaching 52 %.

Siderophore production

We determined the ability of extraintestinal strains of *K. mobilis* to employ different siderophore-mediated strategies of iron acquisition. Biological assays with indicator strains deficient in siderophore synthesis but capable of acquiring exogenous iron chelators indicated that the strains excreted the catecholate siderophore enterobactin, the hydroxamate siderophore aerobactin (with the exceptions of MPU E36 and MPU E61) and hydroxamates other than aerobactin, e.g. coprogen, ferrichrome, rhodotorulic acid or ferrioxamine B. Moreover, all strains were able to cross-feed the indicator strain *Y. enterocolitica* 5030 for yersiniabactin production.

The genes for the yersiniabactin-mediated iron-uptake systems in *Enterobacteriaceae* are often located in a

genomic island known as the *Yersinia* HPI (Bach *et al.*, 2000). To determine whether the isolates had yersiniabactin genes located within the HPI, we carried out PCRs to find out whether the yersiniabactin operon was located in the vicinity of an integrase gene and a tRNA gene, characteristic features of pathogenicity islands. We obtained PCR products with primers specific for the *irp1* gene and primers targeting regions encompassing: (i) the asparagine-specific tRNA gene (*asnT*) and the integrase gene (*int*), and (ii) the *int* gene and the *ybtS* gene involved in yersiniabactin biosynthesis (Karch *et al.*, 1999). The PCR products for the *irp1* gene and the *asnT/int* and *int/ybtS* regions were 240, 1250 and 830 bp, respectively, which corresponded with the expected sizes.

Siderophore production contributes to bacterial virulence, as the ability to acquire iron within the host is crucial during infection. Aerobactin and yersiniabactin have been shown to be virulence factors in many bacterial species, including those from the family *Enterobacteriaceae* (Bach *et al.*, 2000; Mokracka *et al.*, 2004). Enterobactin has a higher affinity for iron than human iron-binding proteins; however, its role in bacterial virulence is not clear, as, for example, the mammalian protein siderocalin binds enterobactin, making it ineffective in bacterial iron acquisition (Nelson *et al.*, 2007). The presence of more than one siderophore-mediated iron-acquisition system may be of benefit for *K. mobilis* strains, as different systems may function in different environments within the host or at different stages during the course of an infection (Torres *et al.*, 2001).

Recently, it has been reported that bacterial iron chelators can activate human hypoxia-inducible factor 1 (HIF-1) during infection. HIF-1 is a key transcriptional regulator during adaptation to oxygen deprivation. Siderophores can activate HIF-1 in a manner independent of hypoxia. This has been proved for yersiniabactin and aerobactin, although currently it is not clear whether the induction of HIF-1 is beneficial or disadvantageous for the host organism (Hartmann *et al.*, 2008).

K. mobilis adhesion to and invasion of human epithelial cells

For many pathogens, adhesion to and invasion of the host epithelial cells have been shown to be the primary steps in colonization. Adhesion to HEp-2 cells and invasion of *K. mobilis* were examined by a gentamicin survival assay. All strains were gentamicin sensitive (0.1 mg ml⁻¹). The results of the adhesion and invasion assays are presented in Table 1. Our results demonstrated that all isolates were able to adhere to HEp-2 cells and had a higher efficiency of adhesion compared with that of the *E. coli* non-invasive control. The adhesion indices ranged from 4.2×10^5 to 62.4×10^5 c.f.u. per 100 HEp-2 cells. The *E. coli* K-12 C600 negative control had an adhesion index of 0.4×10^4 c.f.u., whereas the *Y. enterocolitica* O:8/1B positive control had an adhesion index of 15.4×10^5 c.f.u.⁻¹.

The isolates of *K. mobilis* were able to invade Hep-2 cells. The percentage of associated bacteria that were internalized (invasion index) ranged from 9.7 to 25.3 %, whereas the invasion index of non-pathogenic *E. coli* K-12 C600 was 0.05 %.

