An aflagellate mutant *Yersinia enterocolitica* biotype 1A strain displays altered invasion of epithelial cells, persistence in macrophages, and cytokine secretion profiles *in vitro*

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Despite being classically defined as non-pathogenic, there is growing evidence that biotype 1A *Yersinia enterocolitica* isolates may be aetiological agents of disease in humans. In previous studies, a potential link between motility and the ability of biotype 1A strains to invade cultured epithelial cells was observed. In an attempt to further investigate this finding, a flagella mutant was constructed in a human faecal *Y. enterocolitica* biotype 1A isolate. The flagella mutation abolished the ability of the strain to invade cultured human epithelial cells, although adherence was not affected. The aflagellate mutant was also attenuated in its ability to survive within cultured macrophages, being cleared after 3 h, whilst the wild-type persisted for 24 h after infection. Examination of cytokine secretion by infected macrophages also suggested that the flagella of biotype 1A strains act as anti-inflammatory agents, decreasing production of tumour necrosis factor (TNF)- α whilst increasing secretion of interleukin (IL)-10. Preliminary studies using porcine *in vitro* organ culture (IVOC) tissue suggested that the flagella mutant was also attenuated in its ability to colonize intestinal tissue.

INTRODUCTION

Yersinia enterocolitica is a Gram-negative enteric human pathogen that has been associated with a range of symptoms from mild, self-limiting diarrhoea to systemic complications such as lymphadenitis and reactive arthritis (Bottone, 1999). The organism is classified on the basis of serogrouping and biochemical characteristics into biotypes (BTs) and serotypes (Bottone, 1999). BT1b strains are considered highly pathogenic due to their lethality in a mouse infection model, and are most frequently associated with severe, often fatal, disease in humans (Bottone, 1999). BT2–5 isolates are mildly pathogenic in the mouse infection model, and account for the majority of human yersiniosis cases in northern Europe (Bottone, 1999). Finally, BT1A strains are avirulent in the mouse infection model and as a result have historically been classified as non-pathogenic.

The molecular pathogenesis of *Y. enterocolitica* has been extensively researched, with particular emphasis on BT1b. The recently sequenced *Y. enterocolitica* isolate 8081 is a BT1b O:8 strain, which has been thoroughly characterized. Most research has been conducted on the pYV-encoded type III secretion system, responsible for injection of Yop effector proteins into host cells (Cornelis & Wolf-Watz, 1997). The Yop proteins are responsible for a large number of virulence traits, including the avoidance of macrophage ingestion (Grosdent *et al.*, 2002), immunomodulation (Boland & Cornelis, 1998; Carlos *et al.*, 2004; Denecker *et al.*, 2002; Erfurth *et al.*, 2004) and cytotoxicity (Andor *et al.*, 2001; Zumbihl *et al.*, 1999). The majority of the remaining research on pathogenesis of *Y. enterocolitica* has focused on invasins, and the adhesins Ail and YadA. Invasin has a major role in invasion of epithelial cells by *Y. enterocolitica* (Pepe & Miller, 1993) and also exhibits immunomodulatory properties (Kampik *et al.*, 2000). YadA and Ail are adhesins involved in initial attachment to epithelial cells, as well as subversion of macrophages and immunomodulation (El Tahir *et al.*, 2000; Grosdent *et al.*, 2002; Schmid *et al.*, 2004). All of these virulence determinants are present in BT1b and BT2–5 strains, but absent in BT1A strains (Bottone, 1999). In addition, several key publications have highlighted the key role of LPS and O-antigen variation in *Y. enterocolitica*

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Abbreviations: Amp, ampicillin; BT, biotype; Cm, chloramphenicol; DAPI, 4',6-diamidino-2-phenylindole; IL, interleukin; IVOC, *in vitro* organ culture; Kan, kanamycin; TEM, transmission electron microscopy; TNF, tumour necrosis factor.

pathogenesis (Bengoechea *et al.*, 2003, 2004; Rebeil *et al.*, 2004).

Despite the apparent lack of virulence attributes in BT1A *Y. enterocolitica*, there is growing evidence to suggest that this group of organisms are aetiological agents of human disease (Grant *et al.*, 1998; Morris *et al.*, 1991; Tennant *et al.*, 2003). Recent work has shown that BT1A isolates are capable of invading and escaping from cultured epithelial cells *in vitro*, as well as persisting in cultured macrophages (Grant *et al.*, 1999) and modulating secretion of cytokines by infected macrophages *in vitro* (McNally *et al.*, 2006). In addition, previous work in our laboratory has highlighted the fact that BT1A strains are ubiquitous in the environment, accounting for 53 % of all *Y. enterocolitica* isolated from human disease cases in Great Britain during 1999–2000 (McNally *et al.*, 2004).

The purpose of the present study was to further elucidate the interaction between BT1A *Y. enterocolitica* and epithelial and macrophage cells *in vitro*. Preliminary investigation of the ability of BT1A strains to invade HEP-2 cells uncovered a relationship between motility and invasion. The role of motility in *Y. enterocolitica* has been investigated earlier, suggesting that motility is required for invasion (Young

et al., 2000), and implicating flagella in the secretion of effector proteins (Young *et al.*, 1999a). In order to examine the role of flagella in BT1A isolates, a flagella mutant was constructed in a human faecal BT1A isolate and characterized in a number of *in vitro* pathogenesis assays.

METHODS

Bacterial strains and growth conditions. Thirty-nine BT1A *Y. enterocolitica* isolates were initially screened for invasive ability. These strains were selected from a library held in the Veterinary Laboratories Agency (VLA) culture collection (McNally *et al.*, 2004). From this subset, strain 53/03 was chosen for further analysis, as it was a human isolate, and was motile and invasive. A list of strains, plasmids and primers used in this study is provided in Table 1. All *Y. enterocolitica* isolates were cultured aerobically for 16 h at 28 °C overnight on 5 % sheep blood agar plates, or in LB broth with shaking at 200 r.p.m., unless otherwise stated. *Escherichia coli* DH5α was employed for all cloning procedures and was grown in LB at 37 °C supplemented with ampicillin (Amp, 100 µg ml⁻¹), kanamycin (Kan, 50 µg ml⁻¹) or chloramphenicol (Cm, 25 µg ml⁻¹). *E. coli* S17 λpir was employed for conjugative transfer of plasmids.

Construction of a Δ*fliA* knockout mutant. The *fliA* gene of 53/03 was amplified using primers FliA5 and FliA3 (which were designed using the *Y. enterocolitica* 8081 BT1B genome sequence),

Table 1. Strains, plasmids and primers used in this study

Engineered restriction enzyme recognition sites are indicated in bold type; primer sequences read 5' to 3'.

Strain, plasmid or primer	Details	Source
Strains		
<i>Y. enterocolitica</i> 8081	BT1B O:8. Sequenced strain of human origin from septicaemic patient	Portnoy <i>et al.</i> (1981)
<i>E. coli</i> DH5α	<i>endA1</i> , <i>r⁻m⁺</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA</i> (<i>Naf^r</i>), Δ(<i>lacIZYA</i>)	Woodcock <i>et al.</i> (1989)
<i>E. coli</i> S17	<i>recA thi pro r⁻m⁺</i> RP4::Tc::Mu::Km Tn7 λpir	Parke (1990)
53/03	BT1A O:5	McNally <i>et al.</i> (2006)
AMC2	53/03 Δ <i>fliA</i> (Kan ^r)	This study
Plasmids		
pCR2.1-TOPO	TA cloning vector	Invitrogen
pKNG101	<i>ori6K mobRK2\RP4 sacB str^r</i>	Kaniga <i>et al.</i> (1991)
pUCKan	pUC18 with <i>npt</i> inserted via <i>Pst</i> I	This study
pUCCat	pUC18 with Cm ^r gene inserted via <i>Kpn</i> II	This study
pAM3	pCR2.1 with <i>fliA</i> gene of 53/03 inserted	This study
pAM10	pAM3 with <i>npt</i> gene inserted into <i>fliA</i> via <i>Aat</i> II	This study
pAM16	pKNG101 with inactivated <i>fliA</i> from pAM10 inserted via <i>Bam</i> HI	This study
pMN402	Hyg ^r , ColE1/PAL5000 ori, <i>gfp⁺</i>	Scholz <i>et al.</i> (2000)
pACYC184	Cm ^r , Tet ^r , ColE1 ori	Chang & Cohen (1978)
pAM4	Cm ^r , Tet ^r , pACYC 184 Δ <i>Clal</i>	This study
pAM6	Cm ^r , pAM4 with <i>gfp⁺</i> inserted via <i>Eco</i> RV	This study
pAM19	CM ^r , pAM6 with <i>gyrA</i> promoter inserted via <i>Eco</i> RV/ <i>Clal</i>	This study
Primers		
FliA5	GGATCCGCTATCCGTGGTGGTCAAC	This study
FliA3	GGATCCCCAGACTTGATCATCAATAGG	
GFP5	GATATCGATGAAGGAGATATACATATGGCTAG	This study
GFP3	GCTCGAATTCATTATTTGTAGAG	
GYR5	GATATCCGCGCCATGCTTTTCAGCT	This study
GYR3	ATCGATGAGCCGCTATCCCTCTAC	

sequenced to confirm the correct amplified product, and cloned into pCR2.1-TOPO according to the manufacturer's instructions (Invitrogen) to create pAM3. The *npt* gene, conferring Kan resistance, was excised from pUCKan by *Pst*I digestion, and blunt-end cloned into the *Aat*II site in the middle of the *fliA* gene to give pAM10. The inactivated gene was PCR-amplified and cloned into the *Bam*HI site of pKNG101 by use of engineered restriction sites to give pAM16. The suicide vector pAM16 was then introduced into *E. coli* S17 λ pir, and conjugations performed with 53/03 for 24 h at 28 °C on sterile filters placed on LB plates. The mating filter was resuspended in PBS and serial dilutions plated onto M9 minimal agar plates containing 6% sucrose plus Kan. Colonies were replica-plated and checked by PCR for double recombination events. Potential mutants were confirmed by Southern blotting (using probes for *npt* and *fliA*), PCR, motility test, Western blotting and electron microscopy. The mutant AMC2 was complemented using pAM3 containing the *cat* gene excised from pUCCat, cloned into the *Kpn*I site. The complemented strain was maintained under Amp, Kan and Cm selection to ensure plasmid stability.