Cytotoxic activity

The cytopathic effect caused by cytotoxic toxins was detected by rounding and shrinkage of the HEp-2 cells (Table 1). All strains caused this effect after treating the culture supernatant with 2-ME. Three *K. mobilis* strains (MPU E5, MPU E10 and MPU E36) were found to be cytotoxic to HEp-2 cells at a titre of 1 (i.e. only undiluted supernatants were cytotoxic). Isolates MPU E29 and MPU E61 were more cytotoxic, with titres of 32 and 16, respectively. All strains were cytotoxic to murine J774 cells after 24 h supernatant treatment, as measured by a neutral red assay (Table 1). The highest cytotoxicities (53 and 67 %) were observed for MPU E61 and MPU E10 isolates. All isolates produced cytotoxins that were activated by 2-ME. The non-pathogenic strain *E. coli* K-12 C600 was not cytotoxic to epithelial cells or macrophages.

According to Paraje *et al.* (2005), such thiol-activated toxins have the capacity to damage the membranes of erythrocytes and leukocytes. The culture supernatant of the MPU E10 strain not activated with 2-ME was cytotoxic to

HEp-2 and J774 cells. This indicated that *K. mobilis* can also produce cytotoxins that are not activated by 2-ME. The results suggested that thiol-activated and 2-ME-independent cytotoxic toxins may cause destruction of the epithelial barrier and suppress the innate host immune defence.

Apoptosis of HEp-2 and J774 cells induced by *K. mobilis*

Induction of apoptosis is considered a putative virulence mechanism of bacterial pathogens that may cause tissue damage and facilitate further colonization. Programmed cell death has been defined by both morphological and biochemical criteria (Dockrell, 2001; Nagata, 2005). Morphological assessment of apoptosis is confirmed by staining cells with ethidium bromide and acridine orange and observation under a fluorescence microscope. Acridine orange permeates the cell membrane and stains the nuclei green. Ethidium bromide is taken up only when cytoplasmic membrane integrity is lost, and stains the nuclei red. Live cells have a normal green nucleus, those in the early stages of apoptosis have a bright green nucleus with condensed chromatin, and those in the late stages of apoptosis display condensed or fragmented orange chromatin, whereas cells that have died from necrosis have a structurally normal orange nucleus. Strains of *K. mobilis* provoked apoptosis of HEp-2 and J774 cells (Fig. 1), which displayed morphological changes including cell shrinkage,

Fig. 1. Effect of *K. mobilis* on the morphology of HEp-2 and J774 cells. Cells were infected with *K. mobilis* (a, c) or *E. coli* K-12 C600 (negative control) (b, d). The cells were stained with acridine orange and ethidium bromide and examined with a fluorescence microscope. Live cells had a normal green nucleus (i), apoptotic cells displayed condensed or fragmented orange chromatin (ii) and cells that had died from necrosis had a structurally normal orange nucleus (iii). Original magnifications: $\times 200$ (a–c); $\times 150$ (d).

loss of normal cell-to-cell contact and blebbing at the cell surface at 24 and 48 h after infection. The AIs at 24 and 48 h after infection are shown in Table 2. The highest AI (46.7% in HEp-2 cells at 24 h after infection) was observed for MPU E5. The remaining isolates expressed AIs ranging from 15.7 to 25.6%. The percentage of apoptotic cells increased at 48 h post-infection (p.i.), when AIs of 58.1–77.7% were observed in HEp-2 cells infected with *K. mobilis* MPU E5, MPU E10 and MPU E61. Lower AIs were noted for strains MPU E29 and MPU E36 (32.1 and 34.1%, respectively). The mean AI of the negative control was $4.5 \pm 1\%$, whereas for the positive control it reached $84.6 \pm 5.1\%$. All isolates induced apoptotic morphological changes in J774 cells. The percentage of apoptotic cells ranged from 6.1 to 39% at 24 h and was as high as 58.9% at 48 h after infection. The AI in J774 cells infected with non-pathogenic *E. coli* K-12 C600 was $5.1 \pm 1.2\%$.