Southern blotting. Mutant and wild-type strains were grown overnight in 50 ml LB broth, and cultures harvested by centrifugation. Chromosomal DNA was prepared using the phenol/chloroform extraction method (Sambrook *et al.*, 1989) and Phase Lock Gel tubes (Eppendorf). DNA was digested with *Xmn*I, separated by agarose gel electrophoresis, and transferred to a nitrocellulose membrane (Amersham). Purified *fliA* and *npt* were labelled with the AlkPhos direct labelling kit, with hybridization and detection performed using the CPD-Star detection kit (Amersham).

Flagella secreted protein Western blots. Bacteria were cultured for 16 h aerobically in L broth and then subcultured 1:50 into 50 ml fresh medium. Cultures were grown at 28 °C for 6 h, bacteria were pelleted by centrifugation, and the supernatant was collected and filtered through a 0.2 μ m pore-size filter. Ice-cold TCA was added to a final volume of 10% and the flagella secreted proteins (Fop) precipitated overnight at 4 °C. Precipitates were harvested by centrifugation at 4500 g for 30 min, the supernatants decanted, and the protein pellets suspended in a volume of Laemmli sample buffer (Sambrook *et al.*, 1989) calculated according to the OD₆₀₀ of the original bacterial culture. Proteins were separated on a 10% SDS-polyacrylamide gel and stained by a commercially available silver stain kit (Amersham) or transferred to a nitrocellulose membrane using the Mini-Protein transfer system (Bio-Rad). The membrane was blocked in 0.1 M PBS + 0.05% Tween 20 (wash buffer) with 8% (w/v) dried skimmed milk overnight at 4 °C, then washed in wash buffer. A 1:150 dilution was made of rabbit anti-flagella anti-serum (Bleves *et al.*, 2002) in wash buffer and this was hybridized to the membrane for 3 h at room temperature. The serum is a polyclonal serum raised against the BT1B O:8 type strain ATCC 9610, as used in the Wauters typing scheme (Aleksic & Bockemuhl, 1984; Bleves *et al.*, 2002), and contains antibodies against the somatic and flagellar antigens. Before use in flagella Western blots, serum was pre-absorbed with bacteria that had been grown at 37 °C to prevent flagella expression. The membrane was washed and then probed with a 1:1500 dilution of mouse anti-rabbit horseradish peroxidase (HRP)-conjugated antibody for 90 min. The membrane was washed and then developed using the classical 3-amino-9-ethylcarbazole (AEC)/H₂O₂ method (Sambrook *et al.*, 1989).

LPS and total cell protein profiles. Bacteria were grown on LB agar plates at 28 °C for 24 h, harvested and resuspended in 2 ml PBS to equal densities, as determined by OD₆₀₀. LPS was extracted by adding 2 ml 90% phenol, followed by incubation with constant agitation at 68 °C for 30 min. The supernatant was collected and 0.1 vols 20% (v/v) sodium acetate plus 5 ml absolute alcohol were added. The sample was stored at 4 °C overnight and precipitated LPS harvested by centrifugation. The pellet was resuspended in 2 ml

PBS and dialysed overnight in sterile distilled water. The LPS sample was diluted 1:1 with Laemmli sample buffer and separated by electrophoresis using standard SDS-PAGE techniques. The gel was stained with a commercially available silver stain (Amersham). Total cell protein extracts were prepared by resuspending the cell pellet in Laemmli sample buffer, followed by standard SDS-PAGE and Coomassie Blue staining.

Motility test. Sixteen-hour, aerobic broth cultures were harvested by centrifugation and resuspended in 0.1 M PBS, pH 7.2. Ten microlitres of suspension was used to inoculate the centre of a 0.3% tryptone soya agar plate, which was incubated for 16 h aerobically at 28 °C.

Transmission electron microscopy (TEM) of bacteria. Bacteria were cultured for 16 h aerobically in L broth at 28 °C, harvested by centrifugation and resuspended in 0.1 M PBS. Formvar carbon-coated grids were placed onto a drop of the bacterial suspension for 15 min. The grid was removed, excess liquid drained by blotting paper, and the grid placed onto potassium phosphate tungsten-negative stain solution for ~10 s. Grids were dried and viewed using a Philips CM 10 transmission electron microscope.

Cell cultures. HEp-2 human epithelial cells were cultured in Minimal Essential Medium (MEM, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Life technologies), 1% L-glutamine and 1% non-essential amino acids (Sigma). Cells were grown at 37 °C in a humidified 5% CO₂ atmosphere, and subcultured every 4 days. Two days prior to infection, cells were seeded into a 24-well tissue-culture plate (Nunc) at approximately 2×10^5 cells ml⁻¹. U937 human monocytic cells were maintained in RPMI medium (Sigma) supplemented as above. Seventy-two hours prior to infection, cells were seeded into a 24-well tissue-culture plate (Nunc) at approximately 2×10^5 cells ml⁻¹ and treated with 10 nmol phorbol-myristate-acetate (PMA, Sigma) to trigger differentiation into active macrophages.

Association and invasion assays. Association with and invasion of bacteria into cultured HEp-2 cells was assayed as described elsewhere (Finlay & Falkow, 1988; Miller & Falkow, 1988). Bacteria were cultured for 16 h aerobically in LB broth with shaking, harvested by centrifugation, and resuspended in supplemented tissue-culture medium to $\sim 2 \times 10^7$ c.f.u. ml⁻¹. One millilitre of bacterial suspension was added in triplicate to wells of a pre-seeded 24-well plate at an m.o.i. of 100. The infected cells were incubated for 3 h at 37 °C. Cells were washed three times with 0.1 M PBS, and fresh supplemented tissue-culture medium containing 100 μ g gentamicin ml⁻¹ (Sigma) was added and the cells further incubated for 2 h. Cells were then washed twice in PBS and lysed with a 1% Triton X-100 (Sigma) solution for 5 min. Dilutions of the lysed infected cells were plated onto LB agar in triplicate and incubated overnight, and colonies were counted to give the total number of invasive bacteria per millilitre. In a parallel series of wells, the cells were lysed after the initial 3 h infection period to ascertain the total number of associated organisms per millilitre.

Persistence within U937 human macrophage cell line. Bacteria were grown, harvested and resuspended as for the HEp-2 invasion assay. Differentiated U937 cells were infected at the same m.o.i. as for the HEp-2 invasion assay and incubated for 1 h at 37 °C. Cells were washed three times with 0.1 M PBS, and fresh medium containing 100 μ g gentamicin ml⁻¹ was added with a further incubation for 5 h. Cells were washed twice in PBS and lysed by addition of a 1% Triton X-100 solution for 10 min. Dilutions of the lysed cell infection were plated onto LB agar in triplicate and incubated at 28 °C aerobically for 16 h, and colonies were counted to give the total number of invasive bacteria per millilitre. In a parallel series of

wells the cells were lysed 15 min after the initial addition of gentamicin in order to ascertain the number of organisms internalized by the cells after the 1 h infection.

Secretion of cytokines by infected U937 cells. U937 human macrophages were infected as above for a fixed time point of 3 h. The supernatant was removed and centrifuged at 13 000 *g* for 5 min. Two hundred microlitres of the supernatant was stored at -20°C until required. The levels of secretion for interleukin (IL)-6, IL-10 and tumour necrosis factor (TNF)- α were ascertained by using the Quantikine sandwich ELISA kit (R&D Systems). For assays involving crude flagellin samples, approximately 10 ng protein was added to cells, followed by incubation for 3 h, as for bacterial infections.

Preparation of crude flagellin sample. Bacteria were cultured as described above for the preparation of Fop proteins. Precipitated protein was resuspended in 1.5 M Tris/HCl, pH 8.8, and then subjected to size-exclusion filtration on a YM 30 kDa centrifugation device (Centricon). The filtrate was collected and then centrifuged again using a YM 10 kDa kit (Centricon). The retentate was then stored at -20°C . For use in assays, the protein concentration was determined using the Bio-Rad protein assay kit and a BSA standard dilution series (Bio-Rad). Confirmation of the presence of flagellin in the preparation was performed by Western blotting using unabsorbed serum, confirming the presence of flagellin and the absence of detectable levels of LPS.

TEM of *Y. enterocolitica*-infected U937 cells. U937 cells were infected as above for 1 or 6 h with strains 53/03 or AMC2. Cells were then treated with 3% glutaraldehyde in PBS for 5 min and removed from the tissue-culture well. Cells were pelleted by centrifugation at 1500 r.p.m. for 5 min, washed in 0.1 M PBS and post-fixed in 1% (w/v) osmium tetroxide, dehydrated in ethanol, and placed in hexamethyldisilane for 5 min. Specimens were subjected to critical-point drying with liquid CO_2 , then fixed to aluminium stubs with silver conductive paint and sputter-coated with gold. Specimens were then examined using a Cambridge Stereoscan S250 Mark III transmission electron microscope at 10–20 kV.

Construction of a GFP fusion containing, *Y. enterocolitica*. The *gfp*⁺ gene was amplified from pMN402 (Scholz *et al.*, 2000) using primers GFP5 and GFP3. Plasmid pAM4 was constructed by digesting pACYC184 with *Cla*I, blunt-ended by treatment with the large Klenow fragment of DNA polymerase I, and religated using T4 DNA ligase. Plasmid pAM4 was digested with *Eco*RV, phosphatase-treated, and ligated to the amplified *gfp*⁺ gene to create pAM6. Clones were selected by loss of tetracycline resistance. The *gyrA* promoter was amplified from *Y. enterocolitica* 8081 using primers Gyr5 and Gyr3. The amplified product and pAM6 were digested with *Eco*RV and *Cla*I, the linearized plasmid was phosphatase-treated, and the fragments were ligated to create pAM19. Electrocompetent *Y. enterocolitica* strains were electroporated with 1 μl pAM19 at 1.5 kV, 2.5 μF , 200 Ω .