Strains of *K. mobilis* showed also necrotic activity. HEp-2 cells at 24 h after infection showed an NI ranging from 7.6 to 17.2%, which increased at 48 h to 14.5–28.4%. Simi *et al.* (2003) partially purified a cytolytic enterotoxin from *Enterobacter cloacae* culture supernatant that had haemolytic activity and caused fluid accumulation in rat intestinal loops with erosive necrotic lesions.

Another feature of apoptosis is the fragmentation of DNA into multimers of 180–200 bp. Therefore, we examined *K. mobilis* induction of DNA fragmentation in epithelial cells and macrophages. Fragmentation was observed in HEp-2 cells infected with isolates MPU E5, MPU E10 and MPU E61 for 48 h. Fragmentation was also observed in J774 cells infected with *K. mobilis* MPU E10. The patterns were similar to that obtained with the positive-control *Y. enterocolitica* O:8/1B, which induced DNA fragmentation in epithelial cells and macrophages (Fig. 2). The typical 'comet' reaction was observed when the AI exceeded 58%. Strains of *E. coli* K-12 C200 did not induce DNA

fragmentation in HEp-2 and J774 cells at 24 and 48 h p.i. Barnes *et al.* (2001) suggested that a high concentration of the thiol-activated enterotoxins produced by *Enterobacter cloacae* strains increases the production of reactive oxygen species, which would contribute to toxin-induced inflammation and lysis of leukocytes before triggering apoptosis. Moss *et al.* (2000) reported that *Bordetella pertussis* kills macrophages and epithelial cells by apoptosis by secreting adenylate cyclase-haemolysin toxin, which increases cAMP levels. An increase in the intracellular concentration of cAMP leads to apoptosis of host cells.

Absence of a TTSS

Many Gram-negative pathogenic bacteria, including members of the family *Enterobacteriaceae*, have evolved a TTSS that enables translocation of their proteins into the cells of the infected host. There is no information, however, about a TTSS in *K. mobilis* or other members of this genus. To search for a TTSS, we used two sets of primers targeting *escV* and *ascV*. The *escV* gene codes for an inner-membrane protein that is a part of the TTSS apparatus in *E. coli* (Gauthier *et al.*, 2003). Homologues of *escV* have been also described in strains of other genera of the *Enterobacteriaceae* (Plano *et al.*, 1991; Kubori *et al.*, 2000). The *ascV* gene encodes a protein involved in the TTSS in *Aeromonas* spp. Recently, we reported a TTSS system with a gene showing a high similarity to *ascV* in *Enterobacter cloacae* (Krzymińska *et al.*, 2009b).

Table 2. AI of human epithelial HEp-2 and J774 cells infected with *K. mobilis* strains at 24 and 48 h

The AI values are the means of the percentages of apoptotic cells from two experiments performed in triplicate.

Time p.i./strain	AI of HEp-2 cells (SD)	AI of J774 cells (SD)
24 h		
MPU E5	46.7 (4.2)	6.1 (0.9)
MPU E10	15.7 (1.4)	21.8 (3.1)
MPU E29	16.4 (2.1)	18.5 (2.3)
MPU E36	23.8 (1.8)	39.3 (3.4)
MPU E61	25.6 (3.23)	31.7 (2.9)
48 h		
MPU E5	58.1 (3.1)	25.8 (2.7)
MPU E10	71.7 (4.9)	58.9 (7.8)
MPU E29	32.1 (3.8)	35.7 (3.1)
MPU E36	34.1 (4.8)	57.1 (5.5)
MPU E61	77.1 (6.7)	41.7 (4.1)

Fig. 2. Intranucleosomal breakdown of DNA from HEp-2 and J774 cells infected with *K. mobilis*. Lanes: 1, molecular size marker; 2, DNA extracted from HEp-2 cells infected with MPU E61 at 24 h; 3, DNA from HEp-2 cells infected with MPU E61 at 48 h; 4, DNA from J774 cells infected with MPU E10 at 48 h; 5, DNA from HEp-2 cells infected with *Y. enterocolitica* O:8/1B at 24 h; 6, HEp-2 cells infected with non-pathogenic *E. coli* K-12 C600 at 48 h.