Porcine *in vitro* organ culture (IVOC). Two commercial cross-bred, three-month-old male pigs were euthanized on separate days. Immediately after euthanasia the terminal ileum and spiral colon sections were harvested. The contents of the tissues were emptied and 2 cm^2 sections prepared, washed in pre-warmed 0.1 M PBS, pH 7.2, and placed on sterile gauze. The tissue sections were placed individually in a square Petri dish and partially submerged in pre-warmed DMEM containing 1% L-glutamine and 1% non-essential amino acids. Overnight cultures of bacteria containing the GFP fusion plasmid were harvested by centrifugation and resuspended in PBS. One hundred microlitres of culture was pipetted directly onto the apical surface of the tissue and incubated at 37°C in 5% CO_2 for 6 h, with media changed after 3 h. Tissues were washed vigorously in PBS and fixed in ice-cold 4% paraformaldehyde for 24 h.

Tissue preparation and confocal fluorescence microscopy.

Tissue samples were fixed in 4% paraformaldehyde and snap-frozen in isopentane pre-cooled in liquid nitrogen to between -40 and -60°C . Tissue sections were then cut using a cryostat (-20°C) to a thickness of 4–6 μm and collected directly onto slides. The slides were mounted with Vectashield with 4',6-diamidino-2-phenylindole (DAPI) mounting medium and stored in the dark at room temperature. Confocal fluorescence microscopy was performed on a Leica microscope with an excitation wavelength of 488 nm and an emission filter of 507 nm.

Statistics. *P* values for differences between sets of data for the wild-type, mutant and complemented mutant were performed using Student's *t* test.

RESULTS

Correlation between motility and invasion in *Y. enterocolitica* BT1A isolates

Initially, 39 BT1A strains were assessed for invasiveness and motility. The results of the invasion assay were compared with the sizes of the motility zones measured after 16 h. All but three of the BT1A isolates tested were motile, with motility zone measurements similar to those observed for BT3 and BT4 isolates. There was a slight correlation between invasion and motility, although this was not statistically significant (Fig. 1). However, three of the isolates were completely non-invasive in HEp-2 cells; two were ovine isolates, and one was isolated from an asymptomatic human

Fig. 1. (A) Motility of 39 selected *Y. enterocolitica* BT1A strains. The graph shows the motility of isolates (cm) in relation to the number of invasive bacteria, as determined in an *in vitro* invasion assay utilizing HEp-2 cells (see Methods). (B) Mean zones of motility measured for BT1A, 3 and 4 isolates. Error bars represent SD.

who had been in contact with a confirmed human yersiniosis case, as part of a national intestinal infectious disease survey. All three isolates were serotype O:5, non-motile, and failed to express flagellin (data not shown).

Construction of a $\Delta fliA$ mutant in a clinical BT1A isolate

To further characterize the relationship between motility and invasion, an isogenic flagella mutant in the clinical, motile, invasive isolate 53/03 was constructed. Construction of the mutant (strain AMC2) was confirmed by an increase in size of the PCR product for *fliA* of 1 kb (Fig. 2A) and by Southern blotting to confirm the insertion of the Kan cassette into *fliA* by hybridization, with both a *fliA* (Fig. 2B) and an *npt* (Fig. 2C) probe. The mutant was aflagellate (Fig. 2D), non-motile (Fig. 2E) and failed to express flagellin detectable by SDS-PAGE (Fig. 2F) or by Western blotting of secreted proteins (Fig. 2G). After complementation

of the mutant with pAM3, motility was restored (Fig. 2E), and flagellin secretion was restored as indicated by Western blotting (Fig. 2G). The flagellin detected in BT1A isolates migrated to a different extent to that of strain 8081 and BT3 and BT4 isolates, but was confirmed as flagellin by 2D gel electrophoresis and MS analysis. Any potential regulatory effects as a result of the mutation in *fliA* were investigated by examining whole-cell protein extracts (Fig. 2H) and LPS profiles (Fig. 2I) of the mutant and wild-type strain. The results suggested no obvious changes in protein expression or LPS production between the mutant and wild-type strain.

An aflagellate BT1A *Y. enterocolitica* is attenuated in its ability to invade cultured epithelial cells

The $\Delta fliA$ mutant was characterized in the HEP-2 cell association and invasion assay. The mutant demonstrated an almost complete loss of invasiveness *in vitro* when

Fig. 2. Characterization of the $\Delta fliA$ mutant AMC2 by PCR for the *fliA* gene (A) and by Southern blotting using a probe against *fliA* (B) and the Kan resistance gene *npt* (C). Lane M in (A) contains a 1 kb DNA ladder, with lines indicating the 3, 2 and 1.5 kb markers. Arrows in (B) indicate detected genes in the Southern blot. (D) Characterization of the $\Delta fliA$ mutant AMC2 by TEM; note visible flagella associated with the wild-type strain 53/03 but not AMC2 ($\times 22\,250$; bars, 5 μm). (E) Motility of the mutant, wild-type and complemented strains, as determined by 16 h growth on 0.3% TSA plates. (F) SDS-PAGE and (G) Western blotting of secreted Fop proteins of the control strain 8081, wild-type (WT) 53/03, aflagellate mutant AMC2 (Δ), and AMC2 complemented with pAM3. Arrows indicate the flagellin protein on both the SDS-PAGE gel and the Western blot. Lines to the left of the SDS gel picture indicate molecular mass markers of 50, 37 and 25 kDa, from top to bottom. (H) Whole-cell protein extracts from the mutant and wild-type grown at 28 °C. Lines to the left of the gel indicate molecular mass markers of 150, 100, 75, 50, 37, 25 and 12 kDa, from top to bottom. (I) LPS extracts from the mutant and wild-type grown at 28 °C. Lines to the left of the gel indicate molecular mass markers of 50, 37 and 25 kDa, from top to bottom.

compared with the wild-type and complemented strains ($\sim 10^{2.5}$) ($P=0.000$). Complementation of the mutant resulted in partial restoration of the parental invasive phenotype compared with the wild-type ($P=0.001$), with differences possibly due to overexpression of *fliA* from the plasmid. Numbers of associated bacteria were similar for the wild-type ($\sim 10^5$), mutant ($\sim 10^5$) and complemented strains ($\sim 10^4$), although the complemented strain was slightly reduced in its ability to adhere ($P=0.045$), indicating that the loss of invasiveness was not due to the inability of the mutant to actively swim towards or adhere to the cell layer. Additionally, pre-growth of the organism at 37°C (which is inhibitory to flagella expression) prior to cell infection completely inhibited cell invasion, but had no effect on association with cells (data not shown).

An aflagellate BT1A *Y. enterocolitica* isolate is unable to persist within macrophages *in vitro*

Previous studies have shown BT1A strains to be capable of surviving within cultured macrophages for extended periods

of time (Grant *et al.*, 1999). To assess the effect of flagella on this persistence, human U937 macrophage cell lines were infected with mutant, wild-type and complemented strains, and the number of intracellular organisms after 6 h was compared with the total number of associated organisms recovered after an initial 1 h infection period. The aflagellate strain was completely cleared by the macrophages by 6 h, whilst the mutant and complemented strains persisted ($P=0.000$) (Fig. 3A). A time-course experiment showed that the mutant was ingested by the macrophages to the same extent as the wild-type, but was completely killed by the macrophages between 3 and 4 h post-infection (Fig. 3B). TEM of infected macrophages showed that the mutant strain was contained within a large phagosomal vacuole, whilst the wild-type was present inside the cells in tight vacuoles with no free surrounding space (Fig. 3C, D, E). Additionally, pre-growth of the organism at 37°C (which is inhibitory to flagella expression) prior to cell infection completely inhibited the ability of the organism to survive within macrophages, with no detectable bacteria at 1 h post-infection (data not shown).

Fig. 3. (A) Persistence of wild-type (53/03), mutant (AMC2) and complemented mutant (AMC2 pAM3) in differentiated U937 macrophages over a 6 h period. t_0 indicates number of bacteria present in macrophages after initial 1 h infection; t_5 indicates number of bacteria present 5 h later. (B) Time-course of infection, with bacteria enumerated at hourly intervals after infection of macrophages. All results are displayed as c.f.u. ml⁻¹ of bacteria recovered from an infected well. Experiments were performed in triplicate and error bars represent SEM. In addition, bacteria were visualized by TEM within the macrophages ($\times 38\,750$; bars, 2.5 μm) at t_0 for AMC2 (C), and at t_0 (D) and t_5 (E) for wild-type strain 53/03. Note the differences in intracellular location of the bacterial strains, as indicated by arrows.

Fig. 4. Mean levels of secretion of (A) TNF- α and (B) IL-10 by U937 human macrophages after incubation with wild-type (53/03), mutant (AMC2) and complemented mutant (AMC2 pAM3), as well as crude flagellin preparations from 53/03 (53/03 Flg) and AMC2 (AMC2 Flg). Experiments were performed in duplicate and error bars represent SEM.

Loss of flagella in BT1A *Y. enterocolitica* leads to differences in cytokine secretion by infected macrophages *in vitro*

One explanation for the loss of persistence in macrophages by the aflagellate mutant was the variation in the response of macrophages to infection with the wild-type and mutant. Three cytokine responses were tested: IL-6, IL-10 and