Table 3. Integrons and antibiotic resistance profiles of *K. mobilis* isolates and transconjugant strains

Strain	Class I integron	Gene cassette	Antibiotic resistance profile	Antibiotic resistance profile of transconjugant strain*
MPU 5E	+	<i>aadA1</i>	STR, SPT, NET, CHL, TIC, PIP, TZP, CTR, SUL	STR, SPT, NET, CHL, TIC, PIP, TZP, CTR, SUL, RIF
MPU 10E	–	NA	SUL	NA
MPU 29E	+	<i>aadA1</i>	STR, SPT, CHL, TIC, CIP, SUL	STR, SPT, CHL, TIC, SUL, RIF
MPU 36E	–	NA	SUL	NA
MPU 61E	–	NA	SUL	NA

CHL, Chloramphenicol; CIP, ciprofloxacin; CTR, co-trimoxazole; NA, not applicable; NET, netilmicin; PIP, piperacillin; RIF, rifampicin; SPT, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TIC, ticarcillin; TZP, piperacillin + tazobactam.

*The recipient strain *E. coli* J-53 is resistant to rifampicin.

In this study, none of the isolates of *K. mobilis* gave specific PCR products for either gene. Moreover, the cells of *K. mobilis* grown in conditions supporting the expression of a TTSS (Krzymińska *et al.*, 2009b) and examined under a scanning electron microscope did not exhibit any surface structures resembling the needle complex of a TTSS.

Integrons and antibiotic resistance

K. mobilis strains are naturally resistant to ampicillin, cefalotin and cefoxitin (Grimont & Grimont, 2005). The resistance to other antibiotics may be associated with integrons, which are defined as a natural gene-capture system that contain an integrase gene able to incorporate gene cassettes conferring resistance to antimicrobials. Moreover, integrons are located within mobile genetic elements such as transposons and plasmids, and can be transferred between cells of the same or different species in one genetic event, leading to resistance in bacterial strains that were previously susceptible (Mazel, 2006).

Among the five strains of *K. mobilis*, two yielded a PCR product of 160 bp corresponding to the *intI1* gene. These strains were isolated from a rectal swab (MPU 5E) and from urine (MPU 29E). In both cases, the integron-positive strains had multiple antibiotic resistance, defined as resistance to at least three unrelated antibiotics (Table 3). None of the strains produced extended spectrum β -lactamases or AmpC.

The products of PCR analyses of the variable region of class I integrons were both 1000 bp in size and contained a single gene cassette. In both cases, the sequence analysis identified the *aadA1* gene, which encodes aminoglycoside adenylyltransferase conferring resistance to streptomycin and spectinomycin. Thus, the integrons of the clinical *K. mobilis* strains investigated here differed from those described previously that contained *aac(6')-Ib*, *qacF*, *cmlA2* and *oxa-9* genes (Ploy *et al.*, 1998), *dfrV* and *ereA2* (Peters *et al.*, 2001), *aac(6')-Ib* (Machado *et al.*, 2007) or *dfrA2d* (Frank *et al.*, 2007) within their variable regions.

The integron-positive isolates were checked for rifampicin susceptibility and subjected to a broth mating experiment

with a recipient strain. Both integrons were transferable to *E. coli* J-53 (Rif^R) in the conjugation assay. As a consequence, the recipient strain acquired both the integron and antimicrobial resistance (Table 3), indicating that *K. mobilis* strains can transfer antibiotic resistance genes at least among some members of the same family.

In summary, our study describes a comprehensive evaluation of virulence factors and antibiotic resistance of clinical isolates of *K. mobilis*. The data suggest that adhesion to epithelial cells and invasion by *K. mobilis* induce apoptotic cell death. The process may primarily be a strategy developed by the strains, resulting in tissue destruction and bacterial spread, consequently leading to invasive disease or systemic infection. Strains of *K. mobilis* are able to employ a variety of siderophore-mediated iron-acquisition systems, which allows them to replicate inside the infected host. Moreover, they can harbour integrons in their genomes and are able to spread antibiotic resistance among closely related species.

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