TNF- α . There was no difference between the levels of IL-6 secreted by cells infected with the mutant and those of cells infected with the wild-type (data not shown). Whilst the wild-type strain induced high levels of secretion of the anti-inflammatory cytokine IL-10, U937 infected with the mutant secreted lower levels ($P=0.0014$) (Fig. 4B). Complementation of the mutant partially restored the IL-10 secretion phenotype ($P=0.021$). Similarly, U937 infected with the mutant strain secreted approximately twofold greater amounts of the pro-inflammatory cytokine TNF- α compared with the wild-type ($P=0.0031$) and the complemented strain ($P=0.0045$) (Fig. 4A). These results suggested that flagellin induces some cytokine responses *in vitro*. To investigate this hypothesis, the effect of isolated flagellin on the cytokine response of host cells was tested. Unfortunately, attempts to amplify the *fleABC* operon from BT1A isolates using primers designed against the published sequence (Kapatral & Minnich, 1995), in order to generate recombinant flagellin, proved unsuccessful. Instead, crude flagellin samples (53/03 Flg or AMC2 Flg) were prepared by size-exclusion filtration and used to treat cultured U937 cells. The crude flagellin samples were subjected to Western blotting with serum containing antibodies against the flagellar and somatic O antigens, with no detectable endotoxin present in the sample. The addition of this crude flagellin material had no obvious cytotoxic effect on the cells, as determined microscopically (data not shown). The cells treated with 53/03Flg or AMC2Flg did not secrete IL-10 (Fig. 4B). However, cells secreted approximately twofold lower levels of TNF- α when exposed to flagellin from 53/03 compared with flagellin from AMC2 ($P=0.041$) (Fig. 4A).

An aflagellate BT1A *Y. enterocolitica* is reduced in colonization of porcine terminal ileum and spiral colon *in vitro*

In an attempt to visualize the effect of an aflagellate mutation on binding to intestinal tissue, both the mutant and the wild-type strain were transformed with a plasmid containing a constitutive GFP fusion. Both the wild-type strain and the aflagellate mutant colonized porcine terminal ileum tissue *in vitro* (Fig. 5), but semi-quantitative evaluation

Fig. 5. Colonization of porcine spiral colon intestinal tissue by (A) GFP-tagged wild-type (53/03) and (B) aflagellate mutant (AMC2). Images were captured using a Leica confocal fluorescence microscope and a $\times 63$ objective, which was then magnified a further threefold using the Leica confocal microscope capture software. Bars, 50 μ m. Thick arrows indicate cell nuclei counterstained with DAPI; thin arrows indicate GFP-tagged bacteria.

indicated that the number of wild-type bacteria associated was approximately 50-fold higher than that observed for the aflagellate mutant bacteria. Similarly, although there was less colonization of the spiral colon by wild-type bacteria, it was still approximately 10-fold greater than with the aflagellate mutant bacteria.

DISCUSSION

Although historically defined as non-pathogenic, BT1A *Y. enterocolitica* are increasingly implicated as aetiological agents of disease in humans (Grant *et al.*, 1998, 1999; McNally *et al.*, 2004; Morris *et al.*, 1991; Tennant *et al.*, 2003). Nevertheless, little is known about the interaction of this group of organisms with the infected host, or the potential virulence mechanisms involved. During a preliminary investigation of the phenotypic properties of BT1A strains from a variety of sources (McNally *et al.*, 2006), three isolates were identified as both non-invasive and non-motile. Consequently, the role of flagella as a potential virulence factor in BT1A *Y. enterocolitica* was further investigated by comparison of the characteristics of an aflagellate mutant with those of the wild-type parent.

Although the natural target for mutagenesis would have been the *fleABC* operon, which encodes the structural flagellin genes, attempts at amplifying the *fleABC* operon from BT1A isolates were unsuccessful, using primers based on the previously published operon sequence (Kapatral & Minnich, 1995) or on the recently completed genome sequence of *Y. enterocolitica* 8081 BT1b. Attempts at amplifying the entire *fleABC* operon with primers upstream and downstream, generating a 3.9 kb product, were also unsuccessful, as were attempts at amplifying individual genes or even promoter regions from the area. As there are high levels of homology between *fleA*, *B* and *C*, the failure to amplify individual genes may not be surprising; however, our results seem to confirm that the flagellin region in BT1A isolates is variable (Tennant *et al.*, 2003). Interestingly, the three flagellin genes successfully hybridized with BT1A strains in a comparative microarray study (Howard *et al.*, 2006), suggesting that the sequence variation may be occurring at a low level. The flagellin-coding region of BT1A strains is currently the focus of further investigation.

An alternative strategy adopted was to target the *fliA* gene for mutagenesis. The *fliA* gene is an appropriate target, as the gene product is essential for expression of flagella in a BT1b strain, but its mutation has no effect on expression of other key virulence genes (Iriarte *et al.*, 1995; Young *et al.*, 1999b, 2000). The *fliA* gene was insertionally inactivated with a Kan cassette using the pKNG101 suicide vector. The resulting mutant (AMC2) was confirmed by PCR and Southern blotting, electron microscopy, Western blotting and loss of motility.

Investigations using the $\Delta fliA$ mutant (AMC2) suggested that flagella play a role in invasion of epithelial cells *in vitro*. The aflagellate mutant was almost completely attenuated in

its ability to invade HEp-2 cells, with the phenotype partially restored by complementation with the entire *fliA* gene under the control of its native promoter. Similar observations have been made using a *fliA* mutant constructed in a BT1b strain (Young *et al.*, 2000), and in our study, growth of the wild-type strain at 37 °C, which is inhibitory to flagella expression, prior to cell infection completely inhibited cell invasion. One explanation would be that the lack of motility precludes interaction with the cell monolayer. However, our results indicated that the number of associated bacteria was the same for both the wild-type and the aflagellate mutant after an initial 3 h infection, as well as for the bacteria pre-grown at 37 °C. Thus, it appears that the flagella of BT1A strains play an active role in the invasion of host cells. The active involvement of flagella in invasion by other enteric pathogens, including *Salmonella* (La Ragione *et al.*, 2003), as well as in adherence by enteropathogenic *E. coli* (Giron *et al.*, 2002), has been previously reported.

Comparison of the wild-type and mutant strains also suggests that flagella play a role in survival of BT1A *Y. enterocolitica* within human macrophages *in vitro*. Similar findings have been observed in both naturally occurring and experimentally constructed flagella mutants of *Salmonella* Typhimurium (Schmitt *et al.*, 1994, 2001). However, whether flagella are expressed within macrophages by *Y. enterocolitica* BT1A has yet to be ascertained. The mechanism of this intracellular persistence is unclear. TEM of infected macrophages showed that the wild-type was contained within tight vacuoles, with little free space, whilst the mutant was contained within a large spacious vacuole. Earlier work using murine macrophages has also demonstrated that BT1A bacteria are contained within vacuoles which appear to fuse with each other (Grant *et al.*, 1999). Thus, the vacuolar space surrounding the bacteria may be relevant to intracellular persistence.

As flagella from a wide range of bacterial pathogens have been shown to have immunomodulatory effects on host cells, primarily through interaction with Toll-like receptor (TLR)5 (Donnelly & Steiner, 2002; Wyant *et al.*, 1999; Zhou *et al.*, 2003), the cytokine responses to challenge of U937 cells with the wild-type and aflagellate mutant were compared. Incubation with the aflagellate mutant AMC2 resulted in elevated secretion of the pro-inflammatory cytokine TNF- α , and decreased secretion of the anti-inflammatory cytokine IL-10, compared with the wild-type strain. This suggests that the flagella may play a role in depression of the host inflammatory immune response to infection with *Y. enterocolitica*. Support for this hypothesis was obtained using a crude preparation of secreted flagellin, which induced a twofold reduction in TNF- α secretion compared with the equivalent protein sample prepared from the aflagellate strain.

As the aflagellate mutant was constructed by mutation of a regulatory gene, the possibility exists that other factors which contribute to BT1A pathogenesis were affected, such as outer-membrane protein profiles, or perhaps LPS

composition. The effect of a *fliA* mutation on global gene expression and LPS composition in BT1A strains was partially characterized by examining whole-cell protein extracts and LPS profiles of the mutant and wild-type strains, and there were no observable differences between the two. Whilst *fliA* mutants have been shown to have no effect on expression of virulence genes in pathogenic *Y. enterocolitica*, previous work has shown that there is cross-talk between the flagella operon and other type III secretion systems (Bleves *et al.*, 2002), and that flagella actively export virulence factors (Young *et al.*, 1999a). Whilst we have failed to find any secreted proteins in BT1A strains by SDS-PAGE and silver staining (data not shown), it cannot be discounted that abolition of flagella could result in the absence of an as-yet-unidentified virulence factor.

The aflagellate mutant was also attenuated in its ability to colonize porcine intestinal tissue *in vitro*. This attenuation was more evident with terminal ileal material, which is the area of the porcine gastrointestinal tract that is dense in follicle-associated epithelium, than with spiral colon tissue. This is in disagreement with the association observed with HEP-2 cells *in vitro*, and also with previous work describing the expression of flagella in *Y. enterocolitica* at 28 but not at 37 °C. Clearly, there is a high degree of complexity involved in flagellin production, and the expression of flagella by BT1A *Y. enterocolitica* *in vitro* and *in vivo* is currently under further investigation.

In contrast to our results, earlier studies using flagella mutants of pathogenic biotypes of *Y. enterocolitica* have indicated that flagella have no detectable effect in the mouse model (Young *et al.*, 2000). Given the well-recognized similarity between the pig and human intestinal tracts, our results suggest that such virulence properties are host specific for *Y. enterocolitica* and indicate that the ability of the mouse model to reflect the disease potential of BT1A strains may need to be reconsidered, although a mouse model of colonization has been described (Tennant *et al.*, 2003).

In conclusion, this study reports the successful mutagenesis of a potential virulence factor, flagellin, in a BT1A *Y. enterocolitica* isolate. The aflagellate mutant was attenuated in its ability to invade cultured epithelial cells and colonize porcine intestinal tissue *in vitro*, and was unable to survive within cultured human macrophages over 3 h. The flagellin proteins were also shown to influence secretion of TNF- α by infected macrophages, and potentially induce production of the anti-inflammatory cytokine IL-10. Further work is required to begin to fully understand the potentially pathogenic BT1A *Y. enterocolitica*, but this is hampered by a lack of knowledge of the genome sequence. It is well recognized that at the genetic level BT1A isolates may possess as little as 41 % similarity to BT2–5 isolates (Fearnley *et al.*, 2005). Such differences in sequence similarity have hampered our ability to further confirm our findings by genetic-based methods. Investigation of BT1A isolates as a separate group of

pathogenic bacteria will facilitate understanding of this zoonotic organism.

